Review

Cellular and Molecular Mechanisms of Estrogen Regulation of Memory Function and Neuroprotection Against Alzheimer's Disease: Recent Insights and Remaining Challenges

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This review focuses on recent advances in our knowledge of estrogen action in the brain. The greatest amount of attention was devoted to those studies that impact our understanding of estrogen regulation of memory function and prevention of degenerative diseases associated with memory systems, such as Alzheimer's disease. A review of recent advances in our understanding of estrogen receptors, both nuclear and membrane, is also presented. Finally, these data are considered in regard to their relevancy to the use of estrogen replacement therapy for cognitive health throughout menopause and the development of an estrogen replacement therapy designed for the unique requirements of the brain.

The relationship between memory function and estrogen was first noted clinically as women entering menopause frequently voiced complaints of memory and concentration difficulties (Sherwin 1999). In fact, changes in cognitive functioning have long been associated with menopause (Neurgaren and Kraines 1965). The shift from observation of this phenomenon to scientific human study occurred in the mid-1980's when Sherwin and colleagues began a systematic analysis of the impact of estrogen loss and replacement on memory function (Sherwin 2000). Results of these analyses demonstrated that verbal memory declined with the loss of estrogen and was restored to premenopausal levels when estrogen replacement therapy was instituted immediately following menopause (Sherwin 1988). An earlier important discovery relevant to estrogen effects on memory function came from basic sciences studies in the mid and late 1970's by Luine and colleagues, who found that 17 β -estradiol increased choline acetyltransferase activity which led to increased acetylcholine levels (Luine 1985). Based on these findings, Fillit investigated the impact of estrogen replacement therapy on cognitive function in women with Alzheimer's disease (Fillit et al. 1986). The results of this small clinical trial proved to be pivotal and led to multiple international clinical and epidemiological studies which in turn led to an intense study of the impact of estrogen on the mechanisms of memory in both animals and humans (Brinton 1998). Presented here is a review of the more recent studies, with an emphasis on conceptually link-

ing the many individual findings that have been generated in the past several years. In addition, an analysis of emerging data relevant to our understanding of estrogen regulation of memory function and prevention of Alzheimer's disease is presented.

Estrogen-Induced Neurotrophism and Underlying Mechanisms

Estrogen-induced neurotrophic effects are now well documented in vitro and also in vivo preparations (Fig. 1) (Toran-Allerand 1984, 2000; Brinton 1993; Murphy and Segal 1996; Brinton et al. 1997b, 2000; Woolley, 1999). Investigations to determine the mechanism of estrogen-induced neurotrophism have pointed to a temporal cascade of estrogen-inducible effects that are mediated by separate but potentially interacting pathways. It also appears that blockade of any component of the cascade blocks the neurotrophic effect of estrogen. The earliest phase of estrogen-inducible neurotrophism, filopodial outgrowth, can occur within minutes of exposure (Brinton 1993) and the most likely mechanistic candidate appears to be estrogen receptor dependent activation of a member of the Rho family of GTPases, Rac 1B (Dumontier et al. 2000). The second phase, the development of stable dendritic spines, appears to be mediated by an NMDA receptor dependent mechanism through estrogen activation of a src tyrosine kinase that phosphorylates the NMDA receptor (Yu et al. 1997; Chen and Brinton 2000). The third phase appears to involve stabilization of the spines and an estrogen-induced decrease in the GABAergic input dependent to CA1 pyramidal neurons (Murphy et al. 1998).

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Figure 1 Photomicrographs of control and conjugated equine estrogen-treated hippocampal neurons. (A) Two cultured hippocampal neurons grown for 24 h under control conditions (*upper box*) and in the presence of 1 ng/mL conjugated equine estrogens (CEEs). Note the greater abundance of branching and morphological complexity in the CEEs treated neuron. (B) Two images of the same hippocampal neuron following 3 d of culture. The *upper box* shows the neuron at time 0 and the *lower box* shows the same neuron after 60 min exposure to CEEs (100 ng/mL). The circled area highlights the interaction between the neuron in the *center* and the neuron at the *top* of the microscope field. The merging of the two processes indicates the initiation of synapse development. Note also that several of the processes have elongated over the 60-min observation period. Bar = 15 μ m. Modified from Brinton et al. (2000).

The first step in the estrogen-inducible neurotrophic cascade is the promotion of filopodial outgrowth. Early studies by Brinton showed that 17 β -estradiol induced outgrowth of filopodia within minutes of exposure (Brinton 1993). These filopodia serve as an early guidance support for dendritic spine maturation (Harris 1999). The Rac 1B pathway, a member of the Rho family of GTPases involved in actin organization and expressed in the developing nervous system, was initially found by Dumontier and colleagues to promote the formation of numerous filopodia (Dumontier et al. 2000). Maggi and coworkers found that in SK-N-BE neuroblastoma cells transfected with estrogen receptor alpha (ER α) or estrogen receptor beta (ER β), 17 β -estradiol induced two distinct morphological phenotypes (Chu and Fuller 1997). ER α activation induced an increase in neurite length and number, whereas ER β activation modulated only neurite elongation. ER α -dependent, but not ER β -dependent, morphological changes were observed only in the presence of the active form of the small G protein Rac1B. These data provide the first clear evidence that,

in a given target cell, ER α and ER β may play distinct biological roles and support the hypothesis that 17 β -estradiol activates selected intracellular signaling pathways depending on the receptor subtype bound.

