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Cellular Strategies of Estrogen-Mediated Neuroprotection During Brain Development

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The role of estrogen during brain development is well documented. Estrogen influences cell survival and differentiation and also controls the formation and maintenance of neural networks. Knowledge of trophic estrogen action in the central nervous system (CNS) was the basis for the establishment of research programs directed toward a potential function of estrogen as a neuroprotective factor in the adult brain. Considerable evidence has accumulated over the years supporting this hypothesis. Experimental and epidemiologic studies as well as clinical trials have demonstrated that estrogen is beneficial for the course of neurodegenerative disorders such as Parkinson and Alzheimer diseases but may also protect neurons from postischemic neuronal degeneration. In this article, we aim to unravel potential physiologic responses and cell survival strategies that allow a more detailed understanding of estrogen-mediated neuroprotection in the brain. In particular, we focus on the participation of estrogen in the regulation of apoptotic processes. Furthermore, we present data on reciprocal estrogen–growth factor interactions. Both of these mechanisms were found to operate during brain development and to conciliate estrogen effects on neurons. This makes them likely candidates for taking part in conveying estrogen-dependent neuroprotection in the adult CNS.

Key Words: Estrogen; neuroprotection; apoptosis; growth factors; central nervous system; cellular signaling.

Introduction

Estrogen and Neuroprotection

The steroid hormone estrogen influences a wide variety of developmental and functional aspects in the mammalian central nervous system (CNS). This includes the survival of neurons, the growth and plasticity of neurites, their synaptic connections and transmission, and the neuronal activity *(1–3)*. In addition to these developmental effects, there is increasing evidence that estrogen can also alter higher cognitive functions of the adult CNS such as verbal memory tests, fine motor skills, and performance of spatial tasks *(4, 5)*. The action of estrogen in the CNS has become a topic of keen interest to neuroscientists and clinicians, especially since clinical trials, experimental in vitro and epidemiologic studies, have provided information that estrogen may protect against Alzheimer disease in postmenopausal women *(6)* and may positively affect symptoms of Parkinson disease and tardive dyskinesia *(7)*. The idea of a neuroprotective function of estrogen is supported by observations that females are less vulnerable to acute insults associated with cerebral ischemia, neurotrauma, and hypoxia and that estradiol or estrogen-like compounds protect against stroke *(2,8)*. Several in vitro and in vivo approaches have been applied to study the protective efficacy of estrogen and to decipher the intracellular/intercellular mechanisms whereby estradiol rescues neurons from injuries and/or attenuates the extent of degenerative processes. Culture models including primary cell cultures and cell lines were adopted to analyze the potency of estrogen to defend nerve cells from toxic compounds and excitatory overflow (i.e., glutamatergic action). These in vitro models offer the advantage of manipulating neurons under standard conditions but also allow investigation of the related physiologic and molecular cell responses. In particular, cell culture studies have revealed that estrogen is capable of protecting neurons from oxidative stress *(9)* and from chemically induced apoptosis *(10)*. Major target sites of protective estrogen action are thought to be anti-apoptotic genes/proteins such as Bcl-2 *(11)* and Bcl-xl *(12)* but also the proapoptotic gene nip-2 *(13)*. The expression of these genes appears to be either up- or downregulated, respectively.

One of the intriguing questions at present is how to calculate the cellular mechanism(s) of estradiol-mediated neuroprotection. The findings of several studies have produced not only conflicting results but also provided strong evidence that more than a single signaling mechanism is turned on by estrogen in neural cells *(14)*. Precisely, the effects of estrogen can be either direct (on target neurons) or mediated through an activity control of efferent neurons (contacting neurons

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to be protected), or even involve nonneuronal cells such as astrocytes and microglia, which, in turn, are then able to deliver neuroprotective factors or inactivate toxic compounds. On the cellular level, the effects of neuroprotective estrogen appear to be transmitted by a variety of intracellular signal transduction mechanisms including interactions with nuclear hormone receptors (classic signaling); stimulation of antiapoptotic signaling pathways such as phosphatidylinositol 3-kinase (PI3-K) and mitogen-activated protein kinases (MAPKs) via membrane-associated estrogen receptors (ERs) (*[14–16]*; nonclassic signaling); and, finally, through the potency of estrogen to act as an intrinsic free-radical scavenging antioxidant *(17)*. As a result of the high degree of complexity concerning possible interactions between estrogen and neural cells *(18,19)*, we focus here particularly on two different modes of estrogen action in the CNS. First, we describe the potency of estrogen to interfere with apoptotic processes in the brain and delineate possible cell sites/structures as estrogen targets. Second, we present and discuss data showing that estrogen is a very potent regulator of the expression and availability of trophic factors in the CNS.

