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REVIEW ARTICLE

The Critical Period for Neuroprotection by Estrogen Replacement Therapy and the Potential Underlying Mechanisms

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Abstract: 17β-Estradiol (estradiol or E2) is a steroid hormone that has been broadly applied as a neuroprotective therapy for a variety of neurodegenerative and cerebrovascular disorders such as ischemic stroke, Alzheimer's disease, and Parkinson's disease. Several laboratory and clinical studies have reported that Estrogen Replacement Therapy (ERT) had no effect against these diseases in elderly postmenopausal women, and at worst, increased their risk of onset and mortality. This review focuses on the growing body of data from *in vitro* and animal models characterizing the potential underlying mechanisms and signaling pathways that govern successful neuroprotection by ERT, including the roles of E2 receptors in mediating neuroprotection, E2 genomic regulation of apoptosis-related pathways, membrane-bound receptor-mediated non-genomic signaling pathways, and the antioxidant mechanisms of E2. Also discussed is the current evidence for a critical period of effective treatment with estrogen following natural or surgical menopause and the outcomes of E2 administration within an advantageous time period. The known mechanisms governing the duration of the critical period include depletion of E2 receptors, the switch to a ketogenic metabolic profile by neuronal mitochondria, and a decrease in acetylcholine that accompanies E2 deficiency. Also the major clinical trials and observational studies concerning postmenopausal Hormone Therapy (HT) are summarized to compare their outcomes with respect to neurological disease and discuss their relevance to the critical period hypothesis. Finally, potential controversies and future directions for this field are discussed throughout the review.

Keywords: Estrogen, neuroprotection, ischemic stroke, alzheimer's disease, parkinson's disease, menopause, critical period, hormone therapy.

1. INTRODUCTION: MENOPAUSE AND NEURODEGENERATIVE DISEASES

The process of menopause, whether by natural reproductive senescence, or by surgical removal of the uterus of one or both ovaries, leads to the induction of amenorrhea [1, 2] and corresponding physiological changes in women. Although this change typically occurs around 51 years of age, ovariectomy can lead to indications of menopause in younger women, though some hysterectomized or unilaterally ovariectomized women with one or both ovaries intact will lose menses but not technically undergo menopause until the loss of ovarian follicles occurs naturally, since they continue to produce estradiol (E2) [1]. The hormonal effect of diminished circulating E2 is commensurate with follicular senescence in the ovaries, and halted menstruation [2], accompanied by characteristic perimenopausal symptoms including depression and irritability, hot flashes with intense perspiration and elevated heart rate, vaginal discomfort such as dryness and dyspareunia-associated pain, variable loss of bladder control, and other discomforts [3].

As the duration of time increases from the cessation of E2, the risk increases for potentially deadly or debilitating conditions such as osteoporosis (due to decreases in bone density), heart attack, and atherosclerosis [2, 4]. Furthermore, cardiovascular diseases in post-menopausal women have been correlated with a higher risk for ischemic stroke, cognitive impairment especially following surgical menopause [5, 6], Parkinsonism [7], and Alzheimer's Disease (AD) [8], thus suggesting that E2 deficiency can indirectly lead to cerebrovascular diseases and dysfunction. For example, premenopausal women with circulating estrogen are

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reportedly less susceptible to stroke than men of the same age [9]. Conversely, women who experience premature menopause (≤40 years of age) and receive no further treatment with E2 show five times greater susceptibility to mortality from neurological disorders (*e.g.*, Parkinson's Disease and dementia), stroke, and coronary disease [4, 5, 7, 10] than women who have not experienced premature estradiol deprivation [11]. Furthermore, the incidence of stroke increases with age, and after age 55, 1 in 5 women are likely to develop a stroke, while for men of the same age, this condition afflicts only 1 in 6 [12].

Therefore, the loss of circulating E2 leads to several short-term and long-term physiological and pathological consequences for women, the risks of which are compounded for women who undergo early surgical menopause. Thus, Estrogen Replacement Therapy (ERT) is an appropriate treatment to minimize these risks. However, there is a critical window of opportunity following natural and surgical menopause in which ERT provides the maximum benefit in neuroprotection, delaying the effects of endogenous E2 depletion, but after which, the risks of ischemic stroke and neurodegenerative diseases are heightened by E2 administration. This review discusses experimental and clinical evidence for the critical window for estrogen therapy following menopause, the mechanisms underlying E2 neuroprotection, and the mechanisms governing the duration of the critical window following long-term estrogen deprivation, particularly in the context of cerebrovascular and neurodegenerative diseases such as ischemic stroke, Alzheimer's dementia, and Parkinsonism.

2. NEUROPROTECTIVE BENEFITS OF ERT AGAINST NEURODEGENERATIVE DISEASES

2.1. Benefits and Risks of ERT Identified in Clinical and Observational Studies of Premature- and Post-menopausal Women

Cognitive function in women is often measured using serum E2 as a biochemical marker since high levels of serum E2 have been correlated with significantly enhanced verbal working memory [13, 14]. In postmenopausal women, low levels of serum E2 have been associated with the prevalence of cognitive impairment, while estrogen hormone therapy (HT) in postmenopausal women resulted in stronger performance on neuropsychological cognition tests [14]. Additionally, in postmenopausal women undergoing current treatment with HT (any type of estrogen ± progestogens), the hippocampal volumes were revealed to be larger than those of women who had used HT in the past, women who had never used HT, and men [15]; exogenous E2 administration in these postmenopausal women provided protection against neuronal cell death caused by global ischemia [16].

Research by Schmidt *et al.* showed that the incidence of stroke, as well the degree of neurodegeneration due to ischemic injury, can be greatly reduced in postmenopausal women through estradiol replacement therapy (ERT) [17]. An observational cohort study revealed an association between a reduced risk of incident strokes as well as hemorrhagic strokes and treatment with estrogen therapy 0-5 years after menopause, regardless of regimen and duration, compared to never use patients [18]. Although, ERT has been identified as the most effective treatment for amelioration of menopausal symptoms, randomized controlled trials have verified that this treatment is also related to cardiovascular events. While no correlation was found between the routes of administration (oral, transdermal, or vaginal) or active ingredients (Conjugated Equine Estrogens [CEEs] or estradiol) and an increased risk of stroke when applied during the early stages of menopause, these variables bear careful consideration because later application of CEE was found to be associated with increased risk of hemorrhagic stroke [19].

A population-based study showed a significantly lower incidence of ischemic stroke in women with diabetes aged over 55 years who had been administered CEE than in the control group that had not received CEE [20]. Another study examining the risk of stroke associated with different routes for hormone administration showed that transdermal estrogens alone or combined with micronized progesterone may be the safest option for minimizing the likelihood of stroke [21]. Data from this study also demonstrated an elevated risk of ischemic stroke from dose-dependent oral estrogen treatment, strongly suggesting that the route of administration can affect outcomes.

