CHAPTER

7

Neuroprotective Effects of Estrogen Following Neural Injury

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INTRODUCTION

Approximately 50 million people suffer from neurodegenerative diseases annually in the United States, and nearly 25 percent are newly diagnosed. With almost 1 in 6 Americans suffering from a neurodegenerative disease, the annual financial cost to the US is several billion dollars in treatment and rehabilitation (Brown et al., 2005). The most common neurodegenerative disorders include Alzheimer's disease (AD), stroke, and traumatic brain injury (TBI). Despite the prevalence and enormous financial and physical burden of these conditions, treatment options are limited, and a large amount of research is focused on developing both preventative and acute treatments to limit neurodegeneration.

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While estrogens are most often associated with maintenance of female reproductive function, estrogens also elicit profound effects in the brain. Specifically, estrogens modulate a number of physiological processes that promote neuronal survival, as well as inhibit responses that contribute to cell loss. This chapter describes the common molecular mechanisms underlying neurodegeneration in AD, stroke, and TBI and provides evidence to support the use of estrogens in the treatment or prevention of these conditions.

MOLECULAR MECHANISM OF NEURODEGENERATION AND APOPTOSIS

Neurodegenerative diseases are marked by neuronal death that occurs either rapidly or gradually over time. While rapid neuronal cell loss may result in immediate symptoms and functional deficits, gradual cell loss accounts for a progressive worsening of the disease over time, as in AD. Regardless of the period of time over which neurodegeneration occurs, apoptosis is a common mechanism that mediates cell loss. Molecules that promote neuronal survival by increasing production of neuronal growth factors, reducing oxidative stress, and inhibiting apoptosis may have therapeutic potential that spans multiple neurodegenerative diseases, including TBI, AD, and stroke. While apoptosis is necessary in early stages of neuronal development, the death of mature neurons results in functional deficits in the damaged area. The symptoms of each disease may differ or overlap depending upon the specific populations of neurons affected. For example, a TBI damaging the motor cortex will likely result in motor impairment, whereas AD, which is characterized by loss of neurons in the hippocampus, is characterized by memory loss and cognitive decline, while motor function remains intact in the early stages of AD.

There are numerous signals that can trigger apoptosis, including oxygen deprivation, decreased metabolic rate, i.e., low adenosine triphosphate (ATP) production, oxidative stress, glutamate excitotoxicity, and loss of neurotrophic factors, such as brain-derived growth factor (BDNF) and nerve growth factor (NGF). Several of these signals increase calcium influx into neurons, and excessive intracellular calcium is a key mediator of apoptotic pathways in a cell, in many cases due to mitochondrial dysfunction (Figure 7.1). This calcium-induced ion imbalance activates the pro-apoptotic mitochondrial proteins, Bax and Bad, and while the exact downstream mechanism of Bax and Bad is unknown, Bax and Bad interact with other effector proteins in the cell to disrupt mitochondrial membrane potential. A disturbance in membrane potential triggers the opening of the mitochondrial permeability transition pore (MPTP) and release of cytochrome C into the cytoplasm, where cytochrome C activates pro-apoptotic caspase cascades (see Mattson, 2000 for review). Caspases

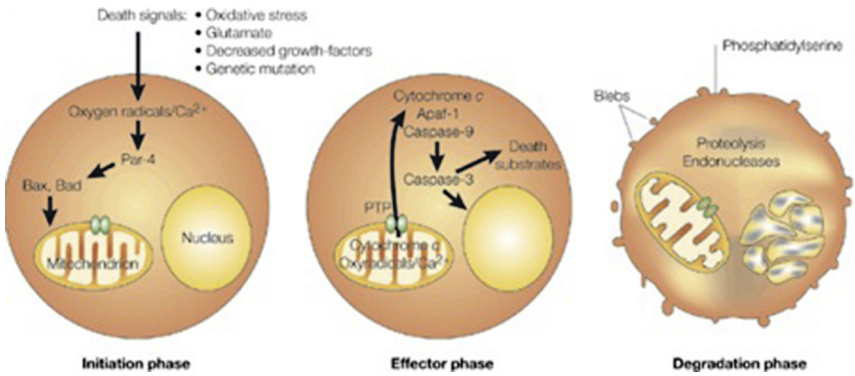

FIGURE 7.1 **Stages of apoptosis.** During the initiation phase of apoptosis, several signals can trigger an intracellular cascade of events that may involve increases in levels of free radicals and Ca^2 and translocation of pro-apoptotic Bcl-2 family members, Bax and Bad, to the mitochondrial membrane. Caspases can also act early in the cell death process before, or independently of, mitochondrial changes. The effector phase of apoptosis involves increased mitochondrial Ca^{2+}, the formation of permeability transition pores (PTP) in the mitochondrial membrane, and release of cytochrome *c* into the cytosol. Cytochrome *c* forms a complex with apoptotic protease-activating factor 1 (Apaf-1) and caspase-9. Activated caspase-9, in turn, activates caspase-3, which begins the degradation phase of apoptosis. *Reprinted with permission from Mattson, 2000.*