Estrogen and Apoptosis

Naturally occurring neuronal death, being essential for proper organ development and tissue homeostasis and representing a defense strategy against pathogens, is generally considered to be apoptotic. Apoptotic cells in the brain, as elsewhere, are characterized by specific morphologic and biochemical changes including cell shrinkage, chromatin condensation, and internucleosomal cleavage of genomic DNA by endonucleases *(20)*. Apoptosis during CNS development has been traditionally viewed as a fate reserved for neuroblasts and differentiating cells that are in the process of making synaptic connections. Therefore, apoptosis enables elimination of "unsuccessful" or "unoriented" neurons, i.e., cells that did not succeed in establishing functional connections and/or dispensable cells that are made due to the natural overproduction of neurons during ontogeny. Neurons within functional circuits are stabilized and protected by mechanisms not fully understood. This qualifies them to escape apoptotic elimination. Recent studies on the embryonic cerebral cortex have shown that many neuroblasts in the proliferative ventricular zone die via apoptosis *(21)*. In another brain region, the cerebellum, apoptosis reaches a maximum early postnatally that correlates with intense synaptogenesis and dendritic remodeling of Purkinje cells *(22)*. Many more examples of apoptotic regulation of neuronal survival during brain ontogeny and in adulthood are discussed in recent reviews *(23,24)*.

Generally, apoptotic cell death can emerge from limited target-derived growth factor supply but can also result from receptor-mediated cell suicide. At the molecular level, apoptosis is regulated by activation of the aspartate-specific cysteine protease (caspase) cascade that depends either on the participation of mitochondria, release of cytochrome-*c*, and stimulation of procaspase-9 or on interactions of death receptors such as FAS (CD95 or Apo-1) with its ligands and the subsequent activation of procaspase-8 *(25)*. Pro- (Bax, Bid, Bad, Bak) and antiapoptotic (Bcl-2, Bcl-xl) members of the Bcl-2 family are known to regulate the mitochondrial pathway of apoptosis allowing or preventing cytochrome-*c* release, respectively (Fig. 1 summarizes both apoptotic cascades). The mature caspase-8 and -9 are considered apoptotic initiators participating in the activation of procaspase-3. Caspase-3, finally, stimulates a specific DNase (DFF40 or CAD) that is responsible for internucleosomal fragmentation of DNA and, ultimately, cell death *(26)*. This protease is believed to function as the main executor of the apoptotic pathway, since caspase-3 knockout mice die soon after birth owing to massive brain abnormalities such as excessive ectopic growth and hyperplasia *(27)*.

Because estrogen has been characterized as a potent trophic factor regulating cell survival during CNS development and has been found to positively influence neurodegenerative processes in the adult brain, the question can be posed, Does estrogen interfere with neuronal apoptosis *(28,29)*? The death cascades may be counteracted by cellular mechanisms that limit the activation of the caspase pathways, control calcium homeostasis and mitochondrial function, modulate excitatory receptor-dependent fatal cell injuries, affect pro/antiapoptotic proteins, and suppress oxyradicals. In general, it seems that estrogen can interfere with all of these death-causing mechanisms (Fig. 2). Thus, estradiol has recently been recognized to inhibit different caspases and prevent caspase-mediated neuronal death by inducing a caspase-inhibiting factor *(30,31)*. This effect appears to be transmitted through estrogen receptors but is nonclassic in action involving the rapid stimulation of the MAPK signaling pathway. Estrogen is also capable of binding to and modifying directly mitochondrial membrane properties by inhibiting calcium efflux *(32)*. *N*-Methyl-D-aspartate–induced toxicity and apoptosis of cortical neurons is antagonized by estrogen *(33)*. Concerning the regulation of antiapoptotic proteins, strong evidence from several laboratories demonstrates that neuroprotection by estradiol in the cortex, hippocampus, and hypothalamus depends on an induction of Bcl proteins *(34–36)*. On the other hand, estrogen may affect apoptosis by suppressing the transcription of proapoptotic genes such as Nip-2 or Bad, as demonstrated for dopaminergic cells in the midbrain *(13)*. A completely different mechanism by which estrogen can mediate neuroprotection is to act as a free-radical scavenger. Behl et al. *(17)* have intensively studied this particular function of estrogen. They have clearly shown that estrogen has antioxidant properties that are sufficient to protect neurons against oxidative stress.