The case for an NMDA receptor dependent mechanism is supported by morphological studies from both in vitro and in vivo preparations, by electrophysiological data from hippocampal slice preparations and by biochemical analyses using intracellular calcium imaging in both dissociated hippocampal neurons and hippocampal slices. A direct effect of estrogens on neuronal process outgrowth was observed by Brinton and colleagues (Brinton et al. 1997a). In these studies, morphological analyses were conducted using dissociated cortical and hippocampal neurons in which synaptic contacts were absent. Results of these studies demonstrated that 17 β -estradiol and other select neurotrophic estrogens induced a significant increase in the outgrowth of both macro- and micro-morphological features (Brinton et al. 1997a,b; 2000). The estrogen-inducible increase in neuronal process outgrowth occurred in neurons that had not yet made synaptic contacts and was completely blocked by an antagonist to the glutamate NMDA receptor. Thus, the effect of the neurotrophic estrogens is direct and not dependent upon synaptic interactions between neurons.

These in vitro findings were paralleled by the in vivo results of Woolley and colleagues. The neurotrophic findings by this group, in which 17 β -estradiol induced a hormone-dependent, estrous-cycle-phase-dependent and NMDA receptor dependent increase in dendritic spines on the apical dendrites of hippocampal CA1 pyramidal neurons (Woolley and McEwen 1994), are now well known. Importantly, Woolley and colleagues went on to determine that the estrogen-inducible spines are associated with an increase in synapses (Woolley et al. 1997). The new estrogen-inducible spines predominantly form synapses with preexisting boutons, to create a greater proportion of multiple-synapse boutons that increases the number of postsynaptic spines synaptically coupled to a single presynaptic bouton (Woolley 1999). That these synapses are excitatory is supported by the data of Weiland et al. (1992) who found that the increase in estrogen-inducible synapses was paralleled by an increase in NMDA receptor agonist binding sites and NMDAR1 subunit immunoreactivity (Gazzaley et al. 1996; Weiland 1992). Although Woolley and colleagues found little evidence for axonal sprouting in their studies, an increase in the presynaptic protein synaptotagmin I mRNA was found to be up-regulated in an estrous-cycle-dependent manner in CA3 pyramidal neurons that make synaptic contacts in CA1 (Crispino et al. 1999). These data indicate the possibility for estrogen induction of both post- and presynaptic modifications.

Several laboratories applied electrophysiological strategies to determine whether the morphological and biochemical evidence implicating a role of glutamate receptors

in estrogen-induced neurotrophism was paralleled by electrophysiological evidence. In slice preparations, Foy and colleagues found that estradiol significantly increased both AMPA glutamate-generated EPSPs—confirming earlier studies by Moss and coworkers (Wong and Moss 1994)—and NMDA receptor mediated EPSPs (Foy et al. 1999). In cultured hippocampal slices, Murphy and Segal found that estradiol induced an increase in both the basal and apical dendritic spines of CA1 pyramidal neurons in cultured slice preparations, and that E2 induced higher peak Ca^{2+} levels in both spines and dendrites of estradiol-treated CA1 neurons which was blocked by an NMDA receptor antagonist (Pozzo-Miller et al. 1999). Brinton and colleagues using dissociated cultured hippocampal neurons devoid of synaptic contacts, found that neurotrophic estrogens significantly potentiated NMDA glutamate-induced rises in intracellular calcium levels that were selectively inducible in the presence of NMDA selective agonists and blocked by NMDA receptor antagonists (Chen et al. 1998). To determine the mechanism of estrogen-induced potentiation of NMDA receptor function, these investigators found that blockade of tyrosine phosphorylation blocked the estrogen-inducible potentiation. These data indicate that estrogen potentiation of the NMDA receptor is through a tyrosine kinase-induced phosphorylation of the NMDA receptor (Figure 2). Baudry and coworkers showed that blockade of src also blocked estrogen potentiation of NMDA-induced long-term potentia-

tion (Bi et al. 2000). Brinton and coworkers went on to show that estrogen receptors α and β both bind to the tyrosine kinase src in an estrogen-dependent manner to activate src, which then phosphorylates the NMDA glutamate receptor (Whitfield et al. 1999). Src phosphorylation of the NMDA receptor results in an increase in calcium influx (Yu et al. 1997) and, presumably, an increase in downstream calcium-dependent responses such as an increase in phospho CREB. In fact, Murphy and Segal (1997) found that estradiol exposure increases phosphorylation of CREB and that blockade of CREB phosphorylation blocked estradiol-induced spine formation in cultured hippocampal neurons.

The case for estrogen regulation of GABAergic inhibition as a partial mechanism for estrogen-induced spine formation is supported by both in vitro and in vivo morphological studies, by electrophysiological studies, and by recent data investigating estrogen regulation of specific isoforms of GAD. Murphy and Segal were the first to demonstrate a role for estrogen-induced decrease in GABA synthesis in hippocampal interneurons (Murphy et al., 1998). Exposure of hippocampal cultures to estradiol for 1 d caused a marked decrease in the GAD content of the interneurons and a concomitant decrease in the number of GAD-positive neurons. Electrophysiologically, they found that estradiol transiently suppressed GABAergic inhibition to CA1 pyramidal cells, which may be an initial step in estrogen regulation of dendritic spines in vivo. These investigators

postulated that the reduction of inhibitory input results in an increase in the relative level of excitatory activity on pyramidal cells. Consistent with their hypothesis, they found that GABAergic miniature IPSCs were reduced in both size and frequency by estradiol, whereas miniature EPSCs increased in frequency (Murphy et al. 1998). Both the Murphy and Woolley laboratories have detected estrogen nuclear receptor alpha in GAD positive interneuron (Murphy et al. 1998; Hart and Woolley 2000). In vivo studies by Woolley and colleagues revealed that estradiol transiently decreased the density of GAD labeled cells in both the stratum oriens and radiatum of CA1 24 h after one injection of estradiol. Within 48 h of a second estradiol injection, the density of GAD-positive cells had recovered. Whole-cell voltage clamp recordings showed changes in synaptic inhibition of CA1 pyramidal cells that paralleled differences in GAD expression. In hippocampal slices derived from estradiol-treated animals, the amplitude of eIPSCs and frequency of mIPSCs were decreased at 24 h following the first estradiol injection and were increased by 48 h after a second estradiol injection (Rudick and Woolley 2000). Results from both the in

