

Novel Mechanisms of Estrogen Action in the Brain: New Players in an Old Story

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Estrogen elicits a selective enhancement of the growth and differentiation of axons and dendrites (neurites) in the developing brain. Widespread colocalization of estrogen and neurotrophin receptors (*trk*) within estrogen and neurotrophin targets, including neurons of the cerebral cortex, sensory ganglia, and PC12 cells, has been shown to result in differential and reciprocal transcriptional regulation of these receptors by their ligands. In addition, estrogen and neurotrophin receptor coexpression leads to convergence or cross-coupling of their signaling pathways, particularly at the level of the mitogen-activated protein (MAP) kinase cascade. 17β -Estradiol elicits rapid (within 5–15 min) and sustained (at least 2 h) tyrosine phosphorylation and activation of the MAP kinases, extracellular-signal regulated kinase (ERK)1, and ERK2, which is successfully inhibited by the MAP kinase/ERK kinase 1 inhibitor PD98059, but not by the estrogen receptor (ER) antagonist ICI 182,780 and also does not appear to result from estradiol-induced activation of *trk*. Furthermore, the ability of estradiol to phosphorylate ERK persists even in ER- α knockout mice, implicating other estrogen receptors such as ER- β in these actions of estradiol. The existence of an estrogen receptor-containing, multimeric complex consisting of hsp90, *src*, and B-Raf also suggests a direct link between the estrogen receptor and the MAP kinase signaling cascade. Collectively, these novel findings, coupled with our growing understanding of additional signaling substrates utilized by estrogen, provide alternative mechanisms for estrogen action in the developing brain which could explain not only some of the very rapid effects of estrogen, but also the ability of estrogen and neurotrophins to regulate the same broad array of cytoskeletal and growth-associated genes involved in neurite growth and differentiation. This review expands the usually restrictive view of estrogen action in the brain beyond the confines of sexual differentiation and reproductive neuroendocrine function. It considers the much broader question of estrogen as a neural growth factor with important influences on the development, survival, plasticity, regeneration, and aging of the mammalian brain and supports the view that the estrogen receptor is not only a ligand-induced transcriptional enhancer but also a mediator of rapid, nongenomic events. **KEY WORDS:** estradiol; estrogen receptor; neurotrophins; neurotrophin receptors, signal transduction; cross-coupling; brain. © 1999 Academic Press

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INTRODUCTION

The cellular and synaptic organization of the central nervous system (CNS)¹ is determined not only by intrinsic, genetic regulation, but by a variety of extrinsic, environmental, or epigenetic influences that operate during development. Hormones, neurotransmitters, neuropeptides, and a host of growth and trophic factors of both neural and extraneural origin are among those influences that appear to act as developmental signals. These molecules have been implicated in cell replication, in neuronal survival, and in such aspects of neuronal differentiation as neurite growth and arborization, synapse formation, and neurotransmitter choice. The development and organization of neural circuits controlling a broad spectrum of sexually differentiated neuroendocrine, behavioral, and cognitive functions in both sexes is permanently influenced by sex-specific, differential exposure of the developing CNS to gonadal steroids such as the estrogens and androgens (49, 62, 128). The importance of the gonadal steroid hormones for sexual differentiation of the CNS is a good example of epigenetic modulation of neural development.

Only recently, however, have studies of gonadal steroid action on the developing mammalian brain focused beyond their roles in the neuroendocrine control of reproductive behaviors and functions. This review expands the usually restrictive view of estrogen action in the developing brain beyond the confines of sexual differentiation and considers estrogen as a neural growth factor with properties and actions similar to the neurotrophin family of growth and trophic peptides, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (NT-4/5).

ESTROGEN

Estrogen has traditionally been referred to as the “female” hormone whose principal source is the ovary and consists principally of three forms: 17 β -estradiol, estrone, and estriol. However, the male also produces estrogen. In fact, while estrogen levels in the male are quantitatively lower than those in the female, many developmental actions of testosterone in the brain of *both* sexes depend upon initial intraneuronal conversion by the cytochrome P450 enzyme aromatase to the estrogenic metabolite 17 β -estradiol. 17 β -Estradiol subsequently binds to high-affinity estrogen receptors within neurons of fore-brain regions such as the basal forebrain, hypothalamus, preoptic area (POA), cerebral cortex, hippocampus, and amygdala (62) to elicit its various effects.

¹ Abbreviations used: ER, estrogen receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; MEK, MAP kinase/ERK kinase; ERKO, estrogen receptor- α knockout; p75^{NTR}, p75 neurotrophin receptor; CNS, central nervous system; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; NT-4/5, neurotrophin-4/5; POA, preoptic area; ERE, estrogen response element; MAP-2, microtubule-associated protein-2; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PKC, protein kinase C; CREB, cAMP response element binding; pCREB, phospho-CREB.

Estrogen has been shown to exhibit growth- or neurite-promoting properties for its target neurons within the developing forebrain. Toran-Allerand *et al.* (123, 124, 128, 132) first demonstrated that estrogen elicits the selective enhancement of neurite growth and differentiation (both axons and dendrites), using cultured slices of the developing rodent hypothalamus, POA, and cerebral cortex (Figs. 1A and 1B). This growth-promoting property of estrogen has been confirmed subsequently in other culture systems (30, 76), in the developing rodent brain (39, 61, 77, 119), in fetal brain transplanted into the eye and brain of adult hosts (65, 78), and in steroid target regions of the steroid-deprived, axotomized, or deafferented adult brain (35, 64). Estrogen stimulation of neurite growth is developmentally regulated and is not seen in the normal adult female brain. However, as a result of the loss of trophic support, whether through estrogen deprivation (ovariectomy), axotomy, or deafferentation, responsiveness to estrogen returns, and estrogen can again be shown to influence the growth and differentiation of neurite-derived structures such as axons, dendrites, dendritic spines, and synapses (35, 64).

THE ESTROGEN RECEPTOR

The classical estrogen receptor has been considered to be a ligand-induced transcriptional enhancer. The regulation of gene expression by steroid hormones such as estrogen is mediated through receptor proteins that dimerize to bind DNA sequences (28) and then interact with components of the transcriptional machinery to initiate transcription (53). There are now at least two estrogen receptor genes, coding for the "classical" receptor, now termed ER- α (67 kDa) and the more recently discovered ER- β (~54 kDa) (52, 75, 79). These estrogen receptors have a highly conserved DNA binding domain (95%), but differ with respect to their ligand binding domain (58% homology) and bear virtually no homology within the N-terminal transactivation domain (51, 52, 75). These receptors also differ in their binding affinities, ligand specificities (51), and tissue distribution (51, 105).