are universal apoptotic proteins present in virtually all cell types, including neurons and glial cells. One example of an apoptotic caspase cascade involves caspases 9 and 3. Cytochrome C binds caspase 9, with the aid of the protein protease-activating factor 1 (APAF1), the APAF1/caspase 9 complex activates the effector caspase, caspase 3, and caspase 3 stimulates DNA degradation and cell degeneration. Dysfunctional mitochondria also release free radicals into the cell that further increases oxidative stress and lipid peroxidation (see Mattson, 2000 for review).

In addition, neurons express several anti-apoptotic proteins, including the bcl-2 family members, bcl-2 and bcl-xL. Also, neurotrophic factors, such as BDNF and NGF, can prevent neuronal apoptosis by inhibiting pro-apoptotic pathways, activating anti-apoptotic signaling pathways, and stimulating production of molecules and proteins that promote cell survival. For example, BDNF and NGF increase the production of antioxidant molecules, as well as proteins that regulate calcium influx and homeostasis (Tamatani et al., 1998).

ESTROGEN RECEPTORS: TYPES AND LOCALIZATION IN THE BRAIN

Estrogens are lipophilic steroid hormones classically associated with regulating female reproductive function. Estrone (E1), estradiol (E2),

and estriol (E3) are the three forms of estrogen synthesized from cholesterol by the ovaries, and to a lesser extent, the adrenal glands. 17-beta-estradiol (17β-E2) is the most potent estrogen. Throughout the course of a woman's reproductive years, 17β-E2 is the most prominent estrogen in circulation compared to E1 and E3; however, during menopause, ovaries cease to produce 17β-E2 from E1, and E1 becomes the principal form of circulating estrogen in postmenopausal women. Estrogens are distributed via the blood to a variety of tissues, including the cardiovascular, immune, and central nervous systems (Gustafson, 2003), and due to their lipophilic nature, estrogens can easily diffuse across cellular membranes, as well as the blood–brain barrier, to elicit their effects.

One way estrogens can elicit their actions is through interaction with estrogen receptors (ER). Currently, there are three known subtypes of ERs: ERα, ERβ, and G-protein coupled receptor 30 (GPR30/GPER). Elwood Jensen discovered ERα in 1958, and the first ERα knockout (KO) mouse was generated in 1993. Prior to 1993, ERα was widely believed to be the sole mediator of estrogenic action; however, this dogma was challenged following the discovery that female ERαKO mice had impaired reproductive function; however, all other vital physiological processes were unimpaired. This led to the hypothesis that more than one ER exists, and the discovery of ERβ in 1996 and GPR30 in 1997 provided rationale for the hypothesis of differential action of estrogens in different tissues. GPR30 is localized to the plasma membrane and was identified following evidence showing estrogenic actions in cells lacking ERα and ERβ (Filardo et al., 2000).

In addition to reproductive tissues, ERs are found in the central nervous system. ERα is widely distributed in the rodent and mouse forebrain (Shughrue and Merchenthaler, 2003). A similar distribution exists in the human brain, where ERα is distributed throughout the forebrain, hypothalamus, and hippocampus, but not in the cerebellum (Shughrue and Merchenthaler, 2003). In both rodents and humans, overall ERβ expression is much lower than ERα in the cerebral cortex; however, compared to humans, the ratio of ERβ/ERα is greater in the rodent midbrain and ERβ is expressed weakly in the cerebellum (Shughrue and Merchenthaler, 2003). GRP30 is expressed in the forebrain, hypothalamus, brainstem and hippocampus in both mice and rats (Brailoiu et al., 2007; Hazell et al., 2009). However, there are many factors that can alter ER expression and/or localization in the brain, including sex, E2 levels, and age. For example, ERα and ERβ expression declines in the hippocampus with age (Ishunina et al., 2007). This altered distribution of ERs in the brain may account, in part, for the

age-associated decline in memory, as well as the differential action of estrogens across different species.