Figure 2 Schematic of estrogen potentiation of NMDA receptor function. Protein-protein interaction between estrogen receptor present at the neuron membrane (Estrogen mReceptor) and the tyrosine kinase, Src, leads to phosphorylation of the NMDA receptor (Yu et al. 1997) to increase calcium conductance through the channel and a rise in intracellular calcium (Chen and Brinton 2000).

Figure 3 Cellular, biochemical, and genetic mechanisms of Alzheimer's disease. Select neuro-protective estrogens have been found to modify multiple aspects of the pathogenesis of Alzheimer's disease. Estrogen can regulate both the generation of and the toxicity of β -amyloid. In addition, estrogen can decrease the damage induced by free radicals generated by β -amyloid and microglial. Estrogen can protect against the secondary insult induced by excessive leakage of glutamate from damaged neurons to reduce intracellular calcium levels. Estrogen can regulate expression of anti-apoptotic genes, such as increasing bcl-2 expression. Lastly, estrogen can increase microglial uptake of β amyloid to potentially decrease β amyloid load in the brain. (See text for further details on each of the mechanisms.) Modified from Ezzell (1995) and Brinton and Yamazaki (1998).

vitro and in vivo preparations indicate that estradiol transiently suppressed GABAergic inhibition to CA1 pyramidal cells; the suppression was followed by a rebound in both GAD synthesis and GABA-mediated inhibition. These data indicate that estrogen inhibition of GABAergic inhibitory influence creates a permissive milieu for the development and transient maintenance of estrogen-induced spines.

Functional Significance of Estrogen-Induced Spines

Definitive analysis of the behavioral significance of estrogen-induced spines has emerged only recently. Findings from the Williams laboratory have provided correlative but convincing data indicating that memory function is linked to estrogen-inducible spine formation. Sandstrom and Williams found that priming with estradiol induced an improvement in memory retention on a delay matching-to-place task and that this improvement was modulated by proges-

terone. The changes in memory function paralleled hormone-induced changes in CA1 pyramidal neuron dendritic spine density, with periods of elevated spine density associated with improvements in memory retention. (Sandstrom and Williams 2001).

The Menopausal Female Brain, Memory Function, and Estrogen-Inducible Spines and Neurogenesis

Analyses of memory function in postmenopausal women indicate that estrogen replacement therapy given immediately following surgically induced menopause can prevent memory deficits induced by a loss in circulating estrogen (Phillips and Sherwin 1992). The findings of Phillips and Sherwin (1992) show that estrogen replacement therapy sustains and preserves memory function at premenopausal levels. A critical factor in determining efficacy appears to be the intervening time period between the loss of estrogen and the initiation of estrogen replacement therapy. Data from the Resnick laboratory indicates that the time window for complete reversal of estrogen-deficit induced memory loss appears to be limited (Resnick et al. 1997).

In a cross-sectional study of human females, Resnick and colleagues found that women who were current users of hormone replacement therapy performed significantly better on a visual memory test that has been shown to predict the onset of Alzheimer's disease, than women who had never received hormone replacement therapy (Resnick et al. 1997). A subset of the women who never used hormone replacement therapy was studied longitudinally. At the first memory testing, none of the women were using hormone replacement therapy. During the intervening six years between the first and second testing periods, a subset of these women began hormone replacement therapy. At the second testing, women who did not receive any hormone replacement therapy showed a significant decline in memory retention compared to their performance on the first test. In stark contrast, the women who received hormone replacement therapy during the intervening six years showed no decline in memory function

relative to their performance on the first test. The disturbing aspect of this study was the observation that the women who elected to receive hormone replacement therapy between the first and second testing, although able to maintain the level of memory function achieved at the first testing (six years before), did not display an increase in the level of memory function to match that of the women who had received hormone replacement therapy earlier in menopause.

Another factor regulating estrogen effects on memory function may be the dosing regimen. Studies by the Williams laboratory of aging female animals showed that long-term gonadal steroid deprivation in aged female rats results in a paucity of dentate granule cell dendritic spines compared with young females deprived short-term. They also found that long-term estrogen replacement (at either high or low levels) in aged females did not reverse the decline in dentate granule cell spine density. In contrast, short-term estradiol replacement increased the dentate granule cell spine density in the aged females to the levels normally observed in young adult females. These data indicate that in females, the response of rat dentate granule cells to aging depends on the time estrogen replacement is instituted and also on the temporal pattern of estradiol replacement. These investigators also observed a sexual dimorphism. Long-term gonadal steroid deprivation in the aged males' dentate granule cells did not decrease dentate granule cell dendritic spines compared with short-term deprived young males. In contrast, gonadectomized aged males treated with short-term estradiol replacement had decreased spine density (Miranda et al. 1999).