As with stroke, the loss of E2 production, either through ovariectomy or menopause, can also significantly increase the likelihood of diseases such as Alzheimer's disease [22-24], Parkinson's disease, decreased cognitive function, and death from neurological pathology and dysfunction [1, 4, 6]. In contrast, oral administration of estrogens was found through meta-analyses to decrease risk by 29-44% [22-24]. In a pilot study of women who participated in the randomized, double blinded, placebo-controlled Kronos Early Estrogen Prevention Study found that at 3 years after a 4-year regimen of either CEE, placebo, or transdermal 17β-estradiol, 17β-estradiol treatment was correlated with lower β-amyloid deposition in subjects who carried the *APOE* ε4 risk factor for AD, but not in non-carriers of *APOE* ε4 allele [25].

The treatment of AD with estrogen has remained controversial since several studies have revealed no clear therapeutic effect of ERT on AD, based on disease progression or the Clinical Global Impression of Change (CGIC) 7-point scale [26-28]. However, observational studies have generally provided strong support for the successful intervention of AD within the critical window after menopause [29]. In particular, two large studies, the Cache County study [30] and the Women's Health Initiative Memory Study (WHIMS) [31] showed no beneficial effects of ERT, and in fact, accelerated the risk of AD from treatment with CEE plus medroxyprogesterone acetate (CEE-MPA) in an older cohort of postmenopausal women. Reexamination of data from the Cache County study revealed that although there was no positive effect of ERT on women who started treatment more than 5 years post-menopause, there was a 30% reduction in risk of AD for women who began ERT treatment without MPA within 5 years of menopause, and if the treatment was extended for 10 years, the risk was lowered further to a 37% decrease [32].

In addition to ERT, treatment with raloxifene, a selective estrogen receptor modulator, has been widely studied and applied for alleviating the risks of osteoporosis and breast cancer that increase in post-menopausal women. A follow-up study at four years after the Multiple Outcomes of Raloxifene Evaluation randomized trial examined the effects of raloxifene on the risk of cardiovascular events in osteoporotic postmenopausal women [33]. Although raloxifene treatment did not detectably affect post-menopausal cardiovascular risks among the overall cohort, for a subset of study participants with increased risk of cardiovascular events, these risks were significantly reduced. Similarly, in healthy post-menopausal women, raloxifene treatment exerted no significant effects on levels of C-Reactive protein, an inflammatory marker for cardiovascular disease. Notably, both HRT and raloxifene were found to comparably lower serum homocysteine levels, an independent risk factor for cardiovascular disease in the same subjects. [34]. In addition to homocysteine, raloxifene has been shown to lower LDL-C, fibrinogen, and lipoprotein(a), while raising HDL2-C but not triglycerides associated with cardiovascular disease [35]. Raloxifene and ERT also improved vascular endothelial function in postmenopausal women [36], though further studies are necessary to determine the full cerebrovascular effects of estrogen receptor modulators in post-menopausal women.

Despite the benefits of ERT, the risks of hormone receptor-positive breast cancer, which comprise a large proportion of breast cancer cases, necessitates treatment with anti-hormone therapeutics that block estrogen stimulation. These anti-hormone treatments, especially third-generation aromatase inhibitors (AIs) such as exemestane (steroidal AI) or anastrozole and letrozole (nonsteroidal AIs), have been shown to provide beneficial effects against both early and advanced hormone-sensitive breast cancer in post-menopausal women [37]. Data provided by a Japanese substudy of the Tamoxifen Exemestane Adjuvant Multinational (TEAM) project revealed that tamoxifen treatments led to a significant alteration in the lipid profiles of post-menopausal women, and in particular TC and LDL-C were significantly decreased, whereas no clinically significant effects were observed on serum lipid profiles following exemestane and anastrozole regimens [38]. Furthermore, tamoxifen may be used to mitigate high cardiovascular risks, such as the development of hyperlipidemia, in breast cancer patients. A meta-analysis of studies examining the effects of anastrozole, exemestane, or letrozole aromatase inhibitors between 2000 to 2009 indicated that, in comparison with tamoxifen, treatment with these compounds potentially led to an elevated risk of cardiovascular disease and bone fractures [39]. However, a comparative study of endocrine adjuvant breast cancer therapies conducted over 10 years used a benefit/risk index to determine that tamoxifen exhibited lower efficacy and benefits than aromatase inhibitors in postmenopausal women [40].

2.2. Neuroprotective Benefits of ERT Demonstrated *in vitro* and in Animal Models of Menopause

Estradiol has been implicated in neuroprotection in post-ovariectomized (OVX) rats prior to stroke induction. Early evidence for the neuroprotective activity of E2 was shown by Hall *et al*. [41]. Several studies examining brain injury caused by focal ischemia found that E2 treatment was effective for mitigating cerebral damage in OVX female rats [42-44] as well as male rats [45]. Pre-treatment with E2 was found to mediate neuroprotection through the up-regulation of antiapoptotic Bcl-2 protein expression mediated by ERα in cultures of rat hippocampal neurons [46]. In experiments with OVX female rats subjected to middle cerebral artery occlusion (MCAO), E2 dosage also showed a significant correlation with stroke infarct size [47], while in contrast, infarct size was increased by pre-treatment with ICI 182 780, a competitive antagonist of Estrogen Receptors α and β prior to stroke induction [48]. Additionally, ischemic stroke was exacerbated by the administration of aromatase inhibitors that block the conversion of androgen into estrogens.

In male gerbils, treatment with E2 prevented the induction of apoptosis signaling cascades in the hippocampal CA1 region. Global ischemia resulted in higher levels of activated caspase-3 in the CA1 cells, as well as induction of proapoptotic neurotrophin receptor p75(NTR), while exogenous E2 mitigated the increase in caspase-3 and prevented upregulation of p75(NTR) [16].

In order to test the hypothesis that higher levels of circulating E2 prior to menopause lead to a decreased risk of Parkinson's disease in women compared to men, Siani *et al.* [18] examined the progression of nigrostriatal damage, activation of microglia and astrocytes, as well as the polarization of microglia. These neurological disruptions were induced by intrastriatal injection of the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) in male, fertile female, OVX female, and OVX mice treated with 17β-estradiol (OVX+E). Observation by immunohistochemistry of tyrosine hydroxylase to measure dopaminergic activity, immunofluorescence of microglial marker IBA1 and astrocyte marker GFAP, as well as triple immunofluorescent staining of DAPI/IBA1/TNFα or DAPI/IBA1/CD206 to identify M1 (cytotoxic) or M2 (cytoprotective) microglial activation states, respectively, revealed that microglia were induced to an M1 state in OVX and male mice in response to 6-OHDA, while in fertile female and OVX+E mice, microglia were polarized to the M2 phenotype [18].