While the temporal and spatial distribution of ER- α protein is relatively well described, the distribution of ER- β protein remains unclear due to the lack of specific antibodies. Preliminary studies suggest considerable overlap in expression of ER- α and ER- β mRNA in various regions of the developing and adult forebrain such as the cerebral cortex and hippocampus (23, 57, 84, 105). In light of this overlap in mRNA expression for these two receptors, it is important to determine what specific functions can be ascribed to each receptor subtype. For example, it is not clear whether the "estrogen receptor" that mediates the growth-promoting properties of estrogen in the developing brain is the classical receptor ER- α , the ER- β receptor, or another, as yet unidentified, receptor subtype. This issue is further complicated by the observation that ER- α and ER- β can exist and act not only as homodimers but also as heterodimers (74, 79, 91), suggesting a functional interaction between ER- α and ER- β .

The classical mechanism of estrogen action inadequately explains the com-

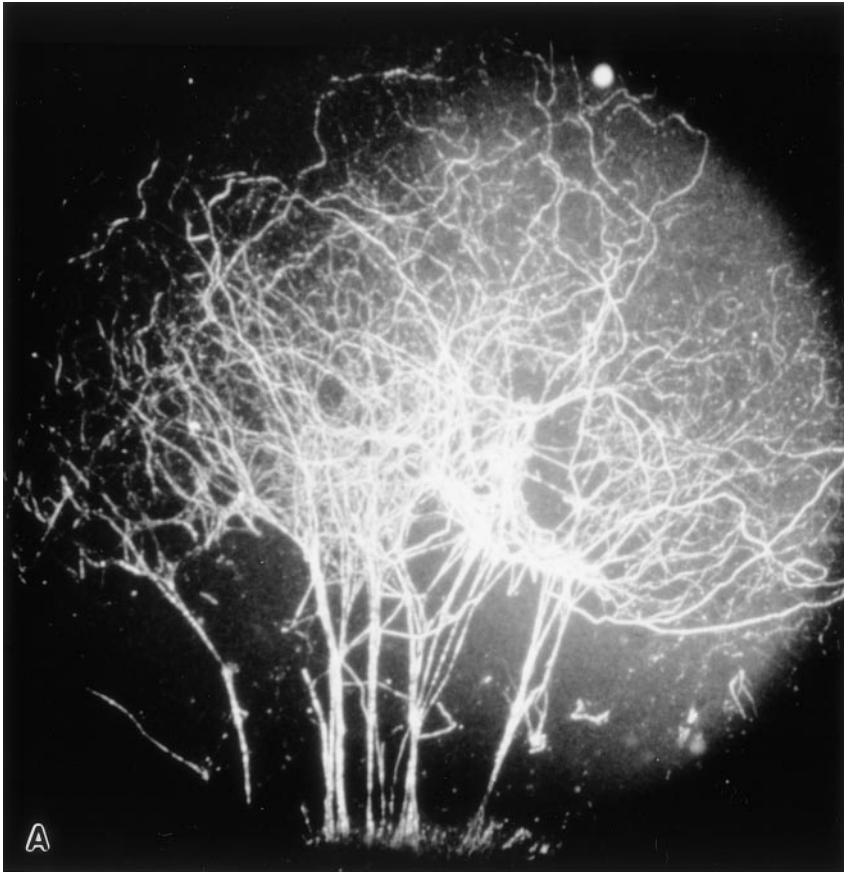


FIG. 1. (A and B) Neurite-promoting effects of estradiol in hypothalamic explant cultures, 19 days *in vitro*. Photomicrographs of right and left homologous coronal halves of a Holmes' silver-impregnated pair of explants from the preoptic area. Darkfield microscopy, $\times 125$. (A). Control exposed only to estrogens endogenous to the horse serum component of the nutrient medium. The silver-impregnated neurofibrils (bundles of neuron-specific, neurofilament proteins) course outward from the margin of the explant. (B). Exogenous 17β -estradiol. There is a significant enhancement of neurite growth from the same region of the homologous explant half, with extensive arborization of neurites in the outgrowth. (Reproduced, by permission of the publisher, from Ref. 124.)

plete and extensive range of estrogen's actions, including the ability of estrogen to regulate genes that do not exhibit an apparent canonical estrogen response element (ERE) (120) and the very rapid (seconds to minutes) effects of estrogen (18, 33, 69). While these "unconventional" effects of estrogen appear inconsistent with transcriptional modulation via an intranuclear estrogen receptor, they could be explained by the existence of various estrogen receptor subtypes.

While the classical estrogen receptor is thought to be largely intranuclear, estrogen binding proteins have also been described within the plasma membrane (3, 89, 92, 134). However, it still remains unclear whether membrane

