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Hormonal Regulation of Hypothalamic Gene Expression: Identification of Multiple Novel Estrogen Induced Genes

Simon W. Law,* Ede M. Apostolakis, Patrick J. Samora, Bert W. O'Malley and James H. Clark

Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, U.S.A.

Estrogen (E) has been shown to play a major role in hypothalamic function and is a prerequisite for progesterone (P) induced sexual behavior in female rats. In the course of studies in search of steroid induced hypothalamic genes, we discovered a surprisingly large number of E-induced genes (21 mRNAs in total). This is the largest number of E-induced genes ever identified in a single organ. Many of these mRNAs exhibit considerable magnitudes of induction and their levels were maintained typically during subsequent P treatment. Among the induced genes, several encode metabolic enzymes and may account for some of the morphological changes observed in hypothalamic neurons in response to E. Since E appears to play a major role in defining the pattern of hypothalamic gene expression in conjunction with its capacity for behavioral modulation, these newly identified cDNAs may serve as genetic markers for correlative studies of E-induced central nervous system behavior.

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INTRODUCTION

The sex steroid hormones estrogen (E) and progesterone (P) control sexual behavior in the rat [1]. E treatment in ovariectomized rats is indispensable for the induction of proceptive and receptive mating behavior upon subsequent administration of P. After pretreatment for 48 h with E, administration of P is a standard protocol for initiating sexual behavior (lordosis). The primary neuroanatomical region that mediates the hormonal control of lordosis behavior is the ventromedial nucleus (VMN) of the hypothalamus [2, 3]. Treatment with E stimulates the expression of P receptor (PR) in the VMN [4, 5]. There is a strong correlation between the induction of PR binding in the VMN and the expression of female reproductive behavior in rats [6, 7]. The action of P is presumed to be a result of its binding to the PR, since the progesterone antagonist RU 38486 blocks the P-induced lordosis [8]. The role of PR is reinforced by the observation that PR antisense oligonucleotides injected into the VMN, can block P-induced lordotic behavior in the rat [1, 9].

Although the E-mediated induction of PR in the hypothalamus is well accepted, the full complement of genomic actions of E is currently unknown. To this end, we initiated a study to discover other novel genes that might be induced by estradiol.

EXPERIMENTAL

Animals

All experiments were in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee. Studies were performed in ovariectomized Sprague–Dawley rats (Sasco, Houston, TX) weighing 180–200 g and housed three per cage in 12 h light and 12 h dark beginning at 0700 CST. Rat chow and water were provided *ad libitum*. Female rats were screened for positive lordosis behavior before admission to the study at 7 days after ovariectomy. Briefly, females were primed with estradial benzoate (E, 100 μg, s.c.) followed by progesterone (P, 100 ng, s.c.) 48 h later. Four hours after the P injection, the female was introduced for 5 min into the test arena containing a sexually proven male. Proceptive, receptive, and lordotic behaviors were observed and recorded [10]. For the experimental protocol, only females exhibiting positive lordotic behavior in response to four males were used.

*Correspondence to S. W. Law.
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Abbreviations: E, estrogen; P, progesterone; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; VMN, ventro-medial nucleus; MOPS, 3-[N-morpholino]propane sulfonic acid.

Tissue collection

On day 21 postovariectomy, females were divided into three groups. One group received only sterile saline and was designated the no-treatment (NT) group. The other groups received either E alone or E + P as described above. Rats were decapitated; the hypothalami were rapidly exposed, dissected, and frozen for storage at −70°C until processing for RNA isolation.

RNA isolation and library construction

RNA was isolated from hypothalami using RNAzolB (Biotecx, Houston, TX) as described in the manufacturer's protocol. Ten hypothalami (50 mg wt each) were pooled together from each group of animals. Each preparation yield about 400 μg of total RNA. Total RNAs were used for all Northern blot analysis. Poly A+ RNAs (mRNA) were used for cDNA synthesis and library construction and were isolated from total RNA using oligo-d(T) cellulose spun column (Invitrogen Corp, San Diego, CA). A cDNA library was constructed from E + P mRNA and was cloned into the λ phage unizap XR vector (Stratagene, La Jolla, CA). Procedures for cDNA library screening were essentially the same as described previously [11, 12].