Estrogen replacement therapy has also been widely studied in the treatment of Alzheimer's dementia. In particular, the accumulation of β-amyloid plaques is one of the hallmarks of Alzheimer's disease. Estradiol treatment was shown to decrease β-amyloid production in embryonic cerebrocortical neurons derived from rats, humans, and mice, as well as in neuroblastoma cells [49]. In the APPswe/PS1dE9 double transgenic mouse model of Alzheimer's disease, treatment of OVX or non-OVX young mice with 17β-estradiol, or its brain-selective prodrug 10β, 17β-dihydroxyestra-1,4-dien-3-one (DHED), resulted in decreased levels of brain β-amyloid for both OVX and intact mice, as well as increased cognitive performance for both OVX double transgenic and intact mice, compared to controls, thereby suggesting a strong potential for both E2 and DHED as early interventions for AD [50]. In human cell lines, cultured astrocytes derived from Alzheimer's patients showed that glutamate

uptake was reduced in patients with AD, and that treatment with E2 increased uptake in a dose-dependent manner. Furthermore, E2 treatment led to transcriptional up-regulation of the GLT-1 and GLAST glutamate transporters, indicating that the E2-mediated regulation of glutamate transport in astrocytes may contribute to neuroprotection against AD [51].

3. MECHANISMS OF ERT NEUROPROTECTION

3.1. The Importance of ERs in Neuroprotection

The vast majority of E2-mediated neuroprotection is conducted through estrogen receptors that participate across a range of signaling pathways to affect very rapid transcriptional and physiological responses (reviewed in [52]). Estrogen signaling is primarily mediated through the two nuclear and membrane-associated estrogen-binding receptor proteins ERα and ERβ, which act as nuclear transcription factors [53-56]. The increased risk of cerebrovascular and neurological disorders experienced by women following estrogen deprivation is due in large part to the loss of estrogen-binding receptors, without which treatment with estrogen cannot effectively activate neuroprotective pathways. Once the receptors have been lost, due to depletion of estrogen which participates in inducing their biosynthesis, so too is the ability to respond to estrogen.

3.2. ER Participates in Maintaining the Blood Brain Barrier

A multitude of studies has examined the neuroprotective effects of estrogen against global and focal ischemia. For example, supra-physiological doses of E2 had protective effects against global cerebral ischemia (GCI)-induced hippocampal injury [57, 58], and GCI in six-month-old female OVX rats, prior estrogen treatment resulted in a significant decrease in the breakdown of the blood-brain barrier [46]. Specifically, E2 activation of ERs in the cytosol has been reported to upregulate transcription of claudin-5 and occludin, two critical proteins necessary for maintaining tight junctions in endothelial cells of the blood brain barrier following global and focal-induced ischemia in rat brains. Burek *et al.* 2014 found that both ERs bind to Estrogen Response Elements and SP1 sites in the cldn-5 promoter [59]. In OVX mice, E2 treatment induced occludin mRNA expression in brain endothelial cells [60], and that agonist-activated ERβ decreased levels of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1α following ischemic stroke [61]. In OVX rats with induced global cerebral ischemia (GCI), treatment with an E2 G-protein coupled receptor GPER-1 agonist after reperfusion resulted in decreased levels of VEGF in the CA1 as well as immunoglobulin extravasation, while increasing levels of claudin and occludin [62]. In addition to maintain the integrity of the blood brain barrier, there is strong evidence that estradiol activates the receptor for insulin-like growth factor-1 (IFG-1R) to activate the anti-apoptotic protein bcl-2 in hippocampal neurons [63]. Dubal *et al.* found, using ovariectomized ERα and β knockout mice with induced ischemia, that ERα was essential for E2 to confer protective effects against brain injury, though ERβ was not [44].

3.3. Genomic Regulation of Apoptosis-related Genes

Both ERs are expressed at relatively high levels in the brain, strongly suggesting a role for ERs in promoting learning and memory function, and subsequently contributing to E2-mediated neuroprotection against Alzheimer's disease [46]. Administration of the selective ER agonists PPT and DPN, for either ERα and ERβ, respectively, increased the survival rate of cultured hippocampal neurons following treatment with excitotoxic glutamate [46]. Treatment with high doses of E2 was also shown to act through ERs α and β in the mitigation of neuronal death *via* inhibition of apoptotic signal cascades in the hippocampal CA1 [16, 64]. Furthermore, long-term treatment with E_2 or tamoxifen (which exhibits estrogen-like effects) was found to protect hippocampal neurons by specifically preventing the downregulation of antiapoptotic Bcl-2 and upregulation of proapoptotic Bax in OVX rats [48]. Furthermore, E2 was shown to ameliorate β-amyloid toxicity and oxidative stress, a hallmark of AD, in hippocampal HT22 clonal cells stably expressing either ERα or ERβ *in vitro,* and that this activity required activation of the MAPK signal pathway [65]. Survivin, a pro-survival inhibitor-of-apoptosis protein and STAT3 target gene, is well-established as an essential component in E2-mediated neuroprotection against global ischemia [66], as demonstrated by the reversal of neuroprotection concurrent with down-regulation of survivin.

3.4. E2-mediated Non-genomic Signaling

Rapid response to cerebral injury or neuronal damage is mediated by so-called "non-genomic" signaling, meaning that E2 can interact with receptors on the plasma membrane to induce a fast, intracellular, neuroprotective response [67, 68] in addition to nuclear signaling pathways governing the transcriptional regulation of apoptosis-related genes. The canonical signal pathways transducing this response are the phosphatidylinositol 3-kinase (PI3K)/Akt/glycogen synthase kinase 3 (GSK3) pathway and through extra-cellular Signal-Regulated Kinases (ERKs) [69-72]. ERKs, for example, have been shown to mediate an E2 neuroprotective response to stroke, ischemic brain injury, and glutamate toxicity associated with AD through the Raf/MEK/ERK pathway [73, 74] Fig. (**1**).

Estrogen Receptor α (ERα) is localized to signalosomes or protein complexes associated with dynamic lipid raft microstructures on the membrane surface [75]. Several recent studies have explored the rapid intracellular signaling response mediated by E2-activated membrane-bound estrogen receptors that protect neurons against degradation, such as those occurring in AD. Reduction in serum E2, for example due to menopause or OVX, may lead to structural changes in signalosomes, thereby disrupting ER functionality, and potentially resulting in loss of E2 neuroprotection, as has been observed in the brains of AD patients [76]. Destabilization of the lipid raft followed by displacement and degradation of ERα leads to a subsequent loss of E2 signaling-mediated neuroprotection. The loss of ERs from the cell membrane, which leads to insensitivity to E2, thus relieves this regulatory mechanism and abolishes the signaling pathway for neuroprotection. This hypothesis is in agreement with research showing that in hippocampal and cortical tissue of AD