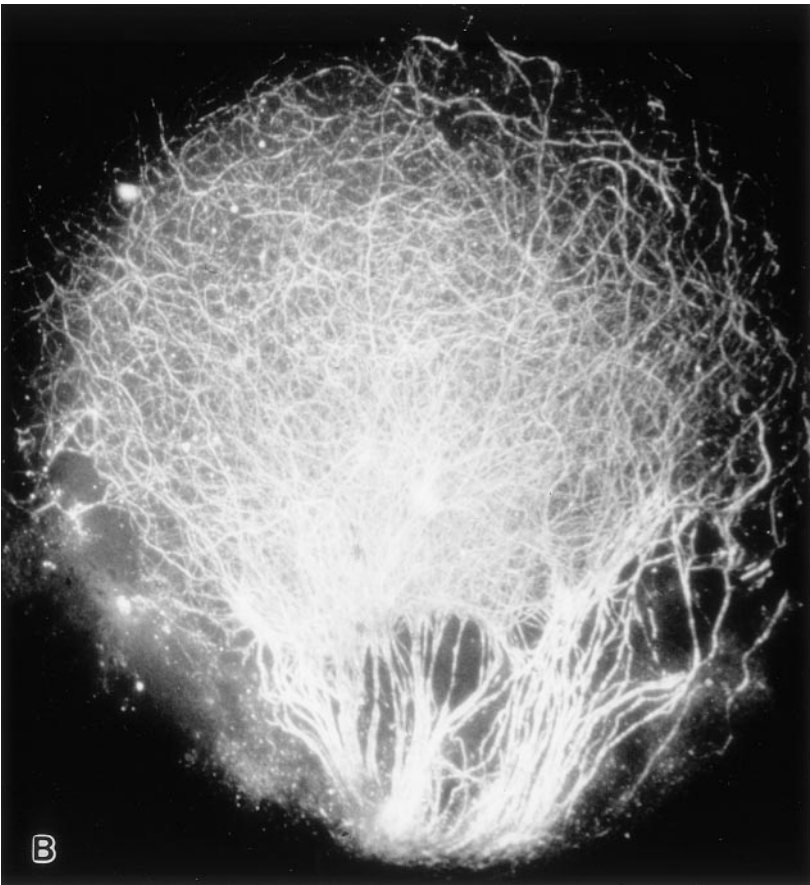


FIG 1—*Continued*

estrogen receptors exist as a subpopulation of already identified estrogen receptor subtypes or represents a novel member of the estrogen receptor family. Some studies (89) suggest that there is a strong structural similarity between the classical (nuclear) estrogen receptor and the putative membrane-associated receptor. Other studies (3), however, suggest that the unliganded membrane estrogen receptor may be a unique membrane-associated estrogen binding site whose primary structure differs from the nuclear estrogen receptor and exhibits tyrosine kinase activity. It is also conceivable that the brain may possess additional subtypes of the estrogen receptor not present in other tissues. In fact, Asaithambi and colleagues (6) have documented a high molecular weight estrogen receptor of 112 kDa in adult murine cerebral cortex that was not found in uterine tissue. Cross-coupling of one or more of these estrogen receptors with growth factor signaling cascades could enable much more rapid effects than might be expected via the classical “genomic mechanism” of estrogen action. In this regard, increasing evidence documents that the estrogen receptor can

mediate extracellular signals in both an estrogen-dependent and estrogen-independent manner, through growth factor signaling pathways (81, 93).

The classical estrogen receptor (ER- α) is a phosphoprotein that becomes hyperphosphorylated by its ligand, estrogen (50, 126). Receptor/ligand interactions trigger a cascade of events including dissociation from heat shock proteins (hsp90 in particular), phosphorylation on tyrosine and serine residues, and dimerization of the estrogen receptor (4, 7, 8, 53, 54, 83). These multiple steps result in the association of the hormone-activated receptor with specific regulatory sequences (EREs) in target genes. Furthermore, phosphorylation is a requisite for estrogen receptor activation and function. The estrogen receptor is the only member of the steroid hormone receptor family that becomes phosphorylated on both tyrosine and serine residues (50, 83). The other steroid receptors are phosphorylated almost exclusively on serine-threonine residues (83). Although the specific function of tyrosine phosphorylation remains unclear, ligand-induced tyrosine phosphorylation makes the estrogen receptor similar to growth factor receptor tyrosine kinases. It is tempting to speculate that the ability of estrogen to elicit tyrosine phosphorylation of its own receptor may relate to the growth- or neurite-promoting actions of estrogen, a property which is characteristic of many growth factors.

ESTROGEN AND NEUROTROPHIN RECEPTOR COEXPRESSION

The neurotrophins are a family of growth factors which, like estrogen, have important influences on the development, survival, plasticity, and aging of neurons in the mammalian forebrain regions that subservise reproductive, cognitive, and various other functions. The biological activities of the neurotrophins are mediated by two structurally distinct classes of cell membrane receptors which are preferentially expressed in neural tissues (16, 17, 95). One class consists of members of the tropomyosin-related kinase (*trk*) family of receptor tyrosine kinases (*trkA*, *trkB*, and *trkC*), each of which mediates neurotrophin signaling through increased tyrosine autophosphorylation of its cognate receptor. NGF binds *trkA*; BDNF and NT-4/5 bind to *trkB*; and NT-3 binds primarily to *trkC*. The second receptor, p75^{NTR}, is a pan-neurotrophin receptor which binds all neurotrophins with low affinity. It is a 75-kDa transmembrane protein which appears to have a modulatory role on *trk* activity and function. p75^{NTR} is related to the tumor necrosis factor/Fas/CD40 receptor family and, when overexpressed or stimulated in the absence of *trk*, has been implicated in the regulation of neuronal apoptosis, the activation of NF κ B, and the production of the ICE-like protease ceramide (14, 17). However, the *trks* can mediate responses to the neurotrophins with or without the participation of p75^{NTR}. Neurons in forebrain regions of both sexes coexpress estrogen and neurotrophin receptors and are also the sites of estrogen and neurotrophin synthesis (72, 127) (Figs. 2A and 2B).