There exist further cellular targets and signaling strategies for estrogen that all may be involved in transmitting neuroprotection in the brain. This includes the control of nitric oxide production in the brain including both the epi-

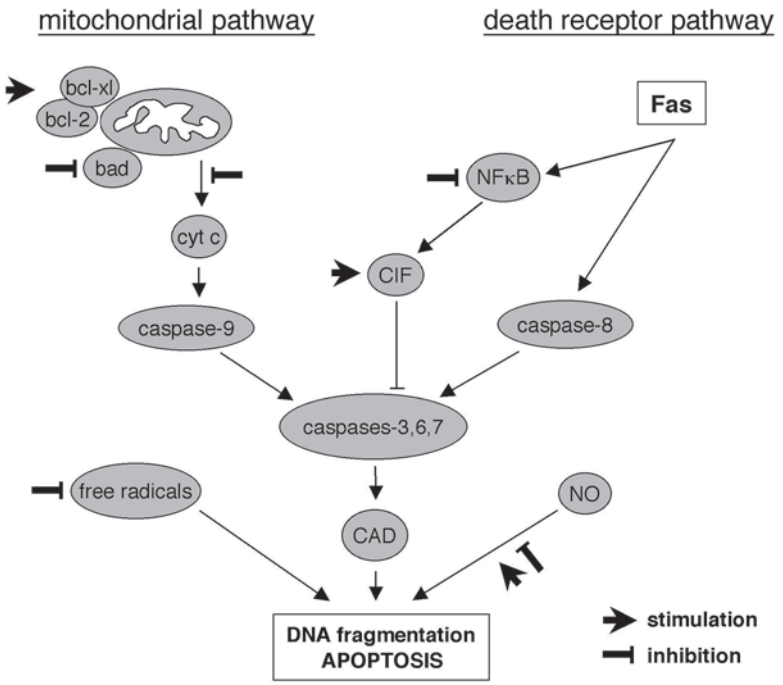

Fig. 1. Hypothetical sites of estrogen interactions with intracellular apototic signaling cascades. These interactions may either depend on conventional nuclear ER activation or are mediated through nonclassic estrogen action. For details see the text. NO, nitric oxide; CAD, caspase 3-activated DNAse; CIF, caspase inhibitory factor.