APOPTOTIC AND NEUROPROTECTIVE GENES AND PATHWAYS MODULATED BY ESTROGEN

There are two ligand-dependent ER signaling pathways: the genomic (classical) pathway or the nongenomic pathway (Hall et al., 2001; Heldring et al., 2007). In the genomic pathway, both ERα and ERβ serve as ligand-activated transcription factors. Once an ER binds estrogen, the active ER can form a homodimer (ERα/ERα or ERβ/ERβ) or heterodimer (ERα/ERβ) and translocate into the nucleus. The ligand-bound ER dimer can bind to estrogen response elements (ERE) in the promoter sequence of target genes, and once bound, the ligand-bound ER can recruit transcription factors or other coregulatory proteins to the promoter. The pool of coregulator proteins and transcription factors present in a cell will dictate the specific genomic action of the ligand-bound ER. Several genes involved in neuronal survival are located downstream of a promoter containing an ERE, and in turn, 17β-E2 promotes transcription of several genes involved in neuronal survival. Following neuronal insult, 17β-E2 administration increases expression of several proteins involved in cell survival, including phosphoinositide 3-kinase (PI3K) (Wang et al., 2006), Akt (Choi et al., 2004; Wang et al., 2006), cyclic-AMP response element binding protein (CREB) (Choi et al., 2004), Bcl-2 (Alkayed et al., 2001; Choi et al., 2004; Dubal et al., 1999; Singer et al., 1998), Bcl-x (Stoltzner et al., 2001), superoxide dismutase (SOD) (Rao et al., 2011), protein phosphatase 2A (PP2A) (Sung et al., 2010), c-fos (Rau et al., 2003), and c-jun (Rau et al., 2003). In addition, 17β-E2 inhibits expression of pro-apoptotic proteins, including Fas, FADD (Jia et al., 2009), and Bax (Choi et al., 2004), thereby inhibiting cytochrome c release (Choi et al., 2004).

In addition to the genomic pathway, estrogens signal through a nongenomic mechanism that occurs within seconds to minutes. A ligand-bound ER dimer can remain in the cytoplasm (ERα or ERβ) or at the plasma membrane (GPR30) and function as a signaling molecule through activation of protein kinases and phosphatases. 17β-E2 promotes neuronal survival through activation of cell survival proteins, such as the mitogen-activated protein kinases (MAPK) (Raval et al., 2009; Singer et al., 1999) Akt, CREB binding protein (Choi et al., 2004; Singer et al., 1999), and calcium-calmodulin-dependent protein kinase (CAMKII) (Raval et al., 2009). 17β-E2 can also inhibit apoptosis through several mechanisms, including increasing caspase-12 activation (Crosby et al., 2007) and inhibiting caspase-3 and caspase-8 activity (Jia et al., 2009).

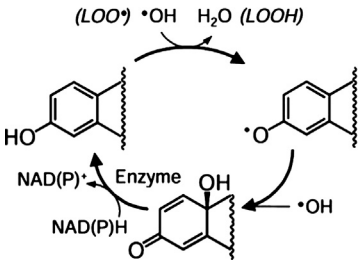

FIGURE 7.2 Estrogen recycling. Estrogens provide a chemical shield to neurons from free radical exposure. After the direct scavenging of radicals, the phenolic A-ring estrogen is reverted to an intermediate quinol molecule that is rapidly recycled to the parent estrogen through an enzyme-catalyzed reductive aromatization process. *Figure taken from Prokai et al., 2003. Reprinted with permission.*

ESTROGENS AS ANTIOXIDANTS

Oxidative stress and lipid peroxidation are common triggers of neurodegeneration in AD, stroke, and TBI. 17β-E2 is protective in several *in vitro* oxidative stress models (e.g., Barnham et al., 2004; Simpkins et al., 1997), including an oxidative stress model of Friedreich's ataxia (Richardson et al., 2011). Interestingly, the ataxia study is the first and only study to demonstrate that 17β-E2's protective action is not mediated by GPR30. The GPR30 agonist, G1, is unable to protect fibroblasts from oxidative stress, and the GPR30 antagonist, G15, does not antagonize the protective action of 17β-E2. These results indicate that the antioxidant activity of estrogen is mediated through ERα, ERβ, or ER-independent pathways.

Neuroprotective steroids must possess an intact phenolic A-ring (Behl et al., 1997; Green et al., 1997), and estrogens with more electron-donating substituent groups on the A ring are increasingly more powerful antioxidants. In accordance, any estrogen analogue that lacks a phenolic A-ring does not protect cells from oxidative stress or lipid peroxidation (Perez et al., 2005). Estrogens can donate an electron from their 3-OH group to neutralize extremely damaging hydroxyl and lipoxyl radicals (Prokai et al., 2003). The phenoxy radical produced is then rapidly reverted/recycled back into the original compound through enzymatic reduction (Figure 7.2). It is unknown how many redox cycles an estrogen can undergo, but this "chemical shield" likely underlies estrogen-mediated inhibition of oxidative stress and lipid peroxidation.