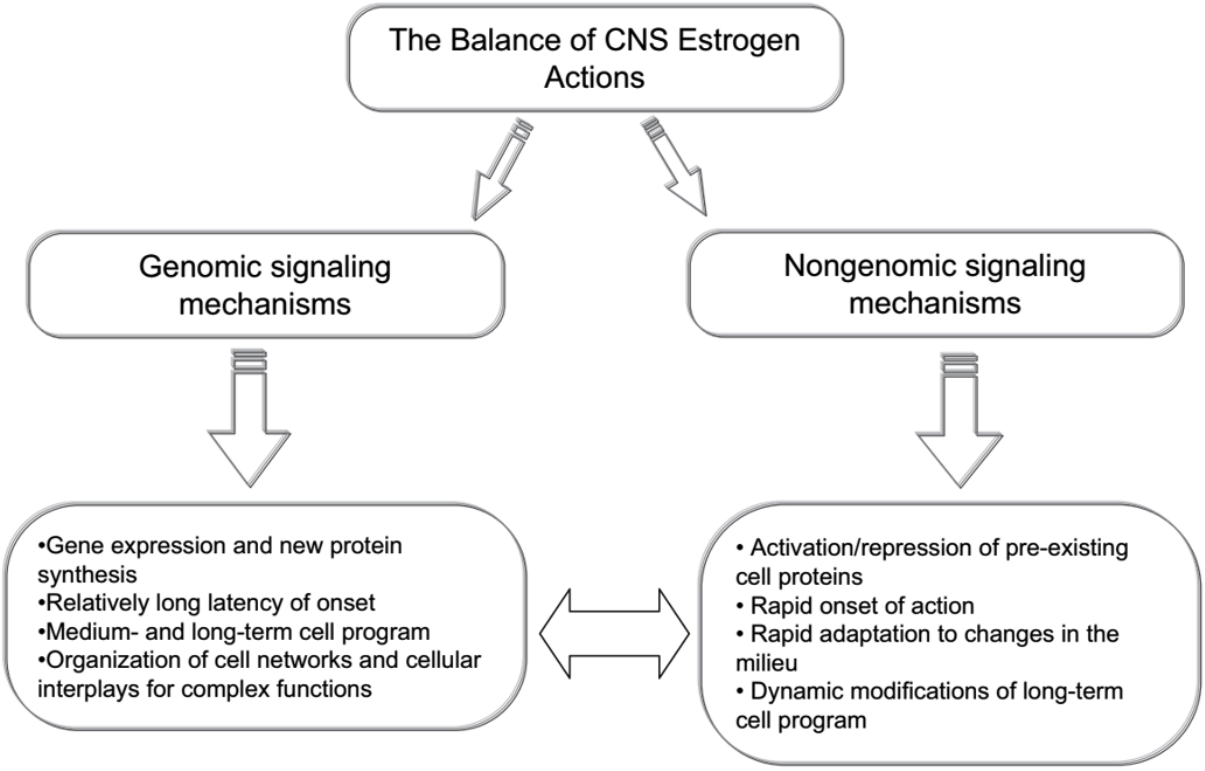

Fig. (1). Differential and integrated effects from genomic and non-genomic estrogen activity in the central nervous system (CNS) [92]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

patients, ERα binds to tau protein and co-localizes to neurofibrillary tangles. Additionally, overexpression of tau protein in M17 human neuroblastoma cells inhibited the transcriptional activity of an ERα-reporter regardless of E2 treatment, indicating a loss of ERα-mediated E2 neuroprotection in AD patients [77].

The G protein-coupled estrogen receptor 1 (GPR30), a 7-transmembrane-spanning G protein-coupled receptor [78], has been shown to participate in the rapid estrogen-mediated response on the scale of seconds to minutes [79]. Several studies have shown that GPR30 activates the cell survival PI3K/Akt pathway across several model systems for neurological disease including Alzheimer's disease (AD) [80], Parkinson's disease (PD) [81], spinal cord injury (SCI) [82], and traumatic brain injury [80]. In addition, activation of PI3K signaling by GPR30 is an essential step in the regulation of neuritogenesis during hippocampal neuron development [83] and in the protection of cognitive function [80]. Activation of GPR30 is also necessary for modulation of the c-Jun N-terminal kinase (JNK) pathway to mediate neuroprotection, during which GPR30 participates in the rapid induction of the pro-survival kinases, Akt and ERK, while mitigating the pro-apoptotic downstream effects of JNK activation [84].

A study by Gangadhara *et al.* (2015) demonstrated that following global cerebral ischemia (GCI), proline-, glutamic acid-, and leucine-rich protein 1 (PELP1), an estrogen receptor co-regulator protein, engages in "rapid signaling" in the hippocampal CA1 region. This co-mediation of rapid signaling is essential for extranuclear signaling, E2 mediation of neuroprotection, and the maintenance of cognitive function [85]. This work revealed that E2 modulates the interactions of PELP1 with GSK3β.

3.5. Initiation of Antioxidant Mechanisms

Several studies consistently show that E2 treatment initiated immediately following OVX resulted in neuroprotection after ischemic stroke through attenuation of central and peripheral production of proinflammatory cytokines [86]. However, the effects of E2 on suppression of the production of deleterious proinflammatory molecules was obliterated when E2 was administered after 10 weeks post-ovariectomy in the rat hippocampal CA1 [86, 87]. Loss of estradiol neuroprotective function for the hippocampal CA1 region in LTED (10 W) animals was also confirmed by NeuN and Fluoro Jade B staining to count the surviving neurons [87]. Additionally, E_2 ceases to suppress activation of NADPH oxidase and O_2^- production in the CA1 region by 3 h after reperfusion in LTED (10 W) animals [87]. Furthermore, E2 treatment following cerebral ischemia inhibited GTPase Rac1 in an Akt-dependent manner, thus preventing the induction of NADPH oxidase *via* NOX2 [87], indicating that E2 can mitigate oxidative stress in neurons through ERα kinase activation rather than binding to DNA responsive elements. Interestingly, Prokai *et al.* (2003) showed that estrogens can directly scavenge hydroxyl radicals [88], converting them into non-phenolic quinols that do not bind to ERs, but are subsequently reduced using NADPH as a co-factor to restore the original estrogen without generating reactive oxygen species [89, 90]. Other studies have indicated that estrogen also induces neuroprotection of dopa-

minergic pathway signaling. More specifically, both *in vivo* and *in vitro* models of PD have been used to show that the benefits of estrogen are mediated by inhibition of the brain Renin-Angiotensin System (RAS) [91]. In the substantia nigra, ERT can ameliorate the effects of diminished levels of ER-α that are concurrent with increased angiotensin activity, NADPH-oxidase activity, and expression of neuroinflammatory markers that result from ovariectomy.

4. CRITICAL PERIOD HYPOTHESIS FOR ERT NEUROPROTECTION

4.1. Overview of the Critical Period Hypothesis

Given that ERT has obvious neuroprotective effects against neurodegenerative diseases for postmenopausal women or OVX female animals, the findings of many basic and clinical studies support a critical period for ERT following menopause. During this period ERT is neuroprotective and appropriate, but after which the neuroprotective benefits are lost and, in some cases, may incur more serious damage. The ''Critical Period Hypothesis'', proposed by Sherwin, Maki, and others, has provided valuable insight into the effective application of hormone therapy following menopause by identifying a precise window of opportunity during which HT is successful for protection against neurological damage and degenerative diseases [92-97]. Specifically, this theory suggests that if HT is initiated soon after natural or surgical menopause, it may prevent cognitive decline [92-96], while administration of estradiol HT shows only limited benefits at best and detrimental effects at worst, if it is initiated outside the window of opportunity, *i.e.*, after a significant menopausal period of time [95, 96].