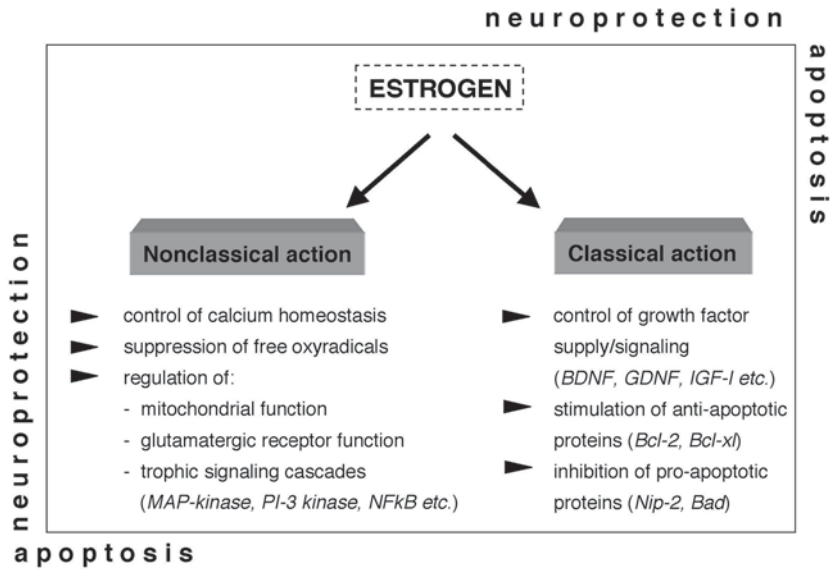

Fig. 2. Schematic summary of hypothetical sites of interactions of estrogen with apoptosis-relevant cell structures and functions in developing mammalian brain classified according to way of estrogen action (i.e., classic through nuclear receptors followed by transcriptional effects and nonclassic not directly involving nuclear mechanisms).

thelial and inducible forms *(37)*, just as attenuating nuclear factor κB (NFκB) expression as well as NFκB function by directly interacting with its p65 subunit *(38)*. In this context, it is worth mentioning that many of the described estrogen effects on apoptosis are not mediated through the classic steroid signaling mechanism that is characterized by the activation of nuclear ERs and the subsequent binding of active receptors to estrogen response elements within the promoter region of target genes. By contrast, the responsible mechanisms can mainly be assigned to nonclassic estrogen action. Nonclassic estrogen effects are principally delineated as rapid in onset (seconds to minutes); insensitive to transcriptional and translational inhibitors; and, finally, by the way the signal is percepted by the cell *(2,19)*. The latter predication means that ERs are located outside the nuclear region and coupled to distinct signaling cascades such as MAPK, PI3-K, and others *(16,19)*. These receptors are mainly found in close association with the plasma membrane. This

particular issue, is beyond the scope of the present article, but has recently been excellently reviewed in great detail by Falkenstein et al. *(19)*.

The data presented in this article reveal a high degree of complexity of estrogen-mediated regulation of apoptotic processes in the CNS. The amazing conclusion from our point of view is in such a way that estrogen can interfere with almost every step of intracellular signal propagation that is implicated in the control of apoptosis. This does not necessarily signify that all of these interactions are comparably important for estrogen-dependent regulation of apoptosis in the CNS. Nevertheless, it highlights the importance of estrogen as a neuroprotectant during brain development as well as its protective role for neurodegenerative diseases in adulthood.

Estrogen and Neurotrophic Factors

Growth factors and estrogen are both important for the regulation of distinct developmental processes in the brain. Interestingly, the two developmental signals display a significant overlap in their cellular action and often share the same target cells *(39)*. This conspicuity provokes the question, Are the activity and cellular action of both biologic determinants intertwined? In fact, we point out herein that estrogen transmits at least some of its developmental effects through the regulation of growth factor expression or signaling. On the other hand, the reverse may also apply. Data from the literature suggest a reciprocal linkage between both factors.

About a decade ago, the research group of Toran-Allerand presented, for the first time, convincing data that suggested a potential interaction between growth factors and estrogen during brain ontogeny. They demonstrated that estrogen and neurotrophins (a family of structurally related growth factors including nerve growth factor, brain-derived neurotrophic factor [BDNF], neurotrophin [NT]-3, and NT-4/5) target the same cell population in the developing cortex *(40)*. In addition, estrogen was found to induce the expression of BDNF in the olfactory bulb and cerebral cortex of rodents *(41)*. Successively, several other reports were published revealing that estrogen–growth factor interactions are important for quite a number of developmental aspects in the CNS of different species. Thus, estradiol regulates BDNF expression in the rodent hippocampus *(42)*, zebra finch forebrain *(43)*, and mouse midbrain *(44)*. The latter observation, deriving from our laboratory, is shown in Fig. 3. First evidence supporting an estrogen-dependent regulation of BDNF expression in the midbrain came from developmental studies that found that the pattern of mesencephalic BDNF expression *(44)* coincides with that of the enzyme aromatase *(45)*, which converts testosterone into 17β-estradiol, and with that of nuclear ERs *(46)* in this brain region. On the other hand, BDNF and estrogen cause similar physiologic cell reactions in the midbrain. In particular, dopamine