NEURAL INJURY AND THE IMMUNE RESPONSE

A balanced immune response is essential to limit tissue damage in neurodegenerative diseases. Damage-associated molecular patterns (DAMPs) released from dying neurons activate local microglia and

promote recruitment of local and circulating leukocytes, and this milieu of immune cells secretes pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα (Kamel and Iadecola, 2012). Inflammation may also predispose individuals to neurodegenerative disease. For example, systemic inflammation is associated with an increased risk of ischemic stroke, with nearly 30 percent of strokes occurring in patients with a current or recent infection (Kamel and Iadecola, 2012). Immune cells and cytokines may be therapeutic targets in neurodegenerative diseases; however, further research is needed to elucidate what constitutes a "healthy" immune response versus a harmful immune response.

ESTROGEN AND THE IMMUNE RESPONSE

While a portion of estrogen's protective actions in brain injury are mediated by induction of neuroprotective pathways in the brain, estrogen is also a powerful immunomodulator. Because the immune response following injury dictates functional recovery and the extent of brain damage, estrogen may be dually protective by also mediating the immune response.

Innate Immunity

There is a rapid, local production of estrogen in the brain following brain injury, indicating that the hormone may be involved in an immediate physiological response to limit tissue damage (Garcia-Segura et al., 1999). This early production of estrogen occurs simultaneously with the innate arm of the immune response. Following injury, neutrophils rapidly migrate into the damaged brain region, and this process becomes more rapid and excessive as the blood−brain barrier becomes disrupted (Kamel and Iadecola, 2012). While adequate neutrophil infiltration is necessary to activate monocytes and macrophages to scavenge cellular debris from the site of injury, excessive neutrophil infiltration can exacerbate tissue damage. Estrogen inhibits the production of the neutrophil chemoattractants CXCL1, CXCL2 and CXCL3, thereby preventing an excessive neutrophil response (Nadkarni and McArthur, 2013). Aside from preventing an excessive neutrophil infiltration into the brain, estrogen also mediates the clearance of neutrophils from the brain after a balanced neutrophil response. Removal of apoptotic neutrophils by monocytes and macrophages is of critical importance for inflammatory resolution. Estrogen also induces an activated macrophage phenotype that secretes anti-inflammatory cytokines, such as IL-10 and TGFβ

(Iadecola and Anrather, 2011). In addition, estrogen prevents the production and secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα, through inhibition of NFkB signaling and transcription of pro-inflammatory genes (Nadkarni and McArthur, 2013).

Adaptive Immunity

Dendritic cells (DC) are often described as bridging the gap between the innate and adaptive immune system. Dendritic cells present antigens to T cells to stimulate the adaptive arm of the immune response, and estrogen promotes DC differentiation and MHC expression, thereby promoting a T cell response in the brain. Further, estrogen modulates the cytokine environment in the brain to control which subset of T cells will be recruited to the site of injury (Iadecola and Anrather, 2011). Cytotoxic T cell (CD8+) infiltration exacerbates tissue damage following brain injury, whereas T regulatory cells (Tregs) suppress pro-inflammatory responses and are often associated with decreased cell loss and better recovery (Nadkarni and McArthur, 2013). Estrogen inhibits production of pro-inflammatory, Th1 and Th17 cytokines, including IFNγ, TNFα and IL-17, as well as increases production of the anti-inflammatory cytokines, IL10 and TGFB, thereby generating a cytokine environment that favors a Treg response (Lakhan et al., 2009). Estrogen is also able to directly promote Treg proliferation through activation of the PI3K/AKT pathway (Iadecola and Anrather, 2011).

ESTROGEN AND TRAUMATIC BRAIN INJURY

TBI resulted in over two million visits to emergency departments in the United States in 2009 and is a significant public health concern at a cost of well over $60 billion each year (Coronado et al., 2012). Defined by the Centers for Disease Control and Prevention as blunt or penetrating trauma to the head associated with at least one of the following: alteration in consciousness, amnesia, neurologic or neuropsychiatric deviations, skull fracture(s), or intracranial lesion, TBI frequently occurs following transportation accidents, falls, and firearm-inflicted injuries (Coronado et al., 2011), but is of increasing concern as a common repercussion of warfare faced by military personnel (Huber et al., 2013). Because there are currently no available treatments that promote a functional recovery of motor function, memory, or cognitive ability, it is vital to investigate potential therapeutics that can alleviate the symptomology associated with brain trauma.