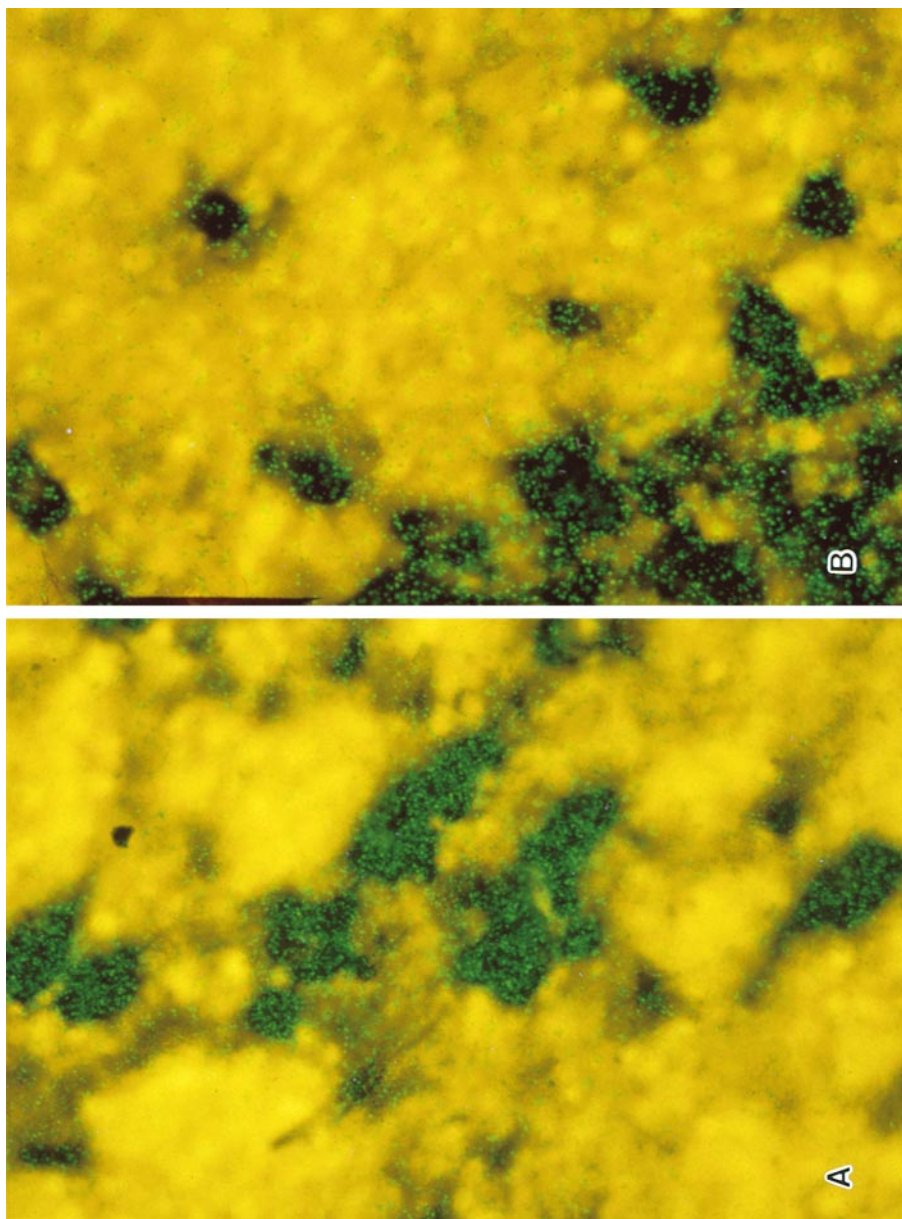


FIG. 2. Colocalization of estrogen receptors with the neurotrophins and their receptors in mouse forebrain by double label in situ hybridization. Nonsitopic blue, digoxigenin-labeled estrogen receptor mRNA, colocalized (A) with isotopically labeled ^{35}S -NGF mRNA, indicated by the green epipolarized silver grains, and (B) with isotopically labeled trkA mRNA, the cognate receptor.

STEROID/NEUROTROPHIN INTERACTIONS

An important question raised by the coexpression of estrogen and neurotrophin receptor systems is whether their ligands interact. Are the developmental actions of estrogen on neurite growth mediated either directly or indirectly, by means of autocrine responses or local paracrine mechanisms (128, 131)? Estrogen and the neurotrophins may influence each other's actions by regulating receptor and/or ligand availability through reciprocal regulation at the level of gene transcription. In this regard, documentation of the presence of estrogen binding sites in both adult female rat sensory neurons *in vivo* (117) and the pheochromocytoma cell line PC12 (116) was associated with differential and reciprocal regulation of estrogen and NGF receptor mRNAs by their ligands. Whereas all sensory neurons coexpressed estrogen receptor, p75^{NTR}, and *trkA* mRNAs, their levels appeared to be influenced differentially by the estrogen state of the animal. As shown by *in situ* hybridization, estrogen receptor mRNA expression was highest in the estrogen-deficient, ovariectomized animal. It was significantly lower during proestrus (117), when estrogen levels are high (115). Estrogen down-regulation of its receptor in sensory neurons is consistent with similar observations in the adult uterus and brain (104, 106). In contrast, at proestrus, high levels of *trkA* mRNA were expressed; while ovariectomy decreased *trkA* mRNA expression (117).

In undifferentiated PC12 cells, NGF was found to increase both estrogen receptor mRNA and estrogen binding significantly (116). Conversely, estrogen elicited a persistent *increase* in *trkA* mRNA expression while transiently *decreasing* p75^{NTR} mRNA in both sensory neurons and PC12 cells. Furthermore, *trkA* mRNA levels were significantly higher when estrogen and NGF were added concurrently than when NGF was added alone (125). The presence of putative EREs in both p75^{NTR} and *trkA* (72) suggests that estrogen may regulate transcription of these genes directly. One can only speculate on the biological and functional significance of differential estrogen regulation of *trkA* and p75^{NTR} mRNAs, since the precise contribution of each receptor is not clearly known. However, the apparent importance of the p75^{NTR}/*trkA* ratio for ligand-dependent autophosphorylation, neurotrophin binding affinities, and ligand specificities has been reported in PC12 cells and cortical neurons (9, 12, 41). By altering their ratios, differential regulation by estrogen might fine tune responses to NGF. Moreover, since p75^{NTR} has recently been implicated in the regulation of neuronal apoptotic death (14), estrogen down-regulation of p75^{NTR} could even contribute to the mechanisms underlying the neuroprotective effects of estrogen.

In organotypic explants of the cerebral cortex, NGF has also been shown to increase estrogen binding sites (71). When explants of the cerebral cortex were exposed to NGF for 8 days in the absence of estrogen, there was a statistically significant increase in the levels of nuclear estrogen binding, which would increase neuronal responsiveness to estrogen. However, there was no corresponding increase in ER- α mRNA, suggesting that NGF's actions on the unliganded estrogen receptor may have been due to a posttranslational mecha-

nism such as phosphorylation. This is a strong possibility, since preliminary studies (126) suggest that the cortical estrogen receptor not only becomes rapidly phosphorylated on tyrosine residues by estrogen, but by the neurotrophins as well. Thus, by altering the responsiveness of estrogen and neurotrophin targets of the CNS to their ligands, these interactions could also promote the growth and differentiation of specific neuromodulatory or neurotransmitter systems, leading to shifts in the developmental patterns of the resulting neural networks.