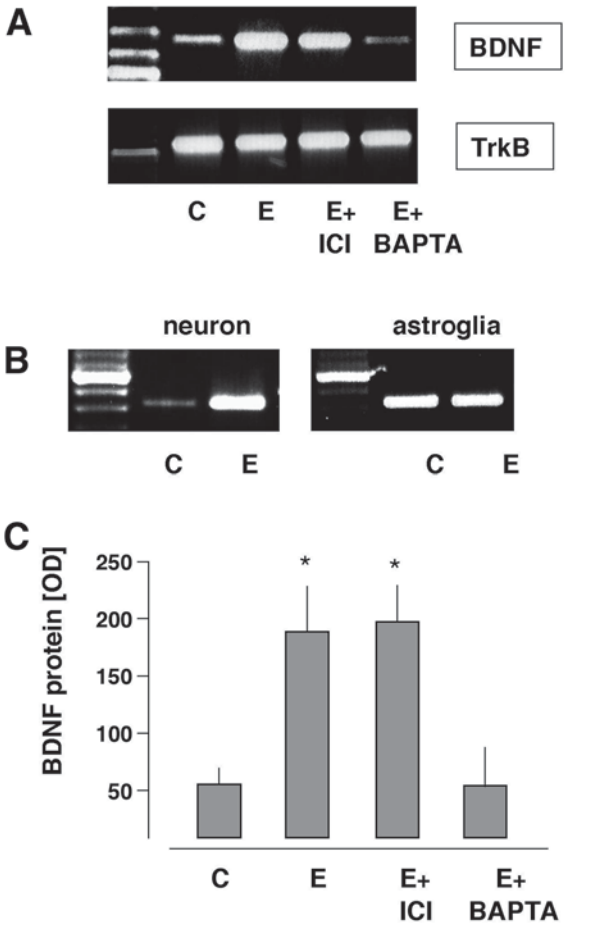

Fig. 3. Effect of estrogen treatment (E, 10 n*M*, 24 h) on BDNF and TrkB mRNA (**A,B**) and BDNF protein (**C**) levels in primary neuronal (A) and astroglial (B) midbrain cultures (prepared from embryonic d 15 mouse fetuses and newborns, respectively). Samples were analyzed by competitive reverse transcriptase polymerase chain reaction (RT-PCR) (A,B) and enzyme-linked immunosorbent assay (C). Estrogen increased BDNF mRNA (A) and protein (C) levels in neuronal cultures. This effect was not inhibited by ICI 182,780 (1 μ*M*) but was completely prevented by the intracellular calcium chelator BAPTA-AM (3 μ*M*). Expression of the native BDNF receptor TrkB was not changed by estrogen treatment (A). In contrast to neurons, no estrogen effect on BDNF levels was seen in astroglial cultures (B). $*p < 0.001$ for treated cultures vs control (C). (Modified from ref. *44*.)

(DA) neurons are targeted by both growth signals. Thus, BDNF and estrogen stimulate both the expression of the DA-synthesizing enzyme tyrosine hydroxylase and neurite growth in this cell population *(44,47,48)*.

Our in vitro and in vivo studies using primary cell cultures and pharmacologic manipulations of fetuses *in utero* revealed that estrogen increases BDNF mRNA and protein levels (Fig. 3A,C). This effect is restricted to neurons, since astrocytes do not respond to estrogen application (Fig. 3B), and is specific for BDNF, since the expression of other neurotrophins such as NT-3, NT-4/5 *(44)*, as well as the native

BDNF receptor TrkB are not changed (Fig. 3B). It is noteworthy at this stage that the cooperation between the two ontogenetic signals (estrogen and BDNF) also takes place in some regions of the adult brain where it affects the survival of seasonally recruited neurons in the canary *(49)* and estrous-dependent fluctuation of hippocampal BDNF expression *(50)*. Thus, the estrogen–growth factor connection appears to be a general phenomenon in the developing and adult brain rather than an exception. Since growth factors not only regulate the survival and plasticity of neurons and neural networks, respectively, but are also crucial for the activity control within neuronal systems *(51)*, estrogen may thus indirectly interfere with nerve cell function and synaptic efficacy.