If it is presumed that estrogen-induced neurotrophism observed in the *in vitro* and *in vivo* animal studies generalizes to the human brain, if estrogen-inducible spine formation was the sole mechanism for estrogen effects on memory function, the level of memory function in the women who delayed receiving hormone replacement therapy should have risen to that of the women who received hormone replacement therapy beginning at the onset of menopause. The data from the Williams and the Resnick laboratories indicate that some factor is lost, as a function of the estrogen-deprived state, or that a degenerative process occurred as a result of estrogen deprivation. Moreover, their data indicate the existence of a critical window for estrogen protection against memory function decline. The recent discovery by Gould and coworkers of estrogen-inducible neurogenesis might provide the missing factor (Tanapat et al. 1999).

Estrogen-Induced Neurogenesis

In addition to estrogen-inducible spines, Gould and coworkers have found that estrogen can induce neurogenesis in the hippocampus (Tanapat et al. 1999). Stereological analyses of the numbers of BrdU-labeled cells revealed that females

produced more cells in the dentate gyrus than did males. The production of new hippocampal cells in females was regulated by ovarian hormone levels; ovariectomy diminished the number of BrdU-labeled cells, but this effect was reversible by estrogen replacement. A natural fluctuation in cell proliferation was also noted; females produced more cells during proestrus (when estrogen levels are highest) compared with estrus and diestrus. Many of these cells acquired neuronal characteristics, including the formation of dendrites and expression of Turned-On-After-Division 64 kDa, a marker of immature granule neurons, and the calcium-binding protein calbindin, a marker of mature granule neurons. However, the increase in neuronal number appears to be transitory. The number of pyknotic cells and the number of BrdU-labeled cells revealed that many of the newly formed dentate granule neurons degenerated. The loss of estrogen-inducible neurons may reflect the generation of a time- and task-specific set of neurons or may reflect the absence of an additional factor necessary for survival. One such factor discovered by Shors and colleagues is learning (Gould et al. 1999). These investigators observed that learning enhanced the survival of neurons generated prior to training. The anti-mortality effects of certain types of learning in adult-generated cells might occur only during a specific "sensitive period" following the production of those new cells. It remains to be determined whether interventions such as learning could reduce the mortality of estrogen-induced neuron formation.

Estrogen-Induced Neuroprotection and Underlying Mechanisms

Estrogen Replacement Therapy and the Risk of Alzheimer's Disease

Multiple epidemiological studies indicate that estrogen/hormone replacement therapy can significantly reduce the risk of Alzheimer's disease (Yaffe et al. 1998; Henderson 2000a). These data, which were rapidly conveyed to the clinical and lay communities, stand as a unique and profoundly important series of observations. Estrogen replacement therapy was the first therapeutic intervention found to significantly reduce the risk of Alzheimer's disease. Moreover, it remains the best characterized of several therapeutic interventions, such as nonsteroidal anti-inflammatory agents, proposed to reduce the risk of Alzheimer's disease (Brinton 1998; Brinton and Yamazaki 1998).

If the epidemiological projections prove to be correct, the widespread use of estrogen replacement therapy during menopause could reduce the number of women in the United States with Alzheimer's disease by more than one million (Henderson 2000a). Thus far the human data are observational; however, several randomized placebo-controlled double-blind clinical trials are currently underway in the United States and the United Kingdom. The best known

of these is the Women's Health Initiative of the National Institutes of Health, which is a largescale long-term (with followup for at least six years) study in which healthy women are randomized to receive conjugated equine estrogens (with or without a progestin) and monitored for health endpoints. It includes 167,000 women aged 50–79; 67,000 in clinical trials and 100,000 in observational studies (<http://www.nhlbi.nih.gov/whi/>). One sector of the Women's Health Initiative will evaluate the role of estrogen replacement therapy in 7525 women for both symptom onset and progression of Alzheimer's disease (Shumaker 1996). The results of this study will begin to emerge ca. 2006. A recent report from Brinton and colleagues has found that the form of estrogen replacement therapy, conjugated equine estrogens, used in the Women's Health Initiative is both neurotrophic and neuroprotective against toxic insults associated with Alzheimer's disease in hippocampal, cortical, and basal forebrain neurons (Brinton et al. 2000).

Although the existing clinical data on estrogen replacement therapy and prevention of Alzheimer's disease are very encouraging, the data on the treatment of women with existing disease are not. Despite the positive benefits reported in earlier small open-label clinical trials (Birge 1997), two randomized, double-blind, placebo-controlled, parallel-group trials found that both short-term (Henderson et al. 2000b) and long-term (one year) estrogen therapy (Mulnard et al. 2000) did not improve the symptoms of most women with Alzheimer's disease. Thus, it would appear that estrogen maintains and sustains neuronal viability to prevent degenerative disease, whereas it appears to be ineffective in reversing the degenerative disease process.

Mechanisms of Estrogen-Induced Neuroprotection

Estrogen has been found to protect against a wide range of toxic insults including free radical generators (Behl et al. 1997; Green et al. 1997; Brinton et al. 2000), excitotoxicity (Singer et al. 1999; Singh et al. 1999; Brinton et al. 2000), β -amyloid-induced toxicity (Brinton et al. 2000), and ischemia (Wise et al. 2000; Green et al. 2001). It is not yet clear whether there is one unifying neuroprotective mechanism induced by estrogen, whether estrogen induces multiple mechanisms that are selectively neuroprotective against select neurotoxins, or whether it is a combination of the two. The available data indicate that it is the latter; that is, estrogen induces a broad class of protective mechanisms that confer protection against a broad spectrum of toxic insults. Which, if any, of these mechanisms are directly responsible for conferring a reduction in the risk of neurodegenerative disease remains to be determined.