Estrogens, known to exhibit neuroprotective qualities, have garnered attention as possible targets for pharmacological intervention for TBI.

17β-E2 and estrone, which are antioxidant, anti-inflammatory, and anti-apoptotic agents (Gatson et al., 2012), show promising neuroprotective effects against the damage associated with traumatic brain injury (TBI). Despite the observation that the incidence of TBI is higher for men (651 per 100,000) than women (429 per 100,000), data on TBI outcome by sex is limited (Moore et al., 2010). However, evidence suggests that women exhibit better performance on post-TBI memory and executive functioning tests (Moore et al., 2010) and overall more favorable outcomes (Yeung et al., 2011) compared to men. Administration of estrogen preceding or immediately following a TBI in rats prevents apoptosis (Chen et al., 2009), and is thus being considered a viable treatment for patients with TBIs. The mechanism of estrogen's neuroprotection is not well characterized, but there is evidence for the role of estrogen in promoting neural regeneration following injury, including both TBI and cerebral ischemia (Chen et al., 2009).

Although *in vitro* models of TBI are less common than *in vivo* models, the few studies evaluating the effect of estrogen administration on *in vitro* models of TBI demonstrate the neuroprotective qualities of estrogen. Using a model of mechanical strain injury whereby the cell culture media is replaced with warm phosphate buffered saline and a 50-ms pressure pulse of compressed gas is introduced into the well, a rotational acceleration/deceleration brain injury can be emulated. With this model, the neuroprotective effects of estrogen were evidenced though decreased post-injury neuronal damage following the addition of 17β-E2 to neuronal-glial cultured cells (Lapanantasin et al., 2006). Protection from injury potentially results from the ability of cells to control intracellular calcium levels (Lapanantasin et al., 2006). Following a TBI, increased intracranial pressure compromises the blood–brain barrier, which then allows the flow of immune cells into the brain, resulting in oxidative injury and cell death (Nortje and Menon, 2004). It is possible that estrogens respond to these signals of cell stress to mediate damage. One proposed mechanism suggests that the secondary injury induced by increased intracranial pressure triggers survival mechanisms, including increased aromatase and estrogen levels (Gatson et al., 2011). When the precursor to estrone, androstenedione, is administered to primary cultured astrocytes before pressure-induced injury, there is a greater rate of conversion from androstenedione to estrone than in the noninjured group. When the cells were incubated with an aromatase inhibitor prior to the addition of androstenedione, estrone production was blocked, suggesting that estrogen levels are moderated by aromatase expression (Gatson et al., 2011). Results of *in vitro* studies demonstrating the neuroprotective value of estrogens have been corroborated by animal models of TBI.

Through a variety of *in vivo* models of TBI, estrogens exhibit neuroprotective qualities. Not only do low physiological levels of estrogen

contribute to larger contusion sizes (Bramlett and Dietrich, 2001), but the administration of estrogens following a TBI can ameliorate negative outcomes. The effects of low estrogen levels can be studied through the use of males, or through the use of females that do not produce endogenous estrogens due to the removal of the female reproductive organs (i.e., ovariectomized). Following a TBI, males and ovariectomized females have greater levels of TUNEL positive cells and caspase-3, indicative of apoptosis and irreversible damage (Gatson et al., 2012; Li et al., 2007). When either estrone or Premarin®, an FDA-approved estrogen therapy, is administered prior to TBI, a significant reduction in two indicators of apoptosis, TUNEL positive cells and active caspase-3, in the cortex and hippocampus is observed (Chen et al., 2009; Soustiel et al., 2005). By decreasing apoptotic activity, there is a greater chance for a favorable outcome given that fewer cells have been irreparably damaged. Administration of Premarin also results in decreased blood glutamate concentrations in male rats that undergo a TBI induced by impact with a silicone-coated rod (Zlotnik et al., 2012). Abnormally high blood glutamate concentrations are associated with adverse outcomes for both TBI and stroke, and high blood glutamate levels are also correlated with poor neurological performance.