Sequencing and analyses

Dideoxy chain termination sequence reactions were performed using the Sequenase version 2.0 (United States Biochemicals, Cleveland, OH) and miniprep plasmid DNA as template. End sequences were obtained from each clone using T3 and/or T7 sequence primers. Sequences were matched to the Genbank using the NCBI blast program.

cDNA isolation and labeling

Recombinant plasmid DNAs were isolated from 3 ml minipreps by a modified alkali lysis procedure [13]. Insert cDNAs were released by digesting with *Eco*RI and *Xho*I (Promega Corp, Madison, WI) and gel purified. DNAs were recovered from agarose gel slices by Geneclean II (Bio 101, La Jolla, CA). Each cDNA (25–50 ng) was labeled with [^{32}P-α]dCTP by random priming [14] and hybridized to Northern blots as described below.

Northern blot analyses

The Northern blot procedure of Kroczek and Siebert [15] was employed in all RNA analyses. Briefly, 20 μg of total RNA was applied to each lane of a 1.2% agarose gel containing 1.1% formaldehyde and 1 × MOPS (0.02 M MOPS, 0.005 M sodium acetate, 0.0001 M EDTA) buffer pH 7.0 and electrophoresed at 100 V for 3 h with no buffer recirculation. After electrophoresis, RNA was transferred to nitrocellulose membrane filters (Schleicher & Schuell, Keene, NH) by capillary action and processed as described previously [17]. RNA molecular size markers at 5 μg per lane were used (Gibco/BRL, Bethesda, MD). Ethidium bromide at 10 μg/ml was included in the RNA samples for better visualization of the fractionated RNA. Blots were prehybridized in a solution containing 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardts (1 × Denhardts = 0.02% ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 0.1% sodium deodecyl sulfate (SDS) 100 μg/ml of sheared and heat denatured salmon sperm DNA and 50% formamide at 42°C for 4 h. After prehybridization, [^{32}P-α]dCTP-labeled cDNA probe was then added (1 × 10^6 cpm/ml) to the same solution and hybridization was continued at 42°C for 18 h. After hybridization, filters were briefly rinsed several times with 2 × SSC followed by two 30 min washes in a solution of 0.1 × SSC and 0.1% SDS at 50°C. Filters were briefly blotted to remove excess liquid, wrapped with saran wrap and autoradiographed at −80°C between two intensifying screens.

RESULTS

Screening and analyses of the cDNA library

In the course of studies in search of P-induced gene (results of which will be reported elsewhere), we screened an E + P cDNA library with probes derived from E cDNA and an E vs E + P subtracted cDNA. The E vs E + P subtraction was performed at a R_0t of 375 mol per s/l, a value much lower than the recommended 1500–3000 mol per s/l [11]. Theoretically, low R_0t hybridization removes (subtracts) abundantly expressed common sequences but not the low copy E-induced sequences. Thus, this is an enrichment procedure that may facilitate recognition of low copy induced sequences by promoting a subset of lower abundancy mRNAs to higher abundance. Screening of the E + P cDNA library using this approach, identified 120 cDNA clones which exhibited more intense hybridization signal to the subtracted E + P cDNA probe and were selected for further analyses. DNA sequences were determined by end sequencing with T3 and T7 primers and were used to search the Genbank database for sequence homology as described in Experimental. Of the 120 clones sequenced, 16 were presently known genes; 70 clones were assumed to be unknown genes as significant sequence homology were not found in Genbank database. Among the remaining 34 clones, some were not viable and some contain mitochondrial genomic sequences and were not analyzed further. None of the identified sequences were known previously to be highly expressed genes in the brain such as actins, tubulins, myelin basic proteins, ribosomal proteins etc. Among the 86 (16 + 70) cDNAs of interest, several shared overlapping or identical sequences and therefore allowed us to focus on 70 clones for Northern blot analyses.