Interactions of estrogen and the neurotrophins may also be involved in the currently unknown mechanisms that underlie the differential regulation of the estrogen receptor by its ligand in the brain. The direction of the responses of the neural estrogen receptor to estrogen appears to be developmental-stage dependent. Thus, although estrogen classically down-regulates ER- α in the adult brain (104, 106), a considerable degree of ER- α mRNA expression is seen in the developing postnatal brain until around postnatal day 28 (73). This occurs despite levels of estrogen normally sufficient for receptor down-regulation in the adult. One interpretation of such regulatory patterns would be that, during the early postnatal period, estrogen up-regulates its receptor (ER- α) or, alternatively, that estrogen is unable to regulate its own receptor (nonregulation) (130). One might further speculate that developmental stage-dependent differences in the direction of estrogen regulation of its own receptor may perhaps be a consequence of interactions with other transcription-regulating molecules, including the neurotrophins. To explain this discrepancy between the responses of the estrogen receptor (at least of ER- α) during development and in the adult, it has been proposed (129) (Fig. 3) that the neurotrophins may serve as regulatory switches. During CNS development, the ability of neurotrophins (either alone or in synergy with estrogen) to significantly increase estrogen receptor binding (71) may be sufficient to influence or override the intrinsic suppressive action of estrogen on its own receptor. Maturation of the CNS, which is associated with significant alterations in the spatial, temporal, and functional expression of the neurotrophins and their receptors, as well as in their physiological role(s) (55), may serve to "free" the estrogen receptor from the regulatory influences of the neurotrophins. However, following axotomy, deafferentation or estrogen deprivation (e.g., ovariectomy, menopause), there may be a "switch" back toward the developmental pattern of estrogen receptor regulation which is manifested by a reexpression of the growth-promoting properties of estrogen not normally seen in the adult, possibly through an increase in neurotrophin ligand and receptor expression (34, 67).

CROSS-COUPLING OF SIGNALING PATHWAYS

Another possible consequence of estrogen and neurotrophin receptor coexpression is the sharing of similar, if not overlapping, sequences of intracellular biochemical events, through convergence or *cross-coupling* of their signaling pathways (101). Cross-coupling of converging estrogen and neurotrophin signal-

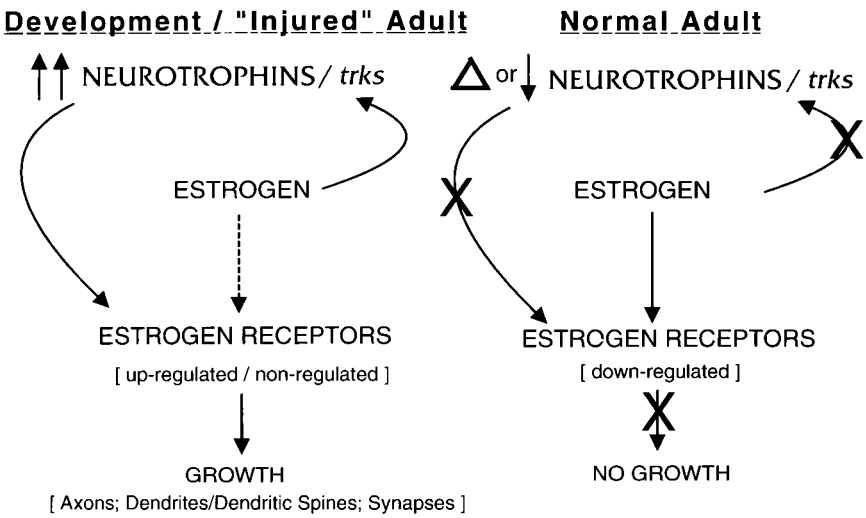


FIG. 3. Neurotrophins as regulatory switches. During development, the ability of NGF, either alone or in synergy with estrogen, to increase estrogen receptor levels may be sufficient to influence or override the intrinsic suppressive action of estrogen on its own receptor. On the other hand, maturation of the CNS is accompanied by alterations in the spatial, temporal, and functional expression of the neurotrophins and their receptors, as well as by age-related changes in the physiological role(s) of the neurotrophins. Such changes may serve to free the estrogen receptor from the regulatory or modifying influences of the neurotrophins, resulting in the emergence of the intrinsic pattern of receptor down-regulation, accompanied by the loss of estrogen's neurite-promoting effects. However, in adult steroid target regions, following injury, such as axotomy or deafferentation, or in the presence of steroid deficiency, there appears to be an apparent "switch" in the direction of estrogen regulation of its receptor back to the developmental pattern that is manifested by a reexpression of the growth-promoting properties of estrogen on axons, dendrites, and synapses which is not seen in the intact adult normally exposed to estrogen. Enhanced neurotrophin sensitivity in the injured or steroid-deficient adult brain may result in a return of neurotrophin modulation of the estrogen receptor and the return of estrogen enhancement of neurite growth. (Redrawn from Ref. 129.)

ing pathways may lead to similar nuclear endpoints which result in the regulation of the same broad array of genes involved in neurite growth and differentiation. In fact, there is a growing body of evidence in both neurons and nonneuronal tissue concerning the interactions between estrogen and peptide growth factor signaling. In neural and nonneural tumor cell lines, estrogen has been shown to elicit very rapid and transient responses, ranging from seconds to minutes (68, 69). These responses are similar to those evoked by mitogenic peptide growth factors such as EGF, IGF-1, and insulin. The time course for these responses is inconsistent with transcriptional modulation and has been postulated to result from the sharing of estrogen and growth factor signaling pathways for cell proliferation and estrogen receptor phosphorylation (15, 42, 43, 46, 47, 68, 69, 90, 97, 101). Consistent with the hypothesis of cross-coupling of estrogen and growth factor signaling are observations from mammary tumor cells, in which estradiol elicited maximal phosphorylation of *src* within 10 s

(69). This effect required only 10–20% estrogen receptor occupancy (68), a level coincidentally corresponding to the percentage of the estrogen receptor estimated to be associated with the plasma membrane (134).

In extraneural estrogen targets such as the uterus and mammary tumor cell lines, there are both *estrogen-dependent* and *estrogen-independent* interactions of the estrogen receptor with other peptide growth factor signaling pathways (e.g., EGF, TGF- α - β , IGF-I). These have been implicated in an increasing number of estrogen-induced or estrogen-like differentiative processes (46, 96). For example, Toran-Allerand *et al.* (131) reported synergism between estrogen and insulin-related peptides in neurite growth enhancement of organotypic slice cultures of fetal mouse and rat brain. The striking enhancement of process formation upon concurrent chronic administration of estrogen and insulin appeared to be restricted to the estrogen receptor-containing regions of explants of the developing olfactory bulb, hypothalamus, preoptic area, and cerebral cortex. Duenas *et al.* (25) subsequently described interactions between estrogen and IGF-I during the differentiation of fetal rat hypothalamic neurons in primary cultures. Administration of either estradiol or IGF-I increased the number and arborization of neurons present in the cultures, assessed by microtubule-associated protein-2 (MAP-2) immunostaining. This effect of estrogen and IGF-I was not additive, and estrogen's effect was significantly weakened by the presence of an antisense oligonucleotide to IGF-I mRNA. The effect of IGF-I was also efficiently blocked by either the antiestrogen ICI 182,780 or an antisense estrogen receptor oligonucleotide. This observation argues for the requirement of the mutual presence of both estrogen and IGF-I to successfully evoke their neurotrophic/differentiative effects documented in this system. In addition, the uterotrophic effects of EGF (42) and the differentiative effects of insulin on neuroblastoma cells (90) were both found to be estrogen receptor dependent. This observed effect of insulin required the presence of p21ras, the initial signaling enzyme of the MAP kinase cascade.