Estrogen neuroprotective effects are multifaceted and encompass mechanisms ranging from the chemical to the genomic. The data on the neuroprotective effects of estrogens indicate three levels of estrogen action (chemical, biochemical, and genomic) that fall within three broad mecha-

nistic categories (antioxidant, defense, and viability). It is not completely determined to what extent each of these levels of action lead to the overall neuroprotective effect; however, each appear to make a significant, if not obligatory, impact on the neuroprotective effects of estrogens. In addition, it is now clear that not all neuroprotective estrogens activate all three sites of action. A broad range of estrogens and estrogenic molecules, such as isoflavones and phytoestrogens, can promote the chemical antioxidant effects of estrogen; a smaller subset of these chemically active estrogens can activate biochemical cascades required for neuroprotection, and an even smaller subset can activate the genomic mechanisms of estrogen-induced neuroprotection (Maruyama et al. 1998; Behl et al. 2000; Brinton and Chu 2000; Nilsen and Brinton 2000). Lastly, neuroprotective estrogens activate mechanisms that have the potential of protecting cells from a broad range of neurodegenerative insults associated with many neurodegenerative conditions including Alzheimer's disease, and also can activate mechanisms specifically relevant to Alzheimer's disease.

Estrogen-Inducible Chemical Antioxidant Properties

An antioxidant effect of estrogens against a wide variety of free radical generators has been described in multiple organ systems and cell types (Behl et al. 2000). 17β -estradiol can attenuate the lipid peroxidation induced by β -amyloid exposure, glutamate toxicity, or FeSO_4 exposure and reduce intracellular peroxides induced by β -amyloid, haloperidol and H_2O_2 (Green et al. 2000). Simpkins and coworkers in the United States (Green et al. 1997) and Behl in Germany (Behl et al. 1997) have identified the C3 position on the phenolic A ring of estrogens as the critical chemical requirement for estrogenic antioxidant function in the central nervous system (CNS). The phenolic A ring estrogens are inhibitors of lipid peroxidation with an efficacy equivalent to α -tocopherol (Green et al. 2000). Behl has shown recently that the phenolic ring structure is both necessary and sufficient for the antioxidant properties of estrogens (Behl et al. 2000). Thus, the phenolic ring structure permits phytoestrogens, isoflavones, and flavones to all exert an estrogenic-like antioxidant property (usually at lower potency) (Chen et al. 1998). Finally, an abundant number of estrogenic molecules can exert protection against free radical damage (Green et al. 1997; Chen et al. 1998; Brinton and Chu 2000). The large array of neuroprotective estrogens contrasts with the very small number of estrogenic molecules that can promote neuronal defense and viability mechanisms (Pike 1999; Brinton and Chu 2000).

A major concern with several earlier studies was the high micromolar concentrations of estrogens used to exert an antioxidant effect (Goodman et al. 1996). Such concentrations make it unreasonable to presume that an antioxidant effect would be physiologically relevant. However, Green et al. (1998) found that the neuroprotective potency

of estrogens could be markedly reduced to well within physiological range in the presence of glutathione. They reasonably suggested synergy of redox cycling between estrogens and glutathione to account for the drastic increase in estrogenic potency in the presence of glutathione.

Estrogen-Inducible Defense Biochemical and Genomic Signaling

Several biochemical mechanisms have been proposed to underlie estrogen-inducible neuroprotection against toxic insults such as β -amyloid and glutamate excitotoxicity. The best known of these is estrogen activation of the MAP kinase signaling pathway leading to an increase in phosphorylated extracellular-signal regulated kinase 1 (ERK1), a component of the mitogen-activated protein kinase (MAPK) pathway. Singh and Toran-Allerand were the first to report that estradiol activated the MAP kinase signaling pathway in brain (Singh et al. 1999). Singer et al. and others (Singer et al. 1999; Nilsen and Brinton 2000) followed up this observation by demonstrating that blockade of this pathway abolished estrogen-induced neuroprotection against glutamate induced toxicity.

The downstream mechanisms by which MAP kinase activation leads to neuroprotection remain to be elucidated. What is clear, however, is that although MAP kinase activation appears to be necessary for neuroprotection it is not sufficient to confer full neuroprotection (Nilsen and Brinton 2000). Another estrogen-activated pathway that potentially interacts with the estrogen-inducible MAP kinase signaling and confers neuroprotection is the Akt/protein kinase B pathway. Recently, Singh (2000) and Wise (Wilson and Liu 2000) reported estradiol activation of the Akt/Protein kinase B kinase, which can mediate anti-apoptotic signaling through increased expression of the anti-apoptotic protein bcl-2. The Akt pathway provides a link with two other estrogen-inducible responses that heretofore have not been clearly linked to a functional outcome. Akt has been shown to induce bcl-2 expression via a CREB-dependent mechanism in PC12 cells (Pugazhenthil et al. 2000). These data provide a framework in which to understand the functional significance of estrogen-inducible bcl-2 expression and increases in phosphoCREB. Bcl-2 contains a cAMP-response element and the transcription factor CREB has been identified as a positive regulator of Bcl-2 expression (Pugazhenthil et al. 2000). Activation of this neuroprotective pathway would provide a unifying signaling cascade that would mechanistically clarify earlier observations of estrogen-induced increases in phospho-CREB and bcl-2 with the recent observations of estrogen-inducible Akt phosphorylation. Interestingly, one mechanism proposed to be involved in the pathogenesis of Alzheimer's disease is mutated presenilin-1 induction of apoptosis and down-regulation of Akt (Weihl et al. 1999). If indeed presenilin-1 does down-regulate Akt in humans and promote apoptosis, it might be possible that

estrogen could at least partially attenuate the action of presenilin-1.