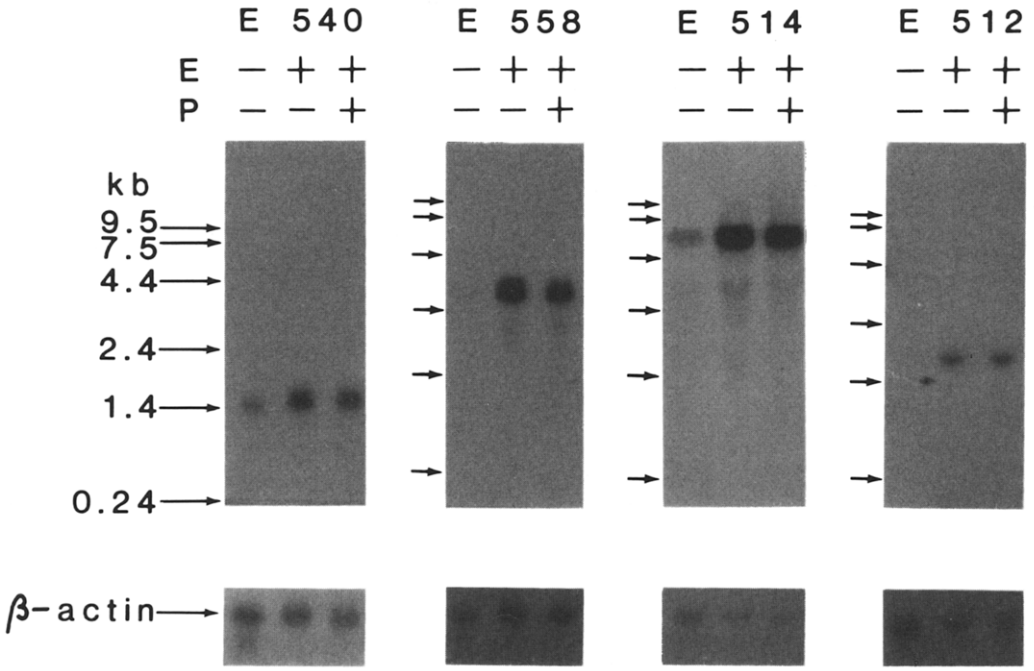

Fig. 1. Northern blot analyses for E-induced cDNAs. Total RNAs (20 μg) isolated from hypothalami of NT, E and E + P treated ovariectomized female rats were analyzed by formaldehyde agarose gels and transferred onto nitrocellulose filter membranes as described in Experimental. Each filter was hybridized to [^{32}P-α]dCTP-labeled cDNA. Results of 4 clones: E540, E558, E514 and E512 are shown here. The bottom panels represent hybridization results of the same filters to β-actin cDNA probe. Molecular size markers are represented in the RNA ladder obtained from BRL. Autoradiography for β-actin was 30 min and 24–72 h for cDNAs.

Table 1. A summary of Northern blot data: only E-induced genes are listed here

Clone no.	Identification	Estrogen induction
VMHE505/509	Unknown	+ + + +
VMHE518	Cytochrome bc-1 complex core P	+ + + +
VMHE552	Unknown	+ + + +
VMHE5103	Unknown	+ + + +
VMHE512	Unknown	+ + +
VMHE514	Na^+, K^+ ATPase B	+ + +
VMHE516	Unknown	+ + +
VMHE541	Unknown	+ + +
VMHE558	MRC OX45Ag deleted form	+ + +
VMHE569	Unknown	+ + +
VMHE583	Unknown	+ + +
VMHE593	Unknown	+ + +
VMHE501/543 /588/5105	GNRP	+ + +
VMHE506/540/549	ADP/ATP transport	+ +
VMHE513	16 Kda H(+)ATPase	+ +
VMHE536	Unknown	+ +
VMHE545	Unknown	+ +
VMHE567	Carbonic anhydrase	+ +
VMHE572	Unknown	+ +
VMHE592/594	Unknown	+ +
VMHE5130–3	C1–13 gene product, neuronal specific gene	+ +

Clones that do not match-up with sequences in the Genbank data base are listed as unknown. The level of induction is arbitrarily assigned as follows: 4+, induced from undetectable level; 3+, markedly induced (i.e. >3-fold); 2+, induced.

Identification of E-induced genes

cDNA inserts were isolated from plasmid DNA and were used to probe Northern blots of rat RNAs extracted from NT or from rats treated with E or E + P as described. We evaluated a total of 64 Northern blots (i.e. 64 cDNA clones) and found that 21 distinct mRNAs (from 28 clones) showed definitive increases in hybridization signal in the E lanes and are referred to as E-induced genes. This represented 43% (28/64) of the 64 cDNAs analyzed. Representative Northern blots and β-actin control blots are shown in Fig. 1. All four mRNAs showed clear induction in the E lanes. Control β-actin hybridization signals were uniform in all lanes and indicated that equal amounts of RNAs were loaded in each lane. The Northern blot data of all clones are summarized in Table 1. Of the induced mRNAs, 50% were induced 3-fold or greater relative to baseline levels in ovariectomized rats. Figure 2 contains the deoxynucleotide sequences obtained from the 5′ ends of members of the three classes of E-induced cDNA clones listed in Table 1.