Concerning the cellular mechanisms that are involved in the mediation of estrogen effects on BDNF expression in the brain, there exists a great diversity depending on the brain region and species studied. We and others have observed that nonclassic estrogen action through presumable membrane ERs and intracellular calcium signaling are responsible for the estrogen-dependent increase in BDNF expression in the rodent midbrain and hippocampus as well as in the songbird telencephalon *(42–44,52)*. By contrast, others have found that nuclear ER signaling (classic estrogen action) is required to regulate BDNF expression in the cortex *(39)*. At present, the best explanation for this discrepancy is the use of different promoters within the BDNF gene in different brain regions *(53)*.

The members of the transforming growth factor-β (TGF-β) growth factor family (glial cell–derived neurotrophic factor [GDNF], artemin, persephin, and neurturin) may also represent potential candidates susceptible to an estrogenic regulation *(54,55)*. All of them can support the survival of a wide variety of neuronal cell populations in both the peripheral nervous system and CNS *(56,57)*. Accordingly, GDNF and its native receptors GFR-α1/2 as well as the tyrosine kinase c-ret are expressed in different regions of the brain *(58)*. We have tested the hypothesis of estrogen-GDNF interactions during development and have chosen for our studies the hypothalamus owing to the fact that hypothalamic DA systems are regulated by GDNF *(59)* as well as estrogen *(60–62)*. Using a cell culture approach, we were able to show that estrogen is capable of inducing GDNF mRNA and protein expression in hypothalamic neurons but not astrocytes (Fig. 4) *(63)*. Additional in vivo studies indicate again an overlap in the developmental expression pattern of hypothalamic GDNF (unpublished) and aromatase *(64)*. Thus, it seems likely that estrogen-mediated effects on developing hypothalamic DA neurons are dependent on an induction of GDNF gene expression in adjacent or DA-contacting afferent neuronal systems. It needs to be mentioned that preliminary data exist suggesting the possibility that gonadal steroids can also interfere with c-ret expression and/or activity *(65)*. This would allow estrogen to control GDNF action not only by regulating GDNF availability but also