The complementary but competing hypothesis of ''healthy cell bias of E2 benefit'' put forward by Brinton, shares the underlying, fundamental concept that E2 only yields neurological benefit if it is applied to healthy neurons [90, 98, 99]. Inasmuch as healthy neurons deteriorate with aging in postmenopausal women, the beneficial role of E_2 declines as the time increases since the onset of menopause, with E2 treatment eventually becoming potentially detrimental and exacerbating neurological injury [90, 98, 99]. We, therefore, review the basic science and clinical evidence for the Critical Period Hypothesis in light of these theoretical mechanisms by which E2 functions in neuroprotection.

4.2. Outcomes for Women Treated within the Advantageous Time Period

The Women's Health Initiative Memory Study (WHIMS), a multi-center, randomized, placebo-controlled trial of over 16,000 postmenopausal women, investigated the relationship between orally administered HT and the incidence of stroke [100]. However, instead of the expected decrease in stroke cases during treatment with ERT, administration of Conjugated Equine Estrogens (CEEs) plus medroxyprogesterone acetate (MPA) significantly increased the risk for ischemic stroke in postmenopausal women (1.8% strokes with a hazard ratio (HR) of 1.44 in CEE plus MPA users compared to only 1.3% incidence of strokes in the placebo group), consequently leading to a premature termination of the WHIMS [1]. Importantly, this study revealed that in late postmenopausal women, initiating hormone therapy led to negative outcomes for subjects. Since this finding contradicted a wealth of previous data supporting the benefits of ERT for postmenopausal women, the ''critical period" and the ''healthy cell bias'' hypotheses were developed and explored to explain the underlying mechanisms for the success of HT in premenopausal women [90, 95, 96, 98].

In order to test the validity of the critical period hypothesis, one study assessed cognitive function in 428 women aged 60 or older who began systemic hormone therapy within a five-year span from the time of menopause (natural or hysterectomy plus bilateral ovariectomy) [101]. Although this study included a smaller sample size than the WHI, the results showed that women who had initiated HT earlier following menopause performed significantly better on cognitive tests than women who had initiated HT later or who had never used HT, thus demonstrating that the timing of E2 replacement following menopause had a significant impact on the neurological benefits of the treatment [101]. Additionally, Dumas *et al.* investigated the cognitive effects of E2 treatment in younger (50-62) versus older (70-81) postmenopausal women in a small, randomized, placebo-controlled trial of oral E2 (17b-estradiol) [84]. This study clearly established the positive effects on cognition conferred by pre-treatment with oral E2 through attenuation of anti-cholinergic drug-induced deficits of episodic memory in the younger, postmenopausal women, while surprisingly it showed the converse was true in older postmenopausal women for whom episodic memory was further hindered [102].

Ensuing research further demonstrated a broad neuroprotective effect by E2 for early menopausal women at high risk of ischemic stroke. Estradiol was thus shown to serve in multiple roles to provide protection against neurological injury when applied during the first 10 years following menopause, though other research shows that E2 treatment can accelerate decline in cognitive ability when administered at 20 years or more after menopausal onset [101]. More recently, Rocca [1, 103] performed meta-analyses of clinical studies on cognitive aging, which further substantiated previous data supporting the E2-mediated critical period hypothesis [1, 103]. Taken together, these analyses show specifically that ERT induces neuroprotection in women who initiated hormone therapy in early menopause (50-60 years of age), but conversely, also suggest that postmenopausal (65-79 years of age) ERT may cause an increased risk of dementia and cognitive decline [1, 103].

Most importantly, the WHI 10-year follow-up study has great clinical significance for determining the actual critical window for ERT because the global index of chronic diseases was decreased in women aged 50-59 (HR 0.85, 95% CI 0.70-1.03), neutral in women aged 60-69 (HR 1.00, 95% CI 0.89-1.13) and elevated in women aged 70-79 (HR 1.15, 95% CI 1.01-1.32) [104]. Thus, the WHI's estrogen-only trial suggests that there is a clear, estrogen-mediated benefit for perimenopausal women aged 50-59, and that this benefit may decline as the time of menopause onset is prolonged. The benefit of estrogen administration eventually converts into a risk when applied in late menopausal women.

In 2016, a clinical study of 643 women treated with either estradiol or placebo, and stratified by early (<6 years) or late (>10 years) stages following menopause, strongly supported the existence of a critical period for estrogen replacement therapy [105]. For women in the early post-menopausal stage, there was a significant decrease in the rate of progression of carotid artery intima-media thickness between estradiol treatment group and the placebo control. This effect was not observed in the late post-menopausal group, indicating a window for E2 treatment of atherosclerosis progression within 6 years of menopause [105].

4.3. *In Vitro* and Model Studies Supporting a Critical Period for ERT

There remains some debate over the specific functional mechanisms by which ERT protects against the neuronal death induced by Global Cerebral Ischemia (GCI) or Medial Cerebral Artery Occlusion (MCAO). Several studies in model animals also indicate that ERT has neuroprotective benefits for recently ovariectomized animals but an increased risk of neurological degeneration when administered to animals following LTED. For example, ovariectomized macaques treated with estradiol and equol following long term estrogen deprivation (≥4 years since OVX) showed significant wane in hippocampal responsiveness, indicating that ERT did not provide therapeutic benefits after that duration of estrogen deprivation [106]. Another study on the critical period for ERT found that middle-aged female rats treated with E_2 immediately after ovariectomy showed enhanced working memory, while this effect was not found in the treatment group subject to 5-month menopause, a standard model of ovarian hormone deprivation [107]. Other similar studies have also clearly demonstrated that chronic E2 treatment improved the cognitive performance of middle-aged, OVX rats, and that administering E_2 after long-term estradiol deprivation directly attenuated this effect [108].

The mechanisms by which E2 treatment mitigates neuronal damage to the hippocampus has also been explored in model organisms *in vitro* and *in vivo*. Two processes involved in learning and memory, long-term potentiation, and dendritic spine density at hippocampal CA3-CA1 synapses, were both increased following E_2 replacement at 15-months post-ovariectomy in young adult rats [109]. However, administration of E_2 on a second treatment group of the same rats after 19-months of estrogen deprivation following ovariectomy resulted in diametrically opposite effects on hippocampal synaptic physiology, thus providing strong support for a critical period of ERT [109]. Furthermore, a separate study on rats with a 5-month E2 deficiency indicated that ovarian hormone deprivation prevented intrinsic membrane excitability and acute sensitivity to estrogen in CA1 hippocampal neurons [110].

Recent work exploring the specific timing and duration of the critical period for postmenopausal E2 treatment examined the effects of estradiol on gene regulatory networks in the arcuate nucleus and medial preoptic area of the hypothalamus of rats [111]. A quantitative low-density PCR array of 48 neuronal genes was examined among eight treatment groups consisting of 4-month-old "mature" rats treated with either the vehicle or E2 for 3 months post OVX, 11-month-old "aging" rats treated for 3 months with either the vehicle or E2 post OVX, aging rats treated with the vehicle or E2 for 6 months post OVX, aging rats treated with the vehicle for 3 months post OVX followed by E2 for 3 months, and aging rats treated with E2 for 3 months post OVX then treated with the vehicle for 3 months. Weighted gene co-expression network analysis revealed that E2 treatment had the largest effect on gene expression, moreso than age, and suites of genes in the hypothalamic tissue related to energy balance, circadian rhythms, and reproduction were differentially affected by these treatments [111].