TBI results in a disruption of the blood–brain barrier that allows the infiltration of inflammatory agents into the brain. *In vivo* evidence corroborates the *in vitro* finding that estrogen may reduce damage incurred through immunological insult. Using a Marmarou TBI technique, a model of diffuse injury in which a weight is dropped onto the head, 17β-E2 administration attenuates the brain edema and blood–brain barrier disruption commonly observed following TBI (Asl et al., 2013). Further evidence for the role of estrogens in ameliorating inflammatory pathways comes from the ability of 17β-E2 to decrease neuroinflammation and apoptosis following a lateral fluid percussion model of TBI (Day et al., 2013) and burn-related injury. TBI and burn injury share a common mechanism in that both result in similar immediate brain changes, including the rapid elevation of pro-inflammatory cytokines, such as TNFα, IL-1β, and IL-6 (Gatson et al., 2009). Administration of 17β-E2 15 minutes after the burn injury significantly reduces levels of damaging cytokines and also blocks the activation of caspase-3, a key player in apoptosis. In addition, estradiol reduces reactive astroglia after brain injury, an effect mediated by the endocannabinoid system (López Rodríguez et al., 2011). In male rats that have undergone a TBI that emulates a stab wound to the brain, cannabinoid receptor antagonists administered just prior to injury blocked the protective ability of estradiol, indicating that cannabinoid receptors may contribute to the ability of estrogen to reduce resulting neuroinflammation (López Rodríguez et al., 2011).

ESTROGEN AND ALZHEIMER'S DISEASE

AD is the most common dementia, affecting well over 5 million individuals in the United States (Hebert et al., 2013) and is three times more prevalent in women than men (Alvarez-de-la-Rosa et al., 2005). The disease is characterized by three main hallmarks: insoluble plaques comprised of aggregated beta-amyloid protein, neurofibrillary tangles formed from hyperphosphorylated tau protein, and neuronal death. The disease progresses over the course of about 4 to 8 years during which time cognitive functioning declines steadily until death (Alzheimer's Association, 2012).

Pharmaceutical treatment of AD, which cannot halt or slow the progression of cognitive decline (Piau et al., 2010), is currently limited to two classes of drugs: noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonists and reversible acetylcholinesterase inhibitors. As the American public ages, there is an urgent need to develop effective treatments that can address the enormous societal and economic burdens of this debilitating disease. Because women are significantly more likely to develop AD, it has been proposed that the postmenopausal decrease in estrogen contributes to molecular, cellular, and hormonal changes that induce pathological changes in the brain (Paganini-Hill and Henderson, 1994). The neuroprotective effect of estrogen has been the target of potential preventative therapies for AD (Simpkins et al., 2009). Both *in vitro* and *in vivo* evidence suggests that estrogen can prevent or reduce the pathological features of AD, including the formation of Aβ plaques and hyperphosphorylation of tau, as well as cognitive decline.

The use of estrogen *in vitro* has revealed neuroprotection against development of all three main pathological features of AD: Aβ plaques, neurofibrillary tangles, and neuronal death. *In vitro* application of estrogen also reduces Aβ-induced toxicity (Fitzpatrick et al., 2002; Marin et al., 2003), potentially by decreasing the production of Aβ or by increasing its degradation. In AD, Aβ plaques form when the amyloid precursor protein (APP) is cleaved into Aβ peptides by the proteolytic enzymes, α-, β-, and γ-secretase. Processing by the α-secretase pathway results in large N-terminal nonamyloidogenic soluble APP (sAPPα), whereas the β- and γ-secretase pathways lead to amyloidogenic Aβ that readily forms toxic aggregates. 17β-E2 decreases the formation of Aβ plaques by increasing α-secretase processing of APP into sAPPα, which does not readily form neurotoxic aggregates like those processed by either β- or γ-secretase (Manthey et al., 2001; Xu et al., 1998). In addition to preventing the formation of Aβ, 17β-E2 also encourages the degradation of Aβ peptides. By upregulating neprilysin (Liang et al., 2010) and insulin-degrading enzyme (Zhao et al., 2011), both enzymes that

degrade Aβ, estrogen slows down the buildup of toxic Aβ fragments. Through slowing the production of Aβ and also increasing the breakdown, 17β-E2 attenuates Aβ-induced toxicity (Nilsen et al., 2006).

Use of estrogen can regulate the *in vitro* hyperphosphorylation of tau protein into neurofibrillary tangles; in human neuroblastoma SH-SY5Y cells treated with tangle-inducing okadaic acid, application of 17β-E2 prevents tau phosphorylation (Alvarez-de-la-Rosa et al., 2005; Zhang and Simpkins, 2010). By decreasing the activity of protein kinases responsible for phosphorylating pathological sites on the tau protein, 17β-E2 can also prevent tau phosphorylation in HEK293 cells expressing full-length tau protein. In particular, a decrease in tau phosphorylation can occur through a reduction in the overactivation of protein kinase A, which plays a crucial role in perpetuating tau pathology (Liu et al., 2008). Similarly, estrogen inactivates glycogen synthase kinase-3β (GSK-3β), another kinase known to phosphorylate tau at pathological sites, in an estrogen receptor-mediated manner (Goodenough et al., 2005). The ability of estrogen to prevent the induction of tau pathology suggests that estrogen may serve a neuroprotective role against the damage associated with neurofibrillary tangle formation.