Estrogen Regulation of β -Amyloid Deposition and Microglial Function

The mechanisms described above activate protective responses that are efficacious against a broad range of neurodegenerative insults associated with Alzheimer's disease but are not unique to it. Gandy and coworkers have found a direct effect of estrogen on a critical component of the cascade of insults that lead to Alzheimer's disease, the generation of the β -amyloid peptide (Petanceska et al. 2000). These investigators found that prolonged ovariectomy resulted in a pronounced increase in brain β -amyloid levels. Total brain β -amyloid in the ovariectomized animals was increased by 1.5-fold on average as compared to intact controls. Estradiol treatment significantly reversed the ovariectomy-induced increase in brain β -amyloid levels. Based on these results, they infer that cessation of ovarian estrogen production in postmenopausal women might facilitate β -amyloid deposition by increasing the local concentrations of β -amyloid 40 and 42 peptides in brain. In addition, their finding that estradiol treatment was associated with diminution of brain β -amyloid levels suggests that modulation of β -amyloid metabolism may be one of the ways by which estrogen replacement therapy prevents or delays the onset of Alzheimer's disease in postmenopausal women.

Another mechanism by which estrogen replacement therapy could impact risk of Alzheimer's disease has been reported by Rogers and colleagues (Li et al. 2000). These investigators found that 17 β -estradiol significantly increased the uptake of β -amyloid by microglia. The expression of ER β was also up-regulated by estrogen treatment (Li et al. 2000).

Estrogen Receptors

In recent years, remarkable discoveries have dramatically advanced our understanding of estrogen sites and mechanisms of action in the brain and in particular in those brain regions responsible for learning and memory. These advances include the discovery of a second estrogen receptor ER β (Kuiper et al. 1998), the discoveries of multiple isoforms of this new estrogen receptor, the discovery of adaptor proteins that regulate estrogen receptor transcription factor function, and an emerging widespread recognition of the importance of membrane sites of estrogen action in the brain. Not surprisingly, old dogmas such as the distinction between nuclear estrogen receptors and membrane estrogen receptors are beginning to fade and in their place is an evolving conceptualization that expands the site of action for estrogen receptor proteins that were traditionally thought to be functional only in the nucleus.

Nuclear Estrogen Receptors

To date there are two well-defined classes of estrogen re-

ceptors (ER), estrogen receptor alpha (ER α) and beta (ER β) (Warner et al. 1999). These two forms of estrogen receptors are encoded by separate genes with the gene for ER α localized to chromosome 6 and the gene for ER β localized to chromosome 14 (Warner et al. 1999). Both receptors are members of the nuclear receptor superfamily of ligand activated transcription factors (Whitfield et al. 1999). The ER- β gene encodes a protein with high homology to ER α in the DNA binding domain (>95%) and lesser homology to ER α in the ligand binding domain (59%) (Warner et al. 1999). Despite the relatively low homology in the ligand binding domain, the binding characteristics for 17 β -estradiol at the two estrogen receptors are remarkably similar (Kuiper et al. 1997). Multiple splice variant isoforms exist for both ER α (seven and counting) and ER β (five and counting) (Chu and Fuller 1997; Hopp and Fuqua 1998; Maruyama et al. 1998; Price et al. 2000). These splice variants have largely been observed in transformed cells and in the ER α knockout (ERKO α), ER β knockout (ERKO β), or in the ER α /ER β double knockout animals. It remains to be determined what role these splice variants play in the brain. However, an intriguing theory is that a subset of the splice variants accounts for cytoplasmic localization of estrogen receptors (DeFranco et al. 1998).

Adaptor/Coregulator Proteins that Interact with Nuclear Estrogen Receptors

The discovery of an increasing number of adaptor proteins that regulate estrogen receptor transcriptional activity has yet to have a major impact on neuroscience research but undoubtedly will do so in the not too distant future. Coregulator or adaptor proteins can modulate the efficacy and direction of steroid-induced gene transcription and are thought to be important determinants of efficacy for estrogenic molecules that bind to the ER (McKenna et al. 1999). Coregulators include coactivators ERAP160 and 140, and RIP160, 140, and 80, steroid receptor coactivator 1 (SRC 1), and transcriptional intermediary factor 2 (TIF 2) which were biochemically identified by their ability to specifically interact with the hormone binding domain of the receptor in a ligand-dependent manner (see Figure 4) (McKenna et al. 1999; Brinton and Nilsen 2001; Kushner et al. 2000). When cotransfected with ER, these coactivators are capable of augmenting ligand-dependent transactivation. For example, the coactivator RIP140 interacts with the ER in the presence of estrogen, and this interaction enhances transcriptional activity between 4- and 100-fold, depending on promoter context. The interaction between ER and coregulators can be negatively regulated by antiestrogens, as in the case of tamoxifen and raloxifene which block the interaction between ER and coactivators (Brzozowski et al. 1997; Shiau et al. 1998).