DISCUSSION

The steroid hormones E and P cooperate in the induction of sexual behavior in the ovariectomized rat [1]. In order to define the molecular events involved in

I. +4 INDUCED GENES

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E509      1  tcgcgtgatc tcagcacgtg ggttcaattt acaatctttt caaaacatat
         51  tnntttncaaa tttacagaat gttgttaatg gaacaagaca aaacatatcc
        101  actttagttt atcaaaaata aaagaagatt taaaaagaag cacatttctc
        151  acaaatagta ttt

E508      1  tcgggatctc agcacgtggg ttcaatttac aaacttttca aaacatattc
         51  ctttcaaatt tacagaatgt tgttaatgga acaagacaaa acatatccac
        101  tttagtttat caaaaataaa agaacattta aaaaatcanc acatttctca
        151  caaatagtat ttattttggt tgttcaatta gtaatatcat aaggttacaa
        201  gtgaacatga tttgtatatt ttaatt

E518      1  caagaagtca atgacagcaa gtggaaactt gggacatacg ccttttcttg
         51  acgagttata acagatgcat gtatccaagt ctcagcccag accagcaaac
        101  acatcagagt caggtttcct aattattaat gttaattcaa catttttctc
        151  aatgaagaaa aaaacttaaa agtatagtcc tcctagctgc catagagcca
        201  ataaaacatt gtatgtaaat gtttttcttg tactcttcct aggcagtcaa
        251  caggtattta ttaaatgctt taaagaaaac agtaatagag taattaagtt
        301  cactaattac atcaattaag aatttcagaa tctgttaaaa tt

E552      1  tttttatgga tgataaatga atgttacttc taantactca ctccctttta
         51  tcaacagtta taacctcagt ccattgtgct acattagcgt gcctctttgt
        101  aataatgtgt agtatgacag ctagatgcct attgggttgt tatagaaggt
        151  acaaatttgt tnncntttna aagtaatttc agttttagga ctgggatttc
        201  acgtggtgtt agaactagag agaacagact ggttatatat acatatagga
        251  acgccatcta caatccttta taaagg

E5103     1  cactcccccc agtcaggacc tccccttgct tcctcagtcc agcgagcagg
         51  attcaggcta acgaaagctg cataccatcc tgagtacagg tgcctgcccc
        101  tgccacacac tagctaggaa agcctgtccc aaatcccagc gatccatt
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II. +3 INDUCED GENES

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E501/543/588/5105
          1  gaggttaaac ggcngcngta tcctgaccgt gcaaaggtag cataatactt
         51  gttccttaat tagggactag aatgaatggc taaacgaggg ttaactgtct
        101  cttactttca atcagtgaaa ttgaccttcc agtgaagagg ctggaatctc
        151  ccaataagac gagaagaccc tatggagctt taatttctag ttcaacttat
        201  ataaaaacaa cctaatgggc taaaacaaaa taaatatgaa ctaaaaaatt
        251  tc

E512      1  ctaaaacacc cctgcacagt ctccacgtac agcagatctt taaggagact
         51  gcccatgtct ccacacagag ctagtcatct gacccacaga gagaggacat
        101  agccctgagc ccccaaaaca taggaaagaa tgagagatgc tcctctgcca
        151  gcctggggag gaggagagca gacaggagta accactccct cccccagtaa
        201  ggtgactgtt atccgtcacc gtgttttaac cacacagagc tggagaggtg
        251  tgtgtaacc

E514      1  gcatggtact ttcccttcct ttctcatttt cttggctctg agatttcaaa
         51  ggtaacggcc catcaacaag catttttaac acactccata gtctttccct
        101  gtggtatcag gtctttacta ttgtttttct ttgtttcctg gggctggggg
        151  gtgggctgtc gtgggggaac tncccctta aattctaa

E516      1  aaataattag tttgggcatt ggtttgctac agtgagctca gatcaaatag
         51  ggaaatactt gaaatgtatg tctcagacca aggcaaactc cattcctcat
        101  attaaacnct ggctgttact tctcactacg tcaccatttt taactgacag
        151  gattagtaaa

E541      1  cagaacctac gaatacctag tgacactcca tacttgatac tgtagaactg
         51  tacagacaaa gcctaaagat gcctgctggt tccactgtca taaaaacggt
        101  cactttgttc tatggtaccc atctttatac agcagaagct tcaatt
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E558      1  ggaatgcatt tatctttata aaaaagagtg atataaacaa cattagtata
         51  atatagccac atgtttttgg taaaaggaat gcatttatct ttataaaaca
        101  gagtgatata aacaacatta gcatgacata gtcacatatt ttggtaaaag
        151  gaatgcattt atcttcacaa aaaggagtga tat