Compelling evidence for the role of estrogen as a neuroprotective factor for AD has also been demonstrated in *in vivo* models. The depletion of estrogen results in increased beta-amyloid (Aβ) levels in guinea pigs (Carroll et al., 2007; Petanceska et al., 2000), and Aβ production and plaque formation in AD mouse models can be reversed with 17β-E2 administration (Zheng et al., 2002). 17β-E2 administered to ovariectomized mice that have AD-related mutations in Aβ (Levin-Allerhand et al., 2002), or both Aβ and tau mutations (Carroll et al., 2007), prevents the worsening of Aβ accumulation that accompanies the loss of estrogen, as well as the associated memory deficits (Carroll et al., 2007). Similarly, estrogen therapy significantly reduces the associated increases in Aβ levels (Levin-Allerhand et al., 2002; Xu et al., 2006).

With respect to tau pathology, the sudden loss of estrogen resulting from an ovariectomy is associated with tau accumulation and hyperphosphorylation. 17β-E2 administration reduces the abnormal tau hyperphosphorylation associated with two risk factors of AD: transient cerebral ischemia (Wen et al., 2004; Zhang et al., 2008) and Down syndrome (Hunter et al., 2004). One possible mechanism for the neuroprotective role of estrogens against tau pathology includes the prevention of signaling pathways that promote neurodegeneration. For instance, 17β-E2 prevents the induction of Dkk1, a neurodegenerative factor that serves as an antagonist for the Wnt/β-catenin signaling pathway (Zhang et al., 2008). In particular, the Wnt/β-catenin signaling pathway protects the CA1 region of the hippocampus from damage. Because the Wnt/β-catenin signaling pathway becomes activated from lack of Dkk1 antagonism, there is subsequent

protection from ischemia-induced neuronal death in the hippocampus, a brain region particularly important for memory. Given that estrogens have been effective at alleviating multiple pathological hallmarks of AD, additional research is warranted to fully characterize the neuroprotective potential of estrogens for therapeutic intervention.

ESTROGEN AND ISCHEMIC STROKE

Stroke is a leading cause of long-term disability and the fourth leading cause of death in the United States following heart disease, cancer, and chronic lower respiratory disease. Approximately 795,000 strokes are reported each year in the US, 87% of which are ischemic strokes (Go et al., 2013). The direct medical cost associated with stroke in 2009 was approximately $22.8 billion, with an additional $13.8 billion in indirect costs associated with lost productivity, unemployment, rehabilitation, and follow-up care (Go et al., 2013).

Despite the significant physical and economic burdens of stroke, treatment options are limited. Tissue plasminogen activator (tPA) is a thrombolytic agent that breaks down blood clots to restore blood flow to the ischemic region of the brain and is the only FDA-approved drug available to treat ischemic stroke. Unfortunately, tPA is only effective if administered within 4½ hours of stroke onset. Many stroke patients do not reach a treatment facility within this time window, and thus are ineligible to receive tPA (Go et al., 2013). As of 2012, 114 drugs have entered clinical trials to evaluate their efficacy in ischemic stroke treatment; however, all have since failed to show efficacy in reducing ischemic damage (Lakhan et al., 2009). Given the incidence of ischemic stroke in the US and the limited therapeutic window of tPA, it is critical to develop a more effective treatment for ischemic stroke.

Women tend to have more severe strokes, more stroke deaths, and increased poststroke functional deficits than men (Appelros et al., 2009). Although lifetime risk of stroke is higher for men than for women, from ages 19–30, and again from ages 45–54, women have an increased stroke risk compared to men. One plausible explanation for the increased risk of stroke during these time periods is alterations in estrogen status. The first stroke surge is likely due to a high rate of childbirth over this age range. During pregnancy, maternal estrogen levels rise due to an increased estrogen production by the placenta. Following childbirth, estrogen levels decrease rapidly, while still remaining elevated compared to prepregnancy levels. These rapid decreases and changes in estrogen levels result in many health issues, including an increased risk of ischemic stroke (Koellhoffer and McCullough, 2013). The second stroke surge is likely due to the menopausal transition.