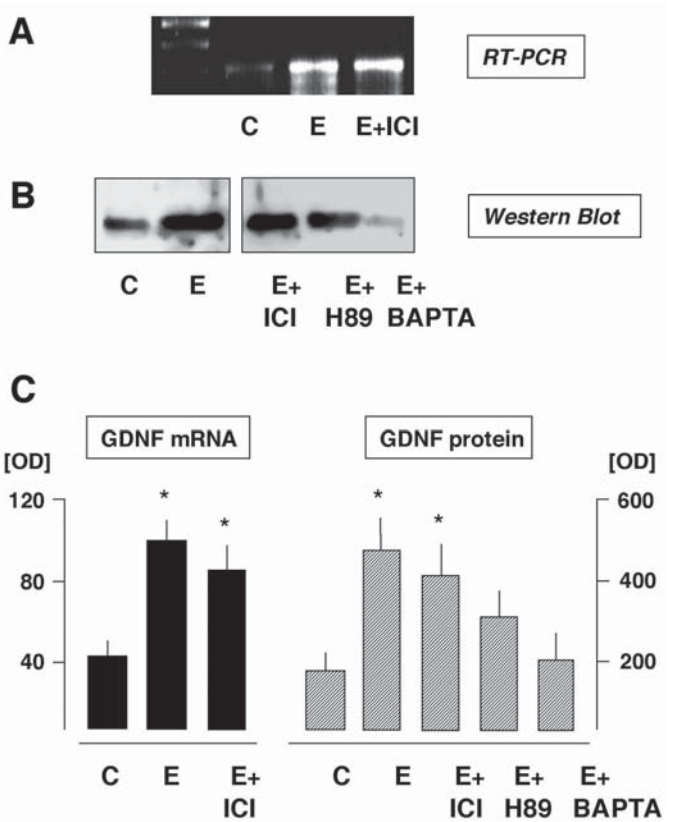

Fig. 4. Effect of estrogen treatment (E, 10 n*M*, 24 h) on GDNF mRNA (**A**) and protein (**B**) levels in primary neuronal hypothalamic cultures (prepared from embryonic day 15 mouse fetuses and grown for 1 wk). Samples were analyzed by competitive RT-PCR (A) and Western blotting (B). Quantification was obtained by densitometric gel scanning. (**C**) Cumulative data (means ± SEM) of eight independent experiments. Note that estrogen exposure increased GDNF mRNA/protein levels. This effect was not inhibited by the nuclear ER antagonist ICI 182,780 (1 μ*M*) but was partly blocked by the PKA inhibitor H89 (100 n*M*) and completely abolished by BAPTA-AM (3 μ*M*), an intracellular calcium chelator. $*p < 0.005$ for treated cultures vs control (C). (Modified from ref. *63*.)

by interfering with GDNF signaling in target cells. Concerning intracellular signal transduction mechanisms that are responsible for mediating estrogenic regulation of hypothalamic GDNF expression, we found that, similar to midbrain BDNF, estrogen action requires binding to membrane-associated ERs and the subsequent stimulation of distinct signaling cascades *(63)*. In contrast to BDNF, two different intracellular signaling cascades are activated simultaneously and are involved in the regulation of GDNF expression: the cyclic adenosine monophosphate/protein kinase A (PKA) pathway and calcium-dependent CaMkinases.

Besides BDNF and GDNF, it has been shown that estrogen and insulin-like growth factor-1 (IGF-1) and IGF-2 also share many similarities concerning their biologic activities. Both regulate cell growth and proliferation. Close interactions

between estrogen and IGFs were reported in neuroendocrine tissues but also seem to play a role in mediating estrogen-dependent promotion of neuronal survival and neuroprotection *(66–68)*. Garcia-Segura et al. *(66)* demonstrated that IGF-1 antisense treatment significantly blocked estrogen-stimulated neuronal survival of cultured hypothalamic neurons induced by serum deprivation. Interestingly, pharmacologic inhibitors of IGF-1-inducible intracellular signaling pathways such as PI3-K and MAPK also blocked the effect of estrogen. Furthermore, estrogen can rapidly activate the PI3-kinase/Akt signaling cascade even in the absence of IGF-1 *(15)*. These data reveal the high degree of complexity of cellular estrogen action. Thus, the coordinated induction of IGF-1 expression and activation of IGF-1 signaling pathways appears to be necessary for the survival-promoting effects exerted by estradiol in the hypothalamus. Originating from in vivo studies that revealed a changing vulnerability of distinct neuronal populations against neurotoxic compounds such as kainic acid during the estrous cycle *(69)*, subsequent investigations supported this concept and demonstrated that estrogen and IGF-1 interactions are important for neuroprotective estrogen action *(70,71)*. These observations, and the fact that the cellular mechanisms downstream of activated IGF-1 signaling that are important for neuroprotection have been intensively studied and largely understood *(66,72)*, make the estrogen/IGF-1 connection an interesting and very promising system for practical medical application in the future.

Conclusion

We have attempted to summarize some of the recent data from our own and other research groups that suggest that estrogen may serve as a neuroprotective substance in the brain. Numerous developmental studies have shown that estrogen stimulates neuronal differentiation and acts as an antiapoptotic factor, thereby promoting cell survival. In addition to these in vitro studies, clinical trials (i.e., estrogen substitution in postmenopausal women) have revealed a positive influence of estrogen on certain neurologic and neurodegenerative diseases. Despite these merits, our knowledge of the cellular mechanisms responsible for transmitting neuroprotective estrogen effects is sparse. Ontogenetic studies suggest that different intracellular signaling cascades are involved in mediating protective estrogen effects. This includes (1) nuclear as well as membrane receptors for estrogen; (2) the regulation/activation of distinct intracellular signal transduction pathways (i.e., MAPK, PI-3 kinase, NFκ-B signaling); and, more important, (3) reciprocal estrogen–growth factor interactions (neurotrophins, TGF-β and TGF-α families, IGFs). A major goal of future research efforts will be to analyze whether and to what extent mechanisms responsible for the establishment of trophic estrogen effects during ontogeny also participate in the mediation of neuroprotection in the adult CNS.

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