E569      1  ctttagtctc cagaacaggc tatgggatag atttttattt cagtttatgg
         51  ccacatnccc taactcactt gcagcttgca gtcttctgtc atttcac

E593      1  attttcacat gtccactaga agtcattaag acgagactgc agtcttcgag
         51  actagctctt cggacggtgt attatcctca ggttcatctg gggaccatta
        101  gtggagctgg aatggtgaga ccaacg

E583      Not Determined
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III. +2 INDUCED GENES

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E506/540/549
          1  caatgtcatc agatacttcc ccacccaggc tctcaacttt gccttcaaag
         51  ataaatacaa gcagatcttt ttgggtggtg tggacaagag gacccagttt
        101  tnncggtact ttgcagggaa cctggcatca ggt

E513      1  ctgggagcca gctctgagga actctctacc ctcccccggc tgttcagggc
         51  tgggggcagg gtggctccat ccaaggagtc tcagcaacag gaaccccaca
        101  aggcaacagt ctactaatga ttactataga gttgggagac agggtgct

E517      1  gatgaacaga gacacctgcc tggcatttca tctgggagcc aggcgacttc
         51  gcaggtgtca tccaacagag ggctccatct gccgggactg tactgtgggg
        101  tcagctccag atctgtgact gctcttggcc gctgactgaa gaggagacnn
        151  tgtcgaggag agtagctttt ccatctggac acaaaacaag ggccctttgt
        201  aggatttttct

E536      1  aaatgcagtt ctgtgtaaag cgatggcttg gcgggtagag gggtgtggct
         51  tacgtttctc tttggatttt aatgaaagat cgatgtggga gcagtttttg
        101  tttgcccttc accgccactt tccaatccgt atgtaccacc atccatttca
        151  gagcattcca gagctgcctg gcttctgttg agaagttaaa ggaacggg

E545      1  aaattggaag tgaggatatg tattgcccaa gggattaaaa aaaataaaaa
         51  gaaaagaaaa gaaaaaataa gaggatgtgt cccatcaggg agggagggg
        101  cggggagaat ccaaatagta tttttgtggg gaaatatcta atatacctte
        151  agtcaacttg accagtcagt cctggatttt agagat

E567      1  gaatgtntna cctggatagt gctcaaggaa cccattactg tcagcagtga
         51  gcagatgtat catttccgta aactgaactt aattcggagn nggannctga
        101  agaactgatg gt

E572      1  tgtacaaaaa gaaaactcta gacaatttta agctagcagc cgcttaacgc
         51  tgctttagtt catttaaatt ctttcccaaa aatacattcc taaatataca
        101  aactatacaa tcttgtcatt ttaatgctgg

E592/594  1  cgcagagggc tgtggctcgg acgctggcca aagaagttcc tagcctttcc
         51  ctttccccta aactgcatca gggaagaatc cttatcgcta gcttggtttt
        101  cacatgaggt tttctgagga aggggactgg gataagaagt ctgtcatgta
        151  gtaaagtaga caagaaatct tattaatcca gtt

E5130-3   1  cttggcttac aagacgccac atcacctatc atagaagaac ttacaaactt
         51  tcatgaccac accctaataa ttgtattcct catcagctcc ctagtacttt
        101  atattatttc actaatacta acaacaaaac taacacacac aagcacaata
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Fig. 2. Sequences of E-induced hypothalamus cDNAs. Only the sequences derived from the T3 primer (5′ end) are listed.

this complex behavioral response, it is important to understand the magnitude of the genetic response to E and to identify specific genes that may be either involved in the behavioral response or serve as gene markers for the response. In the course of a study designed originally to find P-induced genes, we discovered incidentally a surprisingly large number of E-induced genes (21 mRNAs).