ESTROGEN AND THE MAP KINASE CASCADE

The MAP kinase cascade, a major signal transduction cascade involved in differentiative processes, among others, is propagated by Ras activation of Raf, followed by sequential phosphorylation and activation of MEK and ERK (MAP kinase) (63) (Fig. 4). Activated ERK then translocates into the nucleus to interact directly with nuclear transcription factors (CREB, Elk-1, AP-1, etc.) and immediate early genes such as *c-fos* and *c-jun* or, indirectly, through the activation of intermediary signaling proteins such as Rsk. ERK and Rsk translocation are the means by which some signals, arising at the plasma membrane, can regulate gene transcription.

Estrogen has been shown to induce ERK activation in various nonneuronal cell models, including the MCF-7 breast carcinoma cell line (68), human colon carcinoma-derived cells (22), uterine smooth muscle (98), and bone (26). In neurons, however, our knowledge of alternative signaling pathways for estro-

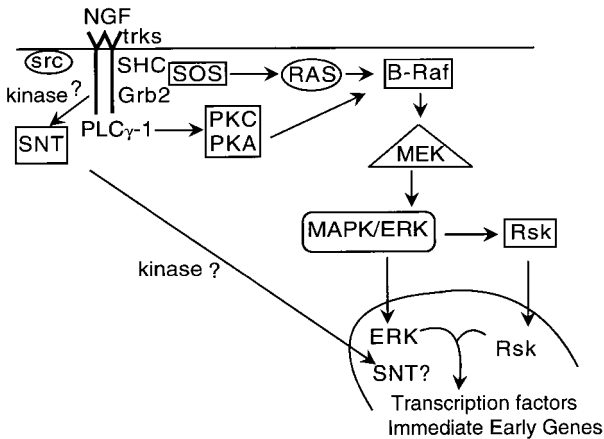


FIG. 4. Neurotrophin signal transduction pathways involved in PC12 cell differentiation. In PC12 cells, treatment with NGF elicits dimerization and activation of its receptor, *trkA*, through tyrosine autophosphorylation. Tyrosine autophosphorylation regulates interactions of the activated *trkA* with multiple intracellular proteins that either have enzymatic functions, such as PLC- γ 1 and c-src, or are docking proteins without intrinsic enzymatic activity, such as Shc and Grb2. Shc and Grb2 with the GTP/GDP exchange protein SOS connect the activated *trkA* to the initial signaling enzyme p21Ras. Ras then activates multiple signal transduction pathways, including the MAP kinase cascade which is propagated by Ras activation of B-Raf, followed by sequential phosphorylation and activation of MEK and ERK, also known as MAP kinase. Activated ERK then either translocates directly to the nucleus or first phosphorylates another kinase, Rsk, which then also translocates to the nucleus. ERK and Rsk translocation are the means by which some signals, arising at the plasma membrane, activate intranuclear transcription factors and genes.

gen action is relatively limited. In explants of the cerebral cortex, estrogen elicited the rapid and sustained activation of ERK1 and ERK2, an effect which requires MEK activation (114), the signaling protein immediately upstream of ERK. Moreover, it was also shown that estrogen is capable of increasing B-Raf kinase activity (114). Taken together, these data suggest that one of estrogen's mechanisms of action may be a consequence of significant convergence of estrogen and neurotrophin signaling pathways.

This ability of estrogen to activate ERK may provide an additional mechanism by which estrogen regulates non-ERE-containing genes, such as the genes for brain creatine kinase (120), β -tubulin, and MAP-2, and could help explain the very rapid effects of estrogen (18, 33, 69).

ERK activation in CNS tissue is much more prolonged (114) than it is in nonneuronal cells such as the MCF-7 cell (68). It has been proposed that the prolonged activation of ERK (MAP kinase) may distinguish the differentiative effect of growth factors such as NGF from the proliferative effect of EGF and other growth factors, which trigger a much more transient activation of ERK (63). Since sustained activation of ERK1 and ERK2 in PC12 cells has been associated with neuronal differentiation, the extended ERK time course which follows estrogen exposure appears to be consistent with its observed differentia-

tive actions on CNS neurites. In addition, the difference in the temporal pattern of activation of ERK in MCF-7 cells versus CNS tissue may suggest that signaling mechanisms elicited following estrogen exposure depend upon cellular type or context.

A clue concerning potential pathways for estrogen-induced ERK activation was initially suggested by reports of a very large (>300 kDa), constitutive multimeric complex in PC12 cells, consisting of at least B-Raf and hsp90 (44). Singh *et al.* (114) documented the presence of this complex in the cerebral cortex and showed that it consisted not only of B-Raf and hsp90, but also contained the estrogen receptor, src (M. Singh *et al.*, unpublished observations) and ERK (102) (Fig. 4). These findings suggest that the complex may be similar to the caveolar-associated complexes that are found in most cells (1).