In order to test the critical period hypothesis in non-human primates, cognitive function was assayed in aged, OVX rhesus monkeys that had been subsequently treated with either the vehicle for two years, cyclic E2 for two years, the vehicle for two years followed by delayed cyclic E2 treatment, or E2 treatment for 1 year, then withdrawn and followed by treatment with the vehicle for 1 year. Among the four treatment regimens, two-year cyclic E2 and the withdrawn cyclic E2 treatment both led to higher cognitive capability in delayed response tasks and delayed nonmatching-to-sample tasks at the two-year mark, compared to untreated and delayed treatment. These findings support the critical period hypothesis since even 1 year of treatment conferred cognitive advantages over monkeys that waited two years before receiving ERT [112].

Other studies in Rhesus monkeys have shown that following menopause, there are morphological changes to the mitochondria of neurons in Brodmann's Area 46 of the dorsolateral prefrontal cortex, which is significant because of the high energy demand of these neurons to mediate working memory, for example measured by delayed response accuracy. Hence, a shift in mitochondrial configuration to donut-shaped, rather than straight or curved, correlates with post-menopausal cognitive decline that can be restored by E2 treatment [113]. Concurrently, there is also a mitochondria-dependent decrease in the density of dendritic spines necessary for synaptic function [113, 114]. Estradiol treatment thus restores spine density in the hippocampus and the prefrontal cortex, and subsequently synaptic integrity, as part of its neuroprotective mechanisms maintaining neuronal mitochondrial and cognitive function following menopause [115]. These findings comport well with other data indicating that postmenopausal neuronal mitochondria undergo a bioenergetic shift toward lower energy ketogenic profile (see section 5.2).

5. THE MECHANISMS UNDERLYING THE CRITICAL PERIOD HYPOTHESIS

Several mechanisms governing the duration of the critical period for effective ERT have been explored through basic and clinical studies. The mechanisms governing pathogenesis of neurodegenerative and cerebrovascular diseases conversely demonstrate how E2 may play a positive or negative role in neuron survival at specific times following menopause. Some of the salient experimental questions concerning the neuroprotective function of E2 include: What governs the specific duration of the critical period for E2 treatment, and what markers can be used to determine if that period has passed? Does E2 alter the homeostatic functions of

neurons in the brain? What molecules and peptides interact with E2? How does E2 and its receptors interact with and regulate other signaling pathways? The neurological consequences of long-term E_2 deprivation include neurodegeneration, cognitive impairment, and E2 neuroprotective loss. Although much remains to be clarified about the genetic and physiological mechanisms involved in the critical period of ERT, the primary mechanisms, as we currently understand them, are summarized below.

5.1. Estrogen Receptor Degradation after Long Term E2 Deprivation (LTED)

LTED, in the absence of early E2 treatment, was found to result in the degradation of the E_2 cognate receptor ERα in the hippocampal CA1 region, the result of which is a decrease in sensitivity to E2-mediated neuroprotective activity [116]. While there are several steps preceding degradation of the ERs, such as dissociation from lipid raft signalosomes, it is the final step in which carboxyl terminus of Hsc70-interacting protein (CHIP)-mediated ubiquitination and proteasomal degradation of estrogen receptors in the brain that occurs following LTED or natural aging that determines the end of the critical period for treatment. Specifically, Fan *et al.* (2005) showed that although the heat shock protein 90 (Hsp90) chaperone complex can prevent degradation of ERα in the absence of E2 [117], CHIP, which targets Hsp90-interacting proteins for degradation, induces proteasomal degradation of ERα and decreases transcription of genes upregulated by ERα, when stably expressed with ERα in ER-negative HeLa cells [118]. Furthermore, shRNA silencing of CHIP or mutation of the U-box and the tetratricopeptide repeat (necessary for ubiquitination and chaperone/ERα binding, respectively) led to increased protein levels of ERα [118]. The significance of this finding is not only that ERα is post-translationally regulated by CHIP, but also that during LTED, in which E2 is absent, CHIP mediates the steady degradation of unliganded ERα, thus leading to the loss of the critical window, and ultimately to desensitization to E2.

In LTED rats, for example, interactions between ERα and the cochaperone BcL-2-associated athanogene 1 (Bag-1) increased 2- to 3-fold over their levels of interaction in short-term E2-deprived rats [116]. Bag-1 reportedly mediates transport of the ubiquitinated estrogen receptor for proteasomal degradation [116, 118-120]. This interaction with Bag-1 strongly suggests a heightened activity by E3 ubiquitin ligase CHIP in binding unliganded ERα in hippocampal CA1 region after long term depletion of E2 [116], which was supported by a 50-60% loss of ERα protein levels and observations of elevated ERα ubiquitination in this hippocampal tissue in old (aged 24 months), but not young (3-month-old) F344 rats. While ERα degradation occurs in both 10-week-old OVX rats and old rats, ERβ degradation was only observed in old rats [116]. This loss of ERβ was commensurate with increased ubiquitination and interaction with CHIP by ERβ that did not occur in young rats. Furthermore, CHIP levels remain relatively constant with age, as do transcription levels of ERα and ERβ, thus indicating that the most likely cause of ER depletion is due to protein degradation, rather than transcriptional downregulation (Fig. **2**).

Several amino acid residues have been implicated in preventing or increasing UBQ-mediated ER degradation in the absence of circulating E2. For example, phosphorylation of SER118 (pSER118-ERα) and SER167 in human ERα induced by reactive oxygen species was reported to inhibit proteasomal degradation [116, 121, 122], which was supported by a clear decrease in pSER118- ERα levels in LTED rat hippocampus tissue [116]. Similarly, work by Berry *et al.* (2008) showed that mutation of the lysine K302 and K303 residues to alanines in the hinge region of ERα expressed in C4-12 breast cancer cells, resulted in a dramatic increase in polyubiquitination *via* association with CHIP and Bag-1 in the unliganded state, which was reversed by the addition of E2. In contrast, WT ERα expressed in the same cells were quickly ubiquitinated when bound to E2, but not so in the unliganded state, thus indicating that these residues interact with E2 to regulate the basal turnover of ERα [119].

As mentioned above, raft-associated ER signalosomes have been shown to participate in the induction of several different neuronal responses [123]. The anchor for ERα, caveolin-1, reportedly interacts with ERα in regions of the brain responsible for memory and cognition and governs ER interactions with lipid raft-associated glutamate receptors in the hippocampus and striatum [124, 125]. Caveolin-1 also mediates coordinated signaling between ERα and insulin growth factor-1 receptor b (IGF-1Rb) wherein E2 and IGF-1 tandem signaling act synergistically in the inhibition of AD onset and age-related neuron dysfunction [126-129].

The loss of circulating E2, loss of E2 neuroprotection, and degradation of ERα is connected to E2 modulation of voltage-dependent anion channel (VDAC) gating, for example during Ab toxicity, and deterioration of ERα signalosome integrity. VDAC channels have been found accompanying ERα lipid raft signalosomes across both murine and human neurons from several brain regions, most notably for the purposes of this review, in the hippocampus [130-132]. Specifically, the loss of E2 may be correlated with the deregulation of VDAC gating, which subsequently precedes AD pathogenesis [133], while E2 activation of the ERα signalosome inhibits the dephosphorylation of VDACs, leading to neuroprotection against Ab-mediated degradation in neurons [104, 134]. Furthermore, shifts in the composition of lipid rafts in the hippocampus and cortex, such as changes in ganglioside content, increases in GM1, GM2, GM3, GM4, and GD3, and decreases in GD1b and GT1b, are correlated with early stages of age-related neurological disorders such as AD and PD [135-138].

The composition of lipid rafts differs dramatically between women with AD and perimenopausal women, whereas the differences in composition are not substantial between the brains of post-menopausal and AD women. More importantly, these changes in composition can result in degradation of ERα and IGF-1Rb receptors due to caveolin-1 dissociation in post-menopausal women [139, 140], ultimately leading to dephosphorylation and a change in VDAC localization to membrane regions outside the lipid raft [140]. This destabilization has been observed by Ramirez *et al*. in lipid rafts of the hippocampus and cortex of AD patients [132].

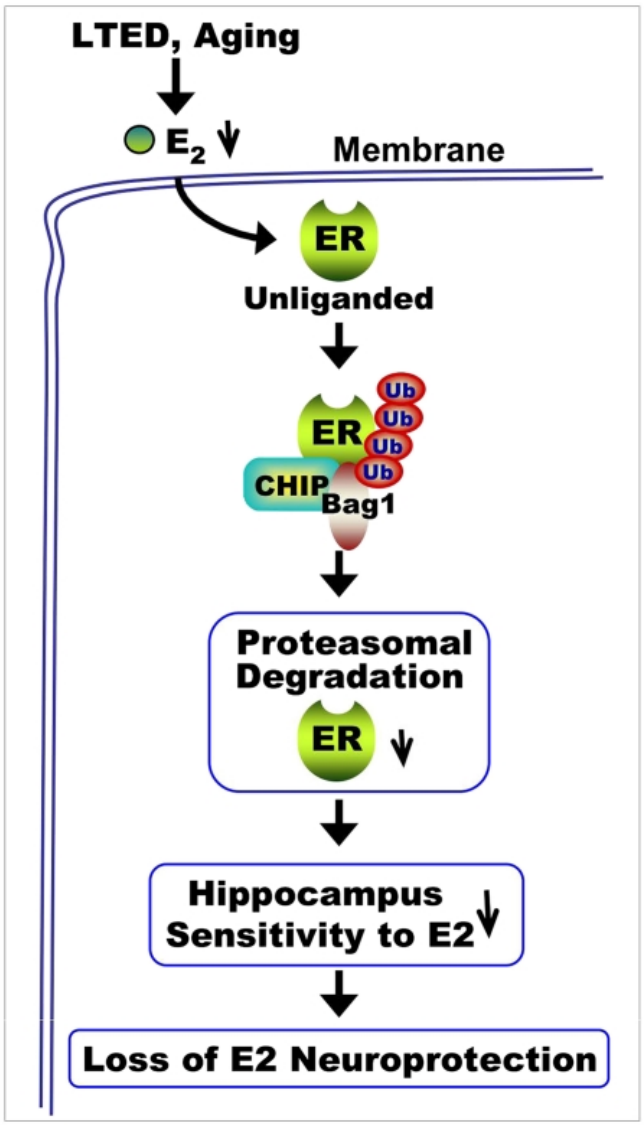

Fig. (2). Long-Term Estradiol Deprivation (LTED) Leads to Brain-Specific Degradation of Unliganded Estrogen Receptor. LTED or natural aging increases levels of unliganded estrogen receptors, which displays enhanced binding with the E3 ubiquitin ligase, CHIP, and the co-chaperone Bag1, leading to accelerated ubiquitination and proteasomal degradation of estrogen receptors. Proteasomal estrogen receptor degradation leads to the loss of estrogen neuroprotection [116]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

In addition, in 9-month-old rats, given E2 treatment at 6 days or 180 days after ovariectomy (equivalent to 4 months or 11 years post-menopause in humans), an ERβ dominant negative splice variant, ERβ2, increased in expression in the hippocampus and white blood cells of all rats, regardless of treatment. However, in rats given E2 shortly after OVX, ERβ2 expression was lower, hippocampal cells showed higher proliferation, and rats showed higher mobility in forced swim tests compared to rats given delayed E2 treatment, indicating that ERβ2 levels in hippocampal and peripheral blood cells could potentially serve as marker for the critical window in postmenopausal women [141].

A relatively recent finding suggests that the activity of ERα, specifically in the hippocampus, dictates the duration of the therapeutic window for ERT by regulating the mechanisms governing synaptic plasticity necessary for memory [142]. Although no improvement to cognitive performance was observed following an increase in ERβ, up-regulation of ERα expression in the hippocampus led to a restoration in the efficacy of E2 and rejuvenation of E2-induced hippocampal NMDAR-mediated plasticity following LTED.

5.2. Mitochondrial Switch to Ketogenic Profile in Neurons

A study conducted by Brinton and colleagues suggests that LTED promotes an energetic switch from glucose to ketogenic profile in the brain [143]. However, less energy is provided for neurons by ketones than is available from glucose, thus decreasing neuronal ATP, the final result of which is neurological impairment [143].

Estrogen also participates in processes critical for maintaining a high bioenergetic state in the mitochondria of hippocampal neurons necessary for strong cognitive function and memory. However, E2 deprivation can lead to a shift in mitochondrial bioenergetics associated with ketogenic rather than glycolytic profile. This change to lower energetic metabolism is characteristic of neurological disorders such as Alzheimer's disease wherein a decrease in mitochondrial respiration is caused by diminished availability of glucose in brain tissue. There is a subsequent shift away from glycolysis through decreased activities of pyruvate dehydrogenase (PDH) and Complex IV cytochrome C oxidase (COX) [143], thus creating a demand for ATP that activates ketogenic beta-oxidation of long chain fatty acids by hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase alpha subunit (HADHA), and ketone oxidation by 3-oxoacid-CoA transferase 1 (SCOT), to produce Acetyl-CoA for ATP biosynthesis [143-145]. The use of ketones for energy production indicates a state of lower mitochondrial bioenergetics that is a hallmark for brains affected by Alzheimer's disease [143].

Estradiol has been reported to stimulate the flow of blood to brain tissue *in vivo* as well as up-regulating neuronal glucose transporters. The resulting increase in glucose availability, therefore, prolongs a higher bioenergetic state in the mitochondria of brain tissue [90, 99, 144]. E2 treatment during the critical window of opportunity can also enhance respiratory oxidative phosphorylation by inducing expression of Complexes I and II in the mitochondrial electron transport chain, presenting another mechanism by which it can maintain a high bioenergetic metabolism in neuronal mitochondria [106]. In mouse model systems, estradiol has thus been demonstrated to inhibit pathways for mitochondrial hypometabolism characteristic of dysbiosis leading to Alzheimer's disease.