Typically occurring near age 50, menopause is the cessation of reproductive fertility in women due to a decreased production of circulating sex hormones, such as estrogen and progesterone (Ritzel et al., 2013). This transition into reproductive senescence and long-term estrogen deprivation is accompanied by symptoms that diminish a woman's quality of life, including hot flashes, night sweats, insomnia, weight gain, and osteoporosis. Women who undergo menopause before age 42 have a doubled lifetime risk of stroke compared to women undergoing menopause after 51 years of age (Go et al., 2013). Evidence also suggests that a decreased length of time between menarche and menopause, resulting in a decrease in overall estradiol exposure, increases the risk of ischemic stroke (De Leciñana et al., 2007).

Ischemic stroke is characterized by an abrupt deprivation of blood flow, oxygen, and nutrients to the brain that quickly leads to cell death in the core of the ischemic brain region. Initiation of the ischemic cascade begins within minutes of ischemia onset and involves several events, such as increased oxidative stress and mitochondrial dysfunction, resulting in apoptosis and a progressive loss of cells in the penumbra that may continue hours to days after stroke. Estrogen has been shown to mediate many events of the ischemic cascade, thereby providing a probable link to sex differences in stroke incidence and progression.

17β-E2 is neuroprotective in *in vivo* models of focal cerebral ischemia. Neuroprotection has been demonstrated in ovariectomized female mice, rodents, and gerbils (Dubal et al., 1998; Rusa et al., 1999; Shughrue and Merchenthaler, 2003; Simpkins et al., 1997; Yang et al., 2000; Zhang et al., 1998), in both young and middle-aged animals (Dubal and Wise, 2001). Gibson et al. (2006) compiled an extensive review of all experimental studies to date that have examined the effects of estrogen treatment on cerebral ischemia. Overall, estrogens reduce lesion volume in a dose-dependent manner when administered up to a week before or up to four hours after transient or permanent cerebral ischemia (Gibson et al., 2006). While most experimental studies demonstrate that both pre- and posttreatment with 17β-E2 reduces lesion size and infarct volume in models of focal cerebral ischemia, there are a limited number of studies that suggest no effect or a more negative outcome as a result of 17β-E2 treatment (Harukuni et al., 2001; Leon et al., 2012).

LIMITATIONS/FUTURE DIRECTIONS

Despite the prevailing amount of experimental evidence supporting the neuroprotective role of estrogens, the Women's Health Initiative (WHI) study was ended early because of findings indicating increased risks of cardiovascular disease, stroke, blood clots, breast cancer, and

dementia for women on estrogen therapy. The WHI began in 1993 and consisted of two double-blind, placebo-controlled clinical trials to determine whether estrogen alone or estrogen given with progestin would reduce the number of cardiovascular events in postmenopausal women. The estrogen-alone trial enrolled 10,739 postmenopausal women between 50 and 79 years of age with prior hysterectomy. The WHI estrogen-alone trial was scheduled to end between October 2004 and March 2005; however, the study was terminated in February 2004 when conjugated equine estrogens did not affect the risk of cardiovascular disease in participants. Aside from failing to reduce the incidence of heart disease, the incidence of stroke was increased by 39 percent in the conjugated equine estrogens group when compared to placebo (Anderson et al., 2004).

Re-evaluation of the surprising findings of the WHI has generated several hypotheses to account for the increased incidence of stroke among women receiving estrogen therapy (Lobo, 2013). First, the increased risk of stroke reported by the WHI is of borderline significance. Eliminating data from subjects with comorbidities, such as obesity and hypertension, may render the increased risk insignificant. Second, the age of the subjects can confound the results of the WHI. While the overall risk of stroke significantly increased, the increased risk of stroke among younger women (<50 years of age) was negligible (Dubey et al., 2005; Grodstein et al., 2008). The average age of WHI participants was 63 years of age, approximately 13 years after the average onset of menopause (Anderson et al., 2004). Overall, 83 percent of WHI subjects were at least 5 years into menopause. The critical window hypothesis suggests that initiation of estrogen therapy near the onset of menopause in younger women has a higher benefit/risk ratio, whereas ET in older women who have been in menopause for an extended period of time may be ineffective or harmful (Coker et al., 2009; Grodstein et al., 2008). Additionally, the route of estrogen administration may impact the results of the WHI. In the WHI, estrogen was given orally, and data suggests that transdermal administration may be safer and more effective than oral administration by avoiding first-pass metabolism and bioactivation (Grodstein et al., 2008). It is also important to consider that in experimental studies, estrogens are generally administered via intravenous or subcutaneous injection, and this difference may shed some light on the discrepancy between experimental stroke models and clinical trials.

CONCLUSIONS

Minimizing the off-target effects associated with the deleterious outcomes of estrogen therapy, while identifying and targeting the sites responsible for estrogen's neuroprