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Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor

(cerebral cortex/olfactory bulb/estrogen replacement)

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ABSTRACT **We have been studying the role and mechanism of estrogen action in the survival and differentiation of neurons in the basal forebrain and its targets in the cerebral cortex, hippocampus, and olfactory bulb. Previous work has shown that estrogen-target neurons in these regions widely coexpress the mRNAs for the neurotrophin ligands and their receptors, suggesting a potential substrate for estrogen–neurotrophin interactions. Subsequent work indicated that estrogen regulates the expression of two neurotrophin receptor mRNAs in prototypic peripheral neural targets of nerve growth factor. We report herein that the gene encoding the neurotrophin brain-derived neurotrophic factor (BDNF) contains a sequence similar to the canonical estrogen response element found in estrogen-target genes. Gel shift and DNA footprinting assays indicate that estrogen receptor–ligand complexes bind to this sequence in the BDNF gene. *In vivo*, BDNF mRNA was rapidly up-regulated in the cerebral cortex and the olfactory bulb of ovariectomized animals exposed to estrogen. These data suggest that estrogen may regulate BDNF transcription, supporting our hypothesis that estrogen may be in a position to influence neurotrophin-mediated cell functioning, by increasing the availability of specific neurotrophins in forebrain neurons.**

Survival, differentiation, and maintenance of forebrain neurons are governed by several classes of local and target-derived neurotrophic factors. One critical class of growth and neurotrophic factors are members of the neurotrophin family of peptides. The neurotrophins, which include nerve growth factor, brain-derived neurotrophic factor (BDNF), and neurotrophin 3, are structurally and functionally related proteins, with distinct temporal and regional patterns of neural expression (1). While certain forebrain neurons respond to the neurotrophins in a ligand-specific manner, all three peptides appear to promote the survival and differentiation of basal forebrain cholinergic neurons *in vitro* (2, 3).

Among the neurotrophins, BDNF, which was first described in 1989 (4), influences several neuronal subpopulations during development and adulthood. BDNF stimulates cell proliferation in the cochleovestibular ganglion (5) and enhances the survival and phenotype expression of basal forebrain cholinergic neurons (3), cerebral cortical neurons (6), and dopaminergic mesencephalic neurons (7). BDNF has also been shown to play a protective role *in vivo*, after injury, in diverse neuronal populations such as the basal forebrain cholinergic neurons (8, 9), nigrostriatal dopamine neurons (10), facial (11) and spinal (12) motoneurons, and retinal ganglion cells (13). In view of the important role of BDNF in the development and maintenance of neuronal populations and its role after neural injury, compounds that can rapidly regulate BDNF mRNA expression are in a critical position to affect developmental/survival outcomes of neurotrophin targets.

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Previous work from this laboratory suggests that estrogen may be an important regulatory influence on the neurotrophin family. The gonadal steroid hormones, the estrogens and the androgens, influence the organization of the developing nervous system and activate adult behaviors related to reproduction, cognition, and aggression, as well as neuronal phenotypic expression (14, 15). The estrogen receptor, which mediates the biological response of the hormone, is a member of the superfamily of steroid/thyroid hormone/vitamin D_3/retinoic acid receptors capable of activating genes by directly binding hormone-specific DNA regulatory elements (16–18). Estrogen response elements (EREs) were first identified in the *Xenopus* (19) and chicken (20) vitellogenin genes, and later in oxytocin (21), prolactin (22), β-luteinizing hormone (23), and c-*fos* (24) genes, among other estrogen-responsive genes. In these instances, estrogen effects are probably mediated through direct activation of target genes.

However, estrogen regulates transcription of a vast array of cellular genes including structural proteins as well as steroids, peptides, neurotransmitters, and their receptors. It is possible that estrogen may not directly stimulate some of these steroid-sensitive genes but act, instead, through intermediate steps via interactions with, or secondary activation of, endogenous transcription-regulating growth factors (15). Estrogenic stimulation of a growth factor intermediary has been observed in extraneural targets of the steroid such as MCF-7 mammary tumor cells (25) and the uterus (26, 27) where estrogen regulation of epidermal growth factor, for example, stimulates, secondarily, cell proliferation and differentiation.

Both estrogen and the neurotrophins have been shown to promote survival and differentiation in their neuronal targets. Neurotrophin ligand and/or receptor expressing neurons of the developing rodent forebrain widely coexpress estrogen receptor systems (28, 29), providing a biological substrate for neural interaction between these classes of growth-promoting factors. In two previous studies, we explored the possibility of regulatory interactions between estrogen and the neurotrophin family by using two prototypical targets of neurotrophins, PC12 cells (30) and adult sensory (dorsal root ganglion) neurons (31). In both cases, estrogen treatment transiently decreased the expression of p75 mRNA, the pan-neurotrophin receptor, and increased mRNA expression for TrkA, the tyrosine kinase receptor that preferentially binds the neurotrophin NGF. By regulating neurotrophin receptor mRNA expression, estrogen may be in a position to regulate neurotrophin-dependent responsiveness in select neuronal groups.

Abbreviations: BDNF, brain-derived neurotrophic factor; ERE, estrogen response element; RT–PCR, reverse transcription–PCR.

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In this report we address a complementary mechanism by which estrogen may regulate neurotrophin-mediated cell functions. We have identified a motif in the BDNF gene that resembles an ERE and determined, by gel-shift and footprinting assays, that estrogen–ligand receptor complexes will bind to this motif. Moreover, estrogen rapidly increases BDNF mRNA expression in two forebrain targets of the cholinergic system, the cerebral cortex and the olfactory bulb. Thus these data suggest that the motif in the BDNF gene may be a functional ERE and that estrogen may regulate transcription of this gene. This finding suggests another mechanism by which estrogen may regulate neurotrophin-mediated cell functions, namely, by increasing the availability of neurotrophins.

MATERIALS AND METHODS

Gel Shift Assays. Synthetic oligodeoxynucleotides, corresponding to nt 49–89 of the rat BDNF gene (32), containing the 19-bp (nt 60–78) ERE-like sequence ($BDNF_{ere}$) and a 42-bp sequence containing the known ERE sequence in the vitellogenin gene (ref. 19; Vit_{ere}) were synthesized on an Applied Biosystems model 380B DNA Synthesizer (Protein Core, Columbia University/Howard Hughes Medical Institute). Sense-strand oligonucleotides were 5′-labeled with [^{32}P]ATP and then annealed with the reverse complementary (antisense) strand. Double-stranded sequences were then incubated with an estrogen-receptor-enriched nuclear protein extract from MCF-7 cells. Estrogen-receptor-containing nuclear protein extract was obtained from MCF-7 cells deprived of estrogen for 1 week and exposed to 2 μM diethylstilbestrol, a synthetic estrogen, 45 min before harvesting for nuclear protein, by using published protocols (33). In control experiments, tubes containing both $BDNF_{ere}$ and nuclear protein samples were exposed concurrently to 100-fold molar excess of unlabeled wild-type ERE (Vit_{ere}) or a nonspecific DNA fragment (Epstein–Barr nuclear antigen 1 consensus binding site), or 10 ng of a rat-specific estrogen receptor antibody (NIH-ER715). Samples were loaded on a 4% nondenaturing polyacrylamide gel and the dried gel was subsequently apposed to film.

DNA Footprinting Assay. Single (sense)-strand $BDNF_{ere}$ oligonucleotides were 5′-labeled with ^{32}P as before and incubated with the complementary strand. Essentially the same procedure was followed as for the gel-shift assay to visualize autoradiographically nuclear-protein-bound $BDNF_{ere}$. The band containing this complex was excised and eluted from the gel plug. Nuclear-bound $BDNF_{ere}$ and control $BDNF_{ere}$ (not incubated with nuclear protein extract) was incubated for 15 min at 37°C with DNase I (5, 25, 50, or 100 ng). The reaction mixture was later electrophoresed through an 8% polyacrylamide/8 M urea gel, and the cleaved product was visualized by film autoradiography.

Tissue Preparation. Cerebral cortex and olfactory bulbs were obtained from ovariectomized or estrogen-treated ovariectomized adult female rats. Bilaterally ovariectomized animals purchased from Zivic–Miller were housed for 10 days before the experiment. Animals were maintained in a 14-h light/10-h dark cycle and given food and water ad libitum. The estrogen-replaced group of animals were injected subcutaneously with 10 μg of estradiol benzoate in sesame oil (1000 hours, day 1) and sacrificed 4 h (1400 hours, day 1) or 52 h (1400 hours, day 3) later. Controls were injected with vehicle (sesame oil) only. Brain tissue was microdissected, frozen rapidly, and later processed for RNA. Blood, collected from the internal carotids, was briefly centrifuged, and the supernatant (plasma) was recovered and assayed for estradiol content by a solid-phase ^{125}I radioimmunoassay (Diagnostic Products, Los Angeles).

Reverse Transcription–Polymerase Chain Reaction (RT–PCR). Total RNA from the cerebral cortex and olfactory bulbs, prepared by the method of Chomczynski and Sacchi (34), was treated with DNase and assayed by RT–PCR for BDNF mRNA. Template cDNA formation was primed by reverse (antisense) primers specific for exon V of BDNF (2.5 μM) and cyclophilin (1.25 μM), at 42°C for 45 min, by using 1 μg of RNA. After heat denaturation of reverse transcription, template cDNA was amplified by the addition of gene-specific ^{32}P-labeled forward (sense) primers at a final concentration of 0.25 μM, with DNA polymerase (2.5 units/100 ml; AmpliTaq, Perkin–Elmer/Cetus). The PCR program, 95°C for 1 min, 55°C for 1 min, and 72°C for 4 min, was cycled 35 times, with a final 15-min extension step at 72°C. Amplified product was size-fractionated on a 5% (nondenaturing) polyacrylamide gel and the product was visualized by film autoradiography. By using a standard morphometrics package (Jandel, Corte Madera, CA), optical density measurements were obtained for bands corresponding to size-appropriate BDNF and cyclophilin products on x-ray film. The amplified BDNF product was normalized to concurrently reverse-transcribed and amplified cyclophilin mRNA. PCR products of BDNF exon V- and cyclophilin-specific primers were confirmed by sequence analyses. Control experiments, using the above PCR program with serial dilutions of RNA, indicated that the amplification of both BDNF and cyclophilin sequences was within the linear range (data not shown). In some experiments, samples were amplified without prior reverse transcription, to preclude the possibility of artifactual DNA amplification. No PCR product bands were seen in these experiments.

RESULTS

Presence of an ERE-Like Motif in the BDNF Gene. Motifs resembling the canonical ERE (GGTCANNNTGACC) were sought in the BDNF gene by using a computerized gene homology program (35). Only one ERE-like motif was observed in the currently known sequence for the BDNF gene, which consisted of a set of pentameric sequences with near perfect nucleotide homology (1-bp mismatch) (Fig. 1). The two pentamers, however, were separated by a nontraditional spacer size, in this case 9 nt. The BDNF gene consists of five exons and Fig. 2 indicates the location of the ERE-like motif within this gene. The motif lies at the 5′ end of exon V that codes for the mature BDNF peptide and, interestingly, includes the start codon for this exon.

Estrogen Receptor–Ligand Complexes Bind and Protect the BDNF ERE-Like Motif from DNase Cleavage. Mobility or gel shift assays were performed to test whether the 19-bp motif, corresponding to an ERE identified in the BDNF gene, would bind the estrogen–receptor complex. The migration of a DNA sequence containing the putative BDNF ERE sequence ($BDNF_{ere}$) was markedly altered when incubated with MCF-7 nuclear protein extract (Fig. 3*A*), suggesting that nuclear proteins bind to this DNA fragment. Oligonucleotide sequences containing the known vitellogenin ERE (Vit_{ere}) were similarly "shifted," indicating that the nuclear protein bound to this motif and to the $BDNF_{ere}$ may be the estrogen receptor. This interpretation is supported by the absence of a shifted band in the presence of excess unlabeled specific (Vit_{ere}) competitor, suggesting that the vitellogenin ERE sequence competitively binds the same nuclear proteins as the $BDNF_{ere}$.

Vitellogenin: GGTCANNNTGACC

BDNF: GGTGAGAAGAGTGATGACC

FIG. 1. Sequences matching the vitellogenin ERE were sought in the BDNF gene by using a sequence analysis program (35). The sequence in the BDNF gene consists of two pentamers (underlined), with close homology to the canonical ERE, with one mismatch (overlined). Unlike the vitellogenin ERE, the two pentamers in the motif from the BDNF gene are separated by 9 nt.

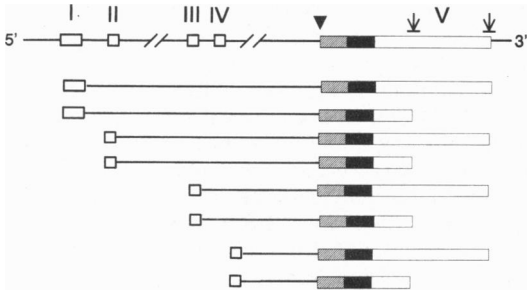

FIG. 2. Schematic representation of the BDNF gene. All BDNF transcripts contain exon V, which codes for the mature peptide (solid bar). Alternate transcripts are formed by the addition of different 5′ untranslated exons (I–IV) with two possible polyadenylylation sites (underlined arrows). Solid arrowhead indicates site of the putative ERE sequence described here, lying at the junction of intron IV and exon V. Underlined arrows indicate region of the peptide coding sequence amplified by primers in RT–PCR experiments. This figure was adapted from Timmusk *et al.* (36).

The addition of a rat-specific estrogen receptor antibody (NIH-ER715) to the reaction mixture results in a supershifted band, which supports the conclusion that estrogen receptor–ligand complexes within the nuclear protein extract bind to the DNA sequence containing the putative BDNF ERE.

Additionally, DNA footprinting assays indicate that the estrogen receptor-bound $BDNF_{ere}$ is resistant to DNase cleavage (Fig. 3*B*). While the unprotected $BDNF_{ere}$ sequence is completely cleaved by the DNase treatment, the protected (receptor bound) $BDNF_{ere}$ has a distinct banding pattern, marked by regions that are not cleaved. At the lowest concentration of DNase, two such regions are clearly visible, which correspond to the complementary palindromic ERE motifs. At higher concentrations of DNase, the lower of these regions appears to be cleaved, while the upper region remains protected.

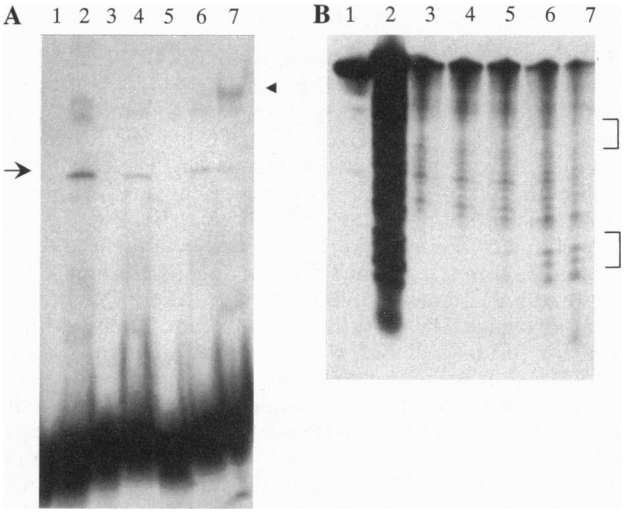

FIG. 3. (*A*) ^{32}P-labeled double-stranded synthetic oligodeoxynucleotides corresponding to nt 49–89 of the rat BDNF gene (32) containing an ERE-like sequence ($BDNF_{ere}$) or a 42-bp sequence containing the known ERE in the vitellogenin gene were incubated with estrogen-receptor-enriched nuclear protein extract from MCF-7 cells. Incubation with MCF-7-cell-derived nuclear protein extract retards the migration (indicated by arrow) of the Vit_{ere} (lane 1 vs. lane 2). A similar migratory shift is seen when MCF-7 nuclear extract is incubated with the $BDNF_{ere}$ (lane 3 vs. lane 4). Nuclear protein binding to the $BDNF_{ere}$ DNA sequence is displaced in the presence of a specific competitor (excess, unlabeled wild-type Vit_{ere}; lane 5) but is unchanged in the presence of a nonspecific competitor (Epstein–Barr nuclear antigen 1 DNA; lane 6). Addition of a rat-specific estrogen receptor antibody (ER715) to the incubation mixture results in a pronounced supershifted band (indicated by arrowhead; lane 7). (*B*) Control $BDNF_{ere}$ (not incubated with nuclear protein extract) was incubated with DNase I. Note that such unprotected $BDNF_{ere}$ is completely cleaved by 5 ng of DNase I treatment (compare lane 1 vs. lane 2), and fragments of various lengths are seen (lane 2). Lanes 3 and 4, containing nuclear protein-bound $BDNF_{ere}$, exhibit two protected regions (indicated by brackets on far right), which correspond to the pentameric homologues of the ERE. At increasing concentration of DNase I, cleaved bands are present in the lower region corresponding to the noncanonical (GGTGA) pentamer (lanes 5–7).

Estrogen Rapidly Up-Regulates BDNF mRNA. Estrogen regulation of BDNF mRNA was detected by semiquantitative RT–PCR analysis with primers (37) designed to amplify a 161-bp region 3′ to the putative ERE. Cerebral cortical and olfactory bulb RNA was obtained from estrogen- and vehicle-treated ovariectomized animals. Earlier studies (30) established that plasma estradiol levels in oil-injected ovariectomized controls were virtually undetectable and are comparable to those seen at proestrus (highest endogenous estrogen levels) when measured 4 h after a single injection of estradiol. At 52 h, plasma estradiol levels decreased by almost 60% of the levels seen at 4 h (30).

Although BDNF mRNA was expressed in ovariectomized animals, its expression was significantly increased in both the cerebral cortex and the olfactory bulb after estrogen treatment (Fig. 4). Estrogen treatment led to a 2- to 2.5-fold increase in BDNF mRNA expression as early as 4 h after a single injection of estrogen (when plasma estradiol levels are in the physiologic range), suggesting a fairly rapid up-regulation of BDNF mRNA in response to estrogen. At 52 h, when plasma hormone levels were low, expression of BDNF mRNA was not significantly different from ovariectomized oil-treated controls.

DISCUSSION

The BDNF gene contains a sequence with close homology to the canonical ERE and our data indicate that estrogen ligand–receptor complexes will bind to this sequence. Moreover, *in vivo*, estrogen rapidly up-regulates mRNA expression of this neurotrophin. These findings strengthen the hypothesis that estrogen may be in a position to increase the availability of this neurotrophin by regulating gene transcription. Our findings also suggest a mechanism by which the two classes of growth-promoting factors may interact to promote cell function in mature and developing animals.

The sequence corresponding to an ERE-like motif in the BDNF gene differs from the canonical ERE principally with respect to the spacer size between the two pentameric palindrome sequences. The canonical ERE, which consists of palindromic pentamers separated by a spacer typically 3 bp long, were first identified in the *Xenopus* (19) and chicken (20) vitellogenin genes and later confirmed (21–24) in other estrogen responsive genes. However, a recent study on the promoter region of the salmon gonadotropin-releasing hormone gene suggests that estrogen receptors will bind ERE-like motifs with an 8- or 9-bp spacer and protect these from DNase cleavage (38). Additionally, pentamers separated as much as 100 bp (39) have also been shown to function as effective EREs. Apart from the noncanonical spacer size, there is also a single base-pair mismatch between the BDNF ERE-like motif and the vitellogenin ERE. Such variations from the canonical pentamers have been seen in other genes as well, notably the ERE identified in the oxytocin gene. The oxytocin ERE has a base mismatch and substitution match similar to the BDNF ERE-like motif, and this variant appears to retain the ability to promote estrogen-dependent transcription in heterologous reporter systems (21).

Our data suggest that mismatches in the canonical pentamer may decrease the binding efficiency of estrogen receptor–ligand complexes. The footprinting assay indicated that while both pentamers are protected from DNase cleavage when

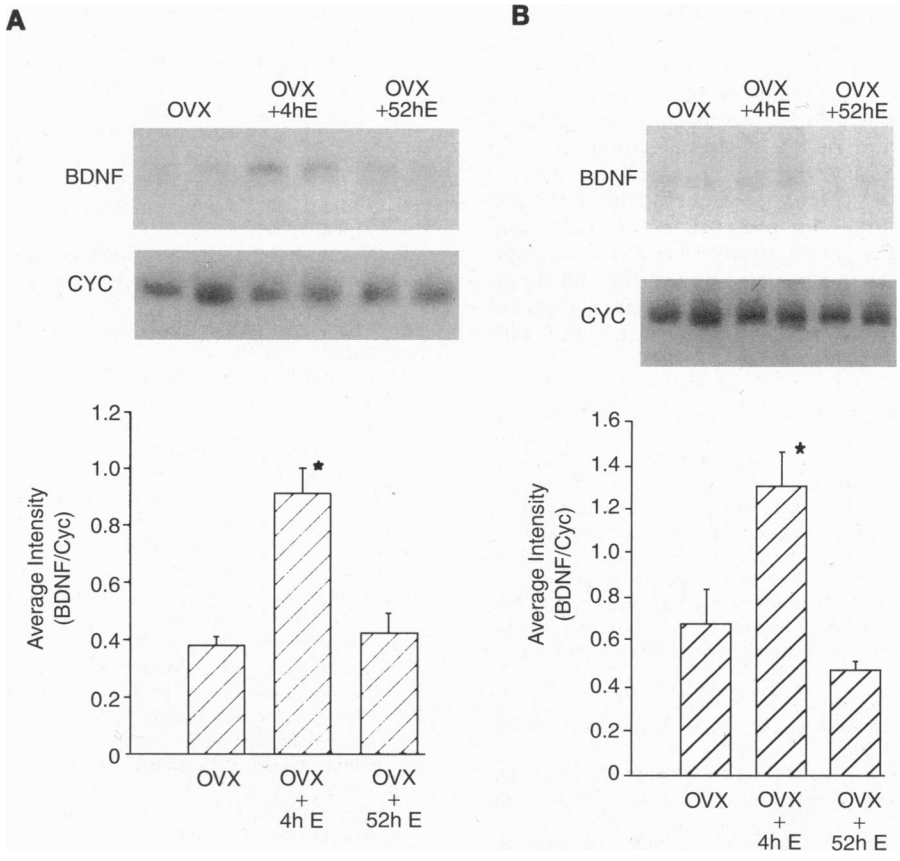

FIG. 4. Total RNA from cerebral cortex (*A*) and olfactory bulb (*B*) was reverse-transcribed and amplified by using primers specific to exon V of the BDNF gene and to the cyclophilin gene. The RT–PCR product of RNA derived from vehicle-treated ovariectomized animals and from ovariectomized animals sacrificed 4 h and 52 h after a single injection of estradiol benzoate was size-fractionated on a polyacrylamide gel. To quantify group differences, optical density of the size-appropriate BDNF product was normalized to that of the cyclophilin product, to control for variations in the amount of total RNA. (*Upper*) BDNF and cyclophilin PCR products from two representative animals from each treatment condition. (*Lower*) Bar graph represents the mean ± SEM of three or four animals from each group. *, Statistically significant ($P < 0.05$) differences compared to the vehicle-treated ovariectomized group. OVX, ovariectomized; E, injection of estradiol benzoate; Cyc, cyclophilin.

bound by estrogen ligand–receptor complexes, the pentamer with a single base-pair mismatch (GGTGA) is less resistant to cleavage at higher DNase concentrations. The poor association of estrogen receptor–ligand complexes with the noncanonical pentamer may explain why this pentamer is cleaved at higher concentrations of DNase and is also consistent with another reported observation that sequentially greater mismatches (from the canonical pentamer) appear not to promote transcription as efficiently (21).

The BDNF gene consists of five exons, and alternate transcripts of mature BDNF mRNA are formed by exon rearrangement and alternate polyadenylylation sites (36, 40). The putative ERE sequence, described here, lies on the 5′ end of exon V of this gene. Individual promoters have been identified for exons I–IV, but not exon V, which codes for the mature BDNF peptide (36, 41). It may be the case that estrogen receptor–ligand complexes, bound to the putative ERE in exon V, collaborate with one or another upstream promoters to regulate transcription. The putative BDNF ERE described here includes the start codon (ATG) for exon V (Fig. 2), and nuclear proteins bound to the start ATG may obscure and perhaps down-regulate gene transcription. However, *in vivo* estrogen treatment clearly up-regulates BDNF mRNA, consistent with the fact that functional EREs identified to date, without exception, appear to enhance transcription of target genes by the gonadal hormone.

Estrogen rapidly and transiently up-regulated the expression of BDNF mRNA in the cerebral cortex and the olfactory bulb. At 4 h after a single injection of estrogen, BDNF mRNA expression was doubled in both forebrain regions and was no different from vehicle-treated controls at 52 h after estrogen treatment. Singh *et al.* (42) have shown that estrogen deficiency (bilateral ovariectomy) for 28 weeks significantly decreased BDNF mRNA, while estrogen replacement over the same period of time protected against this loss of neurotrophin mRNA in specific regions of the cerebral cortex and the hippocampus. In view of the long duration of hormone exposure in this paradigm, however, estrogen action on BDNF mRNA expression need not have resulted from direct activation of the gene. Results from our study provide evidence that estrogen may directly activate this gene. In fact estrogen regulation of BDNF in the cerebral cortex and olfactory bulb occurs within a time frame consistent with the class of early oncogenes. Other studies have also reported that BDNF mRNA can be induced in the brain after hypoxia–ischemia (43) and focal injury (44) as rapidly as immediate early genes such as c-*fos*, c-*jun*, and *junB*. While our studies do not necessarily address the issue of BDNF as an immediate early gene, the rapidity of BDNF mRNA regulation by estrogen is consistent with the observation that estrogen receptor–ligand complexes may directly bind the BDNF gene.

Recent studies have attributed an increasingly important role for BDNF in the development and maintenance of neuronal populations and in the protection of neurons after neural injury. Consequently, compounds that can rapidly regulate the availability of BDNF by stimulating transcription of this gene may crucially influence outcomes related to neural organization and postinjury survival. The data presented here

certainly suggest that estrogen is in a position to initiate such a neurotrophin-mediated cascade of cell regulatory events. Such actions may be important not only during development, when BDNF-sensitive neuronal populations are in the process of differentiating, but also during normal and pathological aging, where the survival of these subpopulations may be affected. In Alzheimer disease, for example, BDNF mRNA is markedly reduced in the cerebral cortex and hippocampus (45). Our data, indicating the presence of an ERE and estrogen's ability to enhance transcription of BDNF, may begin to explain the molecular mechanisms underlying the therapeutic actions of estrogen in the increasing number of recent studies using estrogen replacement therapy in patients with Alzheimer disease (46, 47).

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