Caveolae are flask-shaped, cholesterol and sphingolipid-rich microdomains of the plasma membrane that have been implicated in signal transduction and vesicular trafficking. Caveolae are highly enriched in caveolin, a scaffolding protein; glycosylphosphatidylinositol-anchored proteins; receptor tyrosine kinases such as the neurotrophin, insulin (27), and EGF receptors (20); the p75^{NTR} neurotrophin receptor; and signal transduction molecules such as *Ras*, the *src* family of tyrosine kinases, ERK (56, 99), and protein kinase C (122). This *Ras*-associated caveolar complex is the means by which cytoplasmic *Raf* is thought to be recruited to this membrane domain (70). All components of the MAP kinase cascade are concentrated in the caveolae of quiescent cells. Addition of a growth factor such as PDGF, for example, to either intact fibroblasts or to caveolae isolated from these cells stimulates tyrosine phosphorylation and activation of the MAP kinases (58). Caveolar localization of certain inactive signaling molecules could provide a compartmental basis for their regulated activation and explain cross-talk between different signaling pathways. Since the molecular machinery for activation of the MAP kinase cascade appears to be preorganized at the cell surface of quiescent cells, it is tempting to speculate that association of the estrogen receptor with multimeric caveolar-like complexes of signaling kinases could function as a plasma membrane estrogen receptor and mediate the rapid effects of estrogen. It is still unknown, however, whether our observed complex is constitutive or inducible under different hormonal or growth factor milieus. Nevertheless, these findings further support an association between the estrogen receptor and growth factor signaling (via B-Raf) and could also help explain the dependence of the estrogen receptor on certain actions of specific growth factors (42, 90).

Thus, regardless of whether the ligand is estrogen or a growth factor, phosphorylation within the complex and dissociation of the estrogen receptor may not only result in estrogen receptor activation but in activation of ERK as well, leading in both instances to nuclear translocation of both the activated estrogen receptor and ERK (Fig. 5). Although the dogma of estrogen action has been that the ligand-activated estrogen receptor binds directly to EREs, our findings could also explain how, through the intermediary of nuclear translocation of ERK, estrogen and the neurotrophins could each regulate neurite growth and differentiation through the same broad array of genes such as

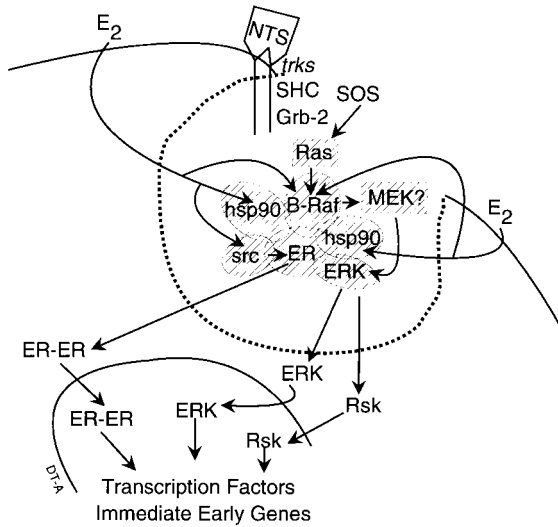


FIG. 5. Alternative pathways of estrogen signaling. The initial route taken to elicit rapid estrogen activation of ERK may involve *direct* activation of the putative ER-containing multimeric complexes associated with caveolae. Following neuronal exposure to estradiol (E_2), dissociation of the estrogen receptor (ER) from the complex, consequent to estradiol-induced tyrosine phosphorylation of *src*, the estrogen receptor, and hsp90, may perhaps elicit conformational changes within the rest of the complex and trigger sequential phosphorylation and activation of the physically associated kinases, such as B-Raf and ERK. Conversely, neurotrophin activation of *trk* receptors, followed by signaling via members of the MAP kinase cascade, would, in turn, provide a reciprocal signaling pathway by which the *unliganded* estrogen receptor, bound through its physical associations with the rest of the complex could become tyrosine phosphorylated by the neurotrophins. In this manner, multimeric complexes of the estrogen receptor, members of the MAP kinase cascade and *src*, could serve as intracellular junctions which link the estrogen and neurotrophin signaling pathways.

β -tubulin (38), MAP-2 (13, 32, 59), *tau*-microtubule-associated protein (24, 30, 66), and GAP-43 (19, 29, 61).

THE RECEPTORS INVOLVED IN NOVEL MECHANISMS OF ESTROGEN ACTION

The identification of a new estrogen receptor gene, the recently cloned ER- β , raises questions as to whether the effect of estrogen on ERK activation may also be mediated either via this novel receptor, a proposed membrane-associated estrogen receptor (45, 89, 92), or even a yet unidentified estrogen receptor. Studies in cerebral cortical explants derived from estrogen receptor- α knockout (ERKO) (21) mice revealed that estrogen is still capable of eliciting ERK activation even in the absence of ER- α relative to their wild-type littermates (113). Interestingly, however, estrogen activation of ERK was consistently stronger in the ERKO cultures. Furthermore, attempts to pharmacologically block the effect of estrogen with the estrogen receptor antagonist ICI 182,780

(133) revealed the surprising finding that estrogen-induced ERK phosphorylation could only be blocked in wild-type and not ERKO cultures (113). These differential responses of wild-type and ERKO cortical explants to the ICI compound suggest that, while estrogen *induction* of ERK phosphorylation does not require ER- α , estrogen *regulation* of ERK activation may somehow involve an interplay between the two known estrogen receptors. Due to the documented existence of ER- α /ER- β heterodimers (74, 79, 91), differences in the relative ratios of ER- α versus ER- β within a given cell could also result in differential responses to estrogen, at least with regards to ERK activation.

SOME OTHER SIGNALING SUBSTRATES FOR ESTROGEN

While these results document the involvement of ERK in estrogen signaling in the developing brain, they do not rule out estrogen activation of other signaling substrates such as adenylate cyclase/cyclic adenosine monophosphate (cAMP), Ca²⁺ channels, protein kinase A (PKA), or protein kinase C (PKC) which could act either in parallel or by converging onto the MAP kinase pathway. In fact, rapid, nontranscriptional actions of estrogen have been documented in both neural and extraneural tissue targets via its interaction with well-characterized protein kinase systems, including PKA and PKC. Ansonoff *et al.* (2) demonstrated that PKC catalytic activity was significantly increased following estrogen treatment of ovariectomized animals in tissue extracts from the preoptic area, but not in the hypothalamus or cortex. However, this increased PKC activity was not accompanied by elevated protein levels of classical PKC isoforms. Estrogen also increased the ability of PKC to potentiate forskolin-induced cAMP accumulation but left receptor-dependent activation of adenylate cyclase unaffected in the same brain region.