The mammalian brain is a highly complex organ. Earlier estimates indicated that one third of the mammalian genome is devoted exclusively to its function [17–19]. According to an estimation by Milner and Sutcliffe [20], the brain expresses approx. 30,000 distinct mRNA species of an average length of 5000 nucleotides. Kinetic [21] and clonal [20] analyses found that between 40–65% of the mass of brain mRNA was brain specific. In other words, roughly 10,000 to 20,000 of the 30,000 brain mRNAs detected in the mammalian brain are specific for that organ. This remarkable complexity means that the bulk of brain mRNAs are rare or extremely low abundant mRNAs and most likely will escape hybridization to high R_0t values. Therefore, the strategy for this study was to use a combination of low R_0t hybridization and differential screening to help identify induced genes. The results using differential library screening to detect P-induced genes will be presented elsewhere. Theoretically, low R_0t hybridization removes abundantly expressed common mRNAs but not the low copy induced sequences. Thus, this is an enrichment procedure that may facilitate recognition of low copy induced sequences by promoting the lower abundancy mRNAs to a level of higher abundancy. This approach was employed to detect E-induced mRNAs. The fortuitous identification of a large number of E-induced genes appears to be the result of this enrichment effect.

Gonadal steroids, especially E, regulate many brain functions and E-concentrating cells are abundant throughout the brain [22–24]. Estrogen has been shown to up-regulate a variety of genes in the hypothalamus, including regulatory proteins such as the PR [4] and c-*fos* [25, 26], rate-limiting enzymes such as tyrosine hydroxylase [27], and neurotransmitter molecules such as neurotensin/neuromedin N [28], somatostatin [29], gonadotropin-releasing hormone (proGnRH-GAP [30]), preproenkephalin [31], and oxytocin receptor [32, 33]. Ironically, these cDNAs were not among the clones identified in hypothalamic tissue samples in the present study. Methodological differences in our approach may explain the different findings. Virtually all previous studies demonstrated mRNA regulation using preselected and targeted cDNAs or antibodies. Also, variation in experimental protocols such as surgery, time of tissue collection, and the amount of hormone treatment may have contributed to differences in the detected gene induction pattern. Finally, a large proportion of the above studies were performed using biochemical methods such as binding [32] and immunocytochemistry [26] or by *in situ* hybridization analyses [4, 27–29, 31]. None of these methods are ideal for measuring relative levels of mRNA expression in the whole hypothalamus.

Other studies that employed more quantitative methods such as Northern [29], dot-blot [31] and RNA protection assays [28], were performed on microdissected subregions of the hypothalamus. Even then, the magnitude of mRNA induction was found to be very small in many instances. For example, an E-induced increase of somatostatin mRNA in the preoptic area and the mediobasal hypothalamus, was detected by *in situ* hybridization studies [35]. However, Northern blot analysis of whole hypothalamus RNA showed only a 0.5-fold E-induced increase in somatostatin mRNA level [29] and required large amount of mRNA (10 μg, poly A+) for hybridization signal. Thus, mRNA induction in specific cell groups when readily revealed by *in situ* hybridization, may not be detected readily by Northern blot analyses due to tissue dilution effects. Consequently, some E-induced mRNAs may have escaped detection due to a tissue dilution effect and remain as very low abundant mRNAs even after enrichment by subtraction.

Morphologically, 30% of neurons in the VMN respond to E by showing an increase in size of cell nuclei, nucleoli and cell somatae [36]; an abundance of stacked RER and polyribosomes indicative of active metabolism are also observed after E administration [36, 40]. Gonadal steroids may induce synaptic remodeling in the adult rodent hypothalamus, since the number of synapses in the arcuate nucleus [35–37] and VMN [38] have been shown to increase with E treatment. A number of the E-induced mRNAs shown in Table 1 encode metabolic enzymes, and are also moderately abundant. Therefore, some of the E-induced genes in this report may be the product of a broad targeting effect of E, thus, providing some molecular basis for the observed morphological changes in the hypothalamus.

Although the present sequence information is limited to 5′ plus 3′-end sequences, it is interesting to note that the vast majority of clones reported herein do not represent known sequences in the Genbank. Considerable interest has been generated recently for obtaining expressed sequence tags, especially from the brain [40, 41]. This study represents the largest number of E-induced genes ever described for a single organ. In the rat uterus, which undergoes a marked growth response to E, only a few specific marker genes have been shown to be under estrogenic regulation. Our finding of a more global response to estrogen, casts a new light on the magnitude and complexity of the effect of this hormone in the rat hypothalamus. Finally, the newly identified cDNAs may serve as markers for E-induced central nervous system behavior and deserve further investigation into their physiologic roles. Some of these gene products could provide

additional insights into the molecular basis of steroid hormone action in sexual behavior, and also may be of use in studies of estrogenic responses in other organs and disease states.

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