In addition to the coregulator proteins, the phospho-CREB-binding protein (CBP) and the related p300 have been

Figure 4 Model of estrogen receptor regulation of gene expression and interaction with coregulator proteins that regulate estrogen nuclear receptor function. Upon binding of estrogen (E) to an inactive estrogen receptor, the receptor is activated and two receptor-ligand monomers dimerize and bind to the estrogen response element (ERE). Once bound to the ERE, the ER uses AF-1 and AF-2 (not shown) to stimulate transcription from the promoter. Coactivators such as RIP140, CBP and SRC-1 bind to and link the hormone receptor with the general transcription factors (GTFs) and RNA polymerase of the transcription machinery to alter gene transcription. Modified from Brinton and Nilsen (2001) and Kushner et al. (2000).

demonstrated to be ER-associated proteins and are involved in ligand-dependent transactivation (Katzenellenbogen et al. 2000). Cell-specific expression of the coregulator and other modifier proteins can have a profound impact on whether ER activates or suppresses gene transcription and whether estrogen alternatives exert an agonist or antagonist effect (McKenna et al. 1999). Moreover, interactions between the estrogen receptor protein and coactivator proteins can also permit the ER to activate transcription at alternative elements such as AP-1 sites (Webb et al. 1999; Kushner et al. 2000). In this circumstance the ER participates as part of the coactivator complex for Jun/Fos. ER binds to the coactivators, CBP and GRIP1, that have been recruited by Jun/Fos and through this contact triggers these coactivators into full activity.

Distribution of ER α and ER β in the Brain and Spinal Cord

Shughrue and Merchenthaler have conducted the most extensive mapping of the mRNA and immunoreactivity for both ER α and ER β in the brain and spinal cord (Shughrue et

al. 1997). The results of their studies revealed the presence of ER α and ER β mRNA throughout the rostral-caudal extent of the brain and spinal cord. Neurons of the olfactory bulb, supraoptic, paraventricular, suprachiasmatic, and tuberal hypothalamic nuclei, zona incerta, ventral tegmental area, cerebellum (Purkinje cells), laminae III–V, VIII, and IX of the spinal cord, and pineal gland contained exclusively ER β mRNA, whereas only ER α hybridization signal occurred in the ventromedial hypothalamic nucleus and subfornical organ. Perikarya in other brain regions (including the bed nucleus of the stria terminalis, medial and cortical amygdaloid nuclei, preoptic area, lateral habenula, periaqueductal gray, parabrachial nucleus, locus ceruleus, nucleus of the solitary tract, spinal trigeminal nucleus, and superficial laminae of the spinal cord) contained both forms of ER mRNA. Although the cerebral cortex and hippocampus contained both ER mRNAs, the hybridization signal for ER α mRNA was surprisingly weak compared with ER β mRNA (Shughrue et al. 1997). This difference in brain localization suggests that these two receptors mediate different functions. Receptor knockout studies in mice of both α and β forms of ER also indicate that these receptors mediate fundamentally different functions. ER α knockouts show significant reproductive deficits, whereas ER β knockout mice do not. At least some of the ER β protein localized by immunohistochemistry appears to be cytoplasmic, even in the presence of ligand (Razandi et al. 1999).

Membrane Estrogen Receptors

The site(s) of estrogen action at the membrane has returned to prominence as an intense area of investigation. The existence of a putative membrane ER has been supported by many functional studies over the past 20 years but research efforts were stymied by the inability to identify and purify a membrane ER (McEwen and Alves 1999). Support for the existence of a membrane ER comes from studies of the rapid, nongenomic effects of E2. Within a few seconds to several minutes, E2 can activate multiple signaling cascades (Aronica et al. 1994; Singh et al. 1999; Nilsen and Brinton 2000; Singh 2000), can induce rapid neuronal electrophysiological changes (Wong and Moss 1994; Foy et al. 1999) and cellular responses (Brinton 1993). Transcriptional modulation, long considered to be the primary function of the nuclear ER, can also be positively or negatively regulated through the membrane-associated ER activation of second messenger signaling pathways (Katzenellenbogen 1996). This action appears to require modification of cytosolic signal transduction pathways, such as the ERK/MAPK (extra-cellular-signal-regulated kinase/mitogen-activated protein kinase) (Aronica et al. 1994; Singh et al. 1999; Nilsen and Brinton 2000; Singh 2000).

Transfection of ER α and ER β cDNAs revealed membrane and nuclear ER derived from single transcripts with near-identical affinities for 17 β -estradiol (Razandi et al.

1999). The density of ER receptors was greatest in the nucleus but clearly detectable at the membrane. Both of the membrane ERs activated G proteins, ERK, and cell proliferation, but there was novel differential regulation of c-Jun kinase activity by ER β and ER α . Dorsa and colleagues have found that both ER α and ER β activated MAP kinase and that these receptors appear to translocate from the cytoplasm to low-density lipid fractions, consistent with localization to the cell membrane (Wade et al. 2000).

For many years, Watson and colleagues have investigated the existence of a membrane receptor for estrogen. Using confocal scanning laser microscopy, they detected the existence of a membrane ER in pituitary tumor cells (Pappas et al. 1995). The development of antibodies to both ER α and ER β led to the discovery that immunologically similar, if not identical, proteins to ER α and ER β are localized within the cytoplasm (Shughrue et al. 1997; Milner et al., 2001).