5.3. Decrease in Acetylcholine Following Estrogen Deficiency

Finally, LTED attenuates the ability of estradiol treatment to increase hippocampal ChAT protein levels [146]. Several studies have shown in the rat model that a prolonged

period following menopausal (natural or surgical) withdrawal of E2 not only inhibits the cognitive and neuroprotective benefits of subsequent ERT, but in fact may exacerbate the effects of cerebral injury such as from experimental middle cerebral artery occlusion (MCAO) or ischemic stroke [147-149]. Estradiol increases expression of cholinergic markers such as brain-derived neurotrophic factor (BDNF) in the cortex and hippocampus [147, 148] and the neurotrophic system, in turn, mediates the effects of E2 on the physiological structure and cognitive functions related to memory in the hippocampus [108, 146-149].

There is a substantial body of evidence showing that estrogen may share overlap in receptors with neurotrophic factors, such as BDNF, indicating that E2 participates in regulating the neurotrophic system [150, 151]. As with receptors discussed above, the loss of serum E2 can ultimately lead to changes in the cholinergic system that are consistent with the critical window hypothesis. In particular, there is evidence suggesting that receptor cross talk leads to either reciprocal- or co-regulation of acetylcholine and E2 [146]. Following LTED, the cholinergic system also undergoes substantial changes (discussed below), after which ERT does not show the same benefits as when applied during the critical window after menopause.

Age-associated estrogen deficiency is concurrent with decreases in hippocampal levels of acetylcholine (ACh), a critical neurotransmitter, and Choline Acetyltransferase (ChAT), which is critical for Ach biosynthesis [146]. ACh is abundant in so-called cholinergic neurons of the CA1 of the hippocampus and pre-frontal cortex [146]. Reduction in acetylcholine and choline acetyltransferase [ChAT] has been historically implicated as an important determinant in Alzheimer's Disease (AD) pathogenesis [152, 153], and similarly, a significant loss of basal forebrain cholinergic projection neurons in the medial septal nucleus (MS) and the nucleus basalis magnocellularis (NBM), necessary for memory and attention [154-156], is associated with symptoms of memory dysfunction in Alzheimer's disease [152-159]. In postmenopausal women given long term treatment with estrogen since initiation of menopause, growth hormone response showed a significant positive correlation with the duration of estrogen treatment following administration of the cholinesterase inhibitor pyridostigmine [160, 161]. Conversely, when estradiol treatment was initiated immediately after ovariectomy in both young adult and middle-aged rats, ChAT levels significantly increased in the hippocampus, though, as with other studies, estradiol treatment initiated at 5 months after surgical menopause did not result in an increase in hippocampal ChAT [146].

The relationship between estradiol neuroprotection and cholinergic function converges on the morphology and density of dendritic spines in the CA1, which mediate spatial memory in cognitive performance [162]. Estradiol treatment has been shown to increase apical spine density by 30% in CA1 pyramidal cells in OVX rats [163], leading to an increase in new excitatory synaptic connections between neurons [164]. Taken together, the facts that a) estrogen receptors are expressed in cholinergic neurons and co-localize with receptors for neurotrophic growth factors [165]; that b) in the MS and NBM at 6 months following OVX mice, there was substantial depletion of tropomyosin-related kinase receptor A [166]; and that c) in the mouse model *in vivo,* estradiol can regulate the expression of neurotrophin receptors [167] suggest to some researchers that estrogen is a regulator of the neurotrophin system in the basal forebrain [168].

CONCLUSION AND FUTURE PROSPECTS

In summary, growing evidence supports the critical role of E_2 in the attenuation of damage from cerebral ischemia and in the delay of onset of cognitive decline in both animals and humans. Unfortunately, a consequence of both natural reproductive senescence and surgical menopause induced by bilateral ovariectomy is a dramatic decline in the level of circulating E_2. Subsequently, a deficiency in estradiol leads to a significant increase in the risk of postmenopausal psychological disorders, osteoporosis, cardiovascular diseases, cerebrovascular diseases, Parkinsonism, and Alzheimer's dementia. To preventatively address these problems by decreasing the risk of disease, ERT is available and well-documented in both basic and clinical studies to provide apparent neuroprotective functions. Although the large-scale Women's Health Initiative (WHI) study brought dramatic changes to clinical practice and laboratory research on estrogen replacement therapy due to its findings that commencing hormone therapy in postmenopausal women 65-79 years old imposed an increased risk of stroke and dementia, the new paradigm shifts were introduced to the field of estrogen replacement therapy.

Closer examination of this study showed that the WHI's timing for initiation of hormone therapy in late menopause, and the types of estrogen used (*i.e.*, CEE + MPA), as well as their routes of administration, may all have been contributing factors in the increased incidence of stroke and other diseases among participants. In response to these findings, the formative "critical period hypothesis of ERT" and the "healthy cell bias of ERT" were proposed to explain the dramatically contradictory effects of E2 treatment between postmenopausal women of different ages. Both of these hypotheses emphasized the importance of timing of ERT at the onset of menopause, and subsequently opened new avenues for exploring the multifaceted and complex role of E2 in the response to ischemia. However, if menopausal women experience a period of long-term E_2 deprivation (*e.g.*, 10 years post menopause) and are then administered a delayed hormone therapy, estrogen may become detrimental to the patient and pose a threat in the form of exaggerated risk of venous thromboembolism, ischemic stroke, and dementia.

In the absence of circulating E2, the receptors and signaling mechanisms at the neuronal membrane are diminished, as are E2-mediated regulation of neurotrophic and anti-apoptotic pathways in the brain (especially in the hippocampus), dendritic spines necessary for synaptic contact in the cholinergic neurons, and potentially some integral proteins such as caudlin-5 and occludin from the tight junctions of endothelial cells necessary for maintaining integrity of the blood brain barrier. In addition, lower levels of E2 also lead to the decreased anti-oxidant capacity for scavenging hydroxyl radicals, and a switch to lower energy, ketogenic metabolic profile for mitochondria in neurons, thus leading to less available energy for excitatory signal transduction.

In brief, our review indicates that ERT exerts profound neuroprotective function both in early menopausal women and in OVX non-human primate and rodent models. The critical period hypothesis of ERT provides a practical and safe bridge between the benefits of ERT for cerebrovascular health in women during early menopause while avoiding the potential dangers of E2 administration late in menopause. There remain many unsolved issues that merit further investigation, and some aspects of ERT which remain controversial among researchers require considerable attention to resolve. ERT is a well-documented treatment, so as clinical physician researchers we strongly advocate for ERT as close to the onset of menopause as possible to alleviate menopausal symptoms and to attenuate postmenopausal risk of diseases, while we build on this foundation to push forward in the development of new treatments for age- and menopause-related diseases such as ischemic stroke, Parkinson's disease, and Alzheimer's disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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