Similarly, estrogen's ability to activate adenylate cyclase and cAMP-mediated gene transcription has been documented in nonneural tissues (4) as well as in the brain. For example, Zhou *et al.* (136) described a rapid (within 15 min) increase in phosphorylated cAMP response element binding (CREB) protein immunoreactivity in the preoptic area and the bed nucleus of stria terminalis following estrogen replacement of ovariectomized adult animals. In addition, Gu *et al.* (37) reported a 50% increase of phospho-CREB (pCREB)-immunoreactive nuclei in the anteroventral periventricular nucleus in 30 min following estrogen treatment of ovariectomized rats, a response that was maintained for up to 4 h. Pretreatment of the animals with nafoxidine, a specific estrogen antagonist, abolished the estrogen evoked pCREB response. In addition, immunohistochemical detection of unphosphorylated CREB and *in situ* hybridization experiments targeting CREB mRNA indicated that estrogen's effect was posttranscriptional and did not arise from *de novo* RNA/protein synthesis. Panickar and colleagues (87) observed that 17 β -estradiol attenuated the decline of CREB-immunoreactive cells in the hippocampus following insulin-induced seizure of ovariectomized rats. The distribution of the CREB decline in

various regions of Ammon's horn was consistent with earlier findings in male rats, following insulin-induced seizures (88).

In cultured hippocampal neurons, estrogen exposure was also shown to concomitantly increase the number of dendritic spines (the primary acceptors of excitatory synaptic stimuli) and the amount of phosphorylated CREB and CREB-binding protein (76). Although these effects were slow in onset, they appeared to be long lasting and the blockade of cAMP-regulated PKA or the application of specific antisense CREB oligonucleotides eliminated them. In addition, the effect of a cAMP analog alone was somewhat weaker than that of estrogen, suggesting that additional mechanisms may be recruited and act in concert to elicit the stronger effect observed following estrogen exposure.

ESTROGEN AND ALZHEIMER'S DISEASE

Numerous studies from humans and animal models suggest that estrogen may be beneficial in preserving cognitive function. In both surgically induced or naturally postmenopausal women, estrogen has been shown to help improve specific aspects of cognitive function (103). Similarly, in ovariectomized rodents, estrogen has also been shown to have significant beneficial effects on learning and memory (60, 111). In addition, decreased estrogen levels in such animals are also accompanied by morphological, neurochemical, and molecular deficits such as structural alterations in hippocampal dendritic spines (135), decreases in NGF (110) and BDNF mRNA expression (112, 118), and reduced cholinergic function (82, 111). Since these changes have also been implicated in the etiology or progression of Alzheimer's disease, a disease whose behavioral hallmark includes profound cognitive dysfunction, estrogen's maintenance of such endpoints may provide a mechanism that links the gonadal hormone milieu to such age-associated neurodegenerative states as Alzheimer's disease.

If interactions between estrogen and the neurotrophins are fundamental to the development and survival of their target forebrain neurons, then the lack of an estrogen stimulus could contribute to the clinical conditions observed in Alzheimer's disease. Although the growth- or neurite-promoting property of estrogen is normally expressed during development, estrogen and neurotrophin interactions, latent since development, may be recruited and reactivated in the "injured" adult brain. Such "injuries" may be fairly severe, such as trauma following axotomy and deafferentation and ischemia or during the course of neurodegenerative diseases such as Alzheimer's disease. Other "injuries" that result in less severe deficits but are of equal importance, include steroid deprivation occurring following ovariectomy, menopause, or tamoxifen treatment for breast cancer.

After menopause, a declining estrogenic stimulus, either from dramatically reduced levels of circulating estrogen or from aromatizable androgen levels, both of ovarian origin in the female, might make estrogen target neurons in the brain more susceptible to age- or disease-related processes such as Alzheimer's disease. In fact, recent evidence strongly suggests that estrogen deficiency may

be a risk factor that contributes to the etiology of Alzheimer's disease in women (31, 48, 80, 85, 86, 107, 121). While Alzheimer's disease is a devastating condition which affects both sexes, women have about a twofold higher prevalence of Alzheimer's disease than men (5). In addition, women with a history of myocardial infarction are five times as likely to develop Alzheimer's disease than those without such a history (5). In contrast, myocardial infarction is not a risk factor for the development of Alzheimer's disease in men, suggesting that the greater risk observed in women is not due to the underlying vascular disease. Since virtually all women who develop Alzheimer's disease are postmenopausal, the precipitous decline in estrogen following menopause could contribute to the neurodegeneration and behavioral deficits seen in Alzheimer's disease. Increasing experimental evidence, in fact, demonstrates that estrogen is neuroprotective (10, 11, 36, 40, 94, 108, 109) and that estrogen replacement therapy may contribute to the prevention, attenuation, or even delay the onset of Alzheimer's disease (31, 48, 80, 85, 86, 121). Furthermore, estrogen replacement may also facilitate other treatments used or being developed for the treatment of Alzheimer's disease. For example, in clinical trials with Tacrine, an anticholinesterase drug currently in use for the treatment of Alzheimer's disease, a greater efficacy of this drug was seen in women receiving estrogen replacement therapy relative to women who were not (100). These observations further emphasize the importance of estrogen in the brain and underscores the ability of estrogen to affect a wide variety of systems in the brain.

CONCLUSION

The effects of estrogen in the CNS can no longer be restricted to those relating to reproductive function. As such, it is also apparent, as reviewed here, that the mechanisms involved in these varying effects of estrogen are equally diverse and extend well beyond the boundaries of transcriptional modulation. The identification of additional estrogen receptors and the depiction of convergence between the estrogen and growth factor-related signaling pathways have further underscored the complexity of estrogen actions within the CNS.

In the developing brain, cross-coupling of estrogen and neurotrophin signaling pathways may be required for estrogen and the neurotrophins to elicit their differentiative effects efficiently, if not completely. Mediation of estrogen actions through interactions with locally synthesized growth factors (such as the neurotrophins), their receptors, and their signaling cascades in the brain may represent a mechanism by which estrogen can exert local control and exhibit both tissue- and developmental-stage specificities. Moreover, during development, reciprocal regulation of the estrogen and neurotrophin receptors by their ligands at transcriptional, translational, and even posttranslational levels suggest the potential for reciprocal regulatory loops that may influence the ontogeny of both receptor systems. Thus, by setting the stage for the ability of estrogen to stimulate the synthesis of proteins required for neuronal differentiation, survival, and maintenance of function, estrogen and the neurotrophins,

acting in concert, may not only have important developmental roles but may also decrease the vulnerability of target neurons to the consequences of neurodegenerative disease processes and enhance the compensatory responses to degenerating neural systems.

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