Milner and colleagues have conducted the most extensive immunolabeling study of ER α localization in neurons (Milner et al. 2001). These investigators found that in addition to interneuronal nuclei, ER α was affiliated with the cytoplasmic plasmalemma of select interneurons and with endosomes of a subset of principal (pyramidal and granule) cells. ER α labeling was found dispersed throughout the hippocampal formation, but was slightly more numerous in CA1 stratum radiatum. Remarkably, almost one-half of the ER α -labeled profiles were unmyelinated axons and axon terminals that contained numerous small, synaptic vesicles. ER α -labeled terminals formed both asymmetric and symmetric synapses on dendritic shafts and spines, suggesting that ER α arise from sources in addition to inhibitory interneurons. Approximately 25% of the ER α was found in dendritic spines, many originating from principal cells. Within spines, ER α was often associated with spine apparatus and/or polyribosomes. This finding indicates that estrogen might act locally through ER α to regulate calcium availability, protein translation, or neuronal outgrowth. The remaining 25% of ER α -labeled profiles were astrocytes, often located near the spines of principal cells.

A third membrane-associated estrogen receptor, ER-X, has been proposed by Toran-Allerand and colleagues. These investigators have conducted extensive biochemical analyses (described previously in the section on neuroprotection) in which neither ER α or ER β appear to activate the MAP kinase ERK. It remains to be determined whether ER-X is a splice variant of either ER α or ER β . Singh and Toran-Allerand have gone on to identify a multi-molecular complex in association with ER, which contains HSP90 and signaling kinases such as Src, B-Raf, MEK, and ERK as well as (surprisingly) amyloid precursor protein (APP) (Toran-Allerand 2000). The association of ER with signaling kinases and other proteins led these investigators to propose that the ER-containing multi-molecular complex may be localized to

the neuronal membrane plasma membrane in association with caveolarlike structures. Localization with the caveolae would provide a compartment in which ER could interact with a host of signaling pathways requiring G proteins and kinases (Toran-Allerand 2000). A protein associated with the caveolae, caveolin-1, potentiates ER α -mediated signal transduction (Schlegel et al. 1999). Coexpression of caveolin-1 and ER α resulted in ligand-independent translocation of ER α to the nucleus as shown by both cell fractionation and immunofluorescence microscopic studies. Similarly, caveolin-1 augmented both ligand-independent and ligand-dependent ER α signaling. These results identify caveolin-1 as a new positive regulator of ER α signal transduction.

Therapeutic Challenge

In the United States in 2000, there were 41.75 million women over the age of 50 (<http://www.menopause.org/>) (North American Menopause Society, 2001). Approximately 31.2 million women are over the age of 55 yr; by the year 2020, this number is expected to be 45.9 million. Currently, a woman's life expectancy is estimated at 79.7 yr; today, a woman who reaches the age of 54 can expect to survive to the age of 84.3 yr. Approximately two-thirds of the total US population will survive to age 85 or older. Most women spend one-third to one-half of their lifetime in postmenopause. Currently, there are >470 million women over the age of 50 worldwide, and 30% of those will live to the age of 80 (North American Menopause Society 2001).

Age remains the greatest risk factor for developing Alzheimer's disease (Whitehouse 1997). Based on current epidemiological data, of the 18 million aged American women, 40–50% can be expected to manifest the histopathological changes of Alzheimer's disease (Henderson 2000a). The increasing number of women vulnerable to developing Alzheimer's disease is even more staggering when one considers that globally 800,000 people reach the age of 65 every month (Holden 1996; Whitehouse 1997). Alzheimer's disease is the most frequent cause of dementia and the leading cause of the loss of independent living and of institutionalization (Birge 1997; Whitehouse 1997). The care and treatment of persons afflicted with Alzheimer's disease results in more than 100 billion dollars in health care costs (Birge 1997; Whitehouse 1997).

Despite the encouraging epidemiological data indicating a reduced risk of developing Alzheimer's disease in women who have received estrogen replacement therapy (Yaffe et al. 1998), only 25% of eligible postmenopausal women elect to receive prescribed estrogen replacement therapy; of that number, ~50% discontinue use within the first year of therapy and >70% of those for whom it has been prescribed are not compliant (Hammond 1994). Moreover, only 20% of women prescribed estrogen are still compliant three years later (<http://www.asrm.com/Patients/BCHRT.html>; American Society for Reproductive Medicine 2001).

The principal reason that women forego estrogen replacement therapy is the fear of developing breast cancer (Hammond 1994). Therein lies the challenge.

One strategy devised to address this challenge is to develop estrogen alternatives that exert estrogen agonist properties in the brain, bone, and cardiovascular system while exerting estrogen antagonist action in the breast and uterus. Several such compounds exerting a mixed estrogen receptor agonist/antagonist profile have been developed, such as tamoxifen and raloxifene (Grese et al. 1998; Gustafsson 1998). Many more are in the pipeline. The question of whether estrogen alternatives such as phytoestrogens and selective estrogen receptor modulators (SERMs) are effective estrogens for the promotion of memory function and the prevention of degenerative disease in postmenopausal women remains unanswered, and will remain unanswered for decades due to the long periods of observation necessary to determine a reduced risk of developing Alzheimer's disease. To the extent that basic science data can be predictive, it is clear that phytoestrogens, tamoxifen, and raloxifene do not fulfill all the criteria of full estrogen agonists (Brinton et al. 1998; Chen et al. 1998; O'Neill et al. 1999). The challenge remains for neuroscientists to fully understand the mechanisms of estrogen action in the brain that lead to the promotion and maintenance of memory function and to the protection against degenerative diseases such as Alzheimer's. This mechanistic challenge is paralleled by the requirement to translate this basic scientific knowledge into a safe and efficacious estrogenic therapeutic for the maintenance of cognitive function and neural health for millions of women potentially at risk for developing Alzheimer's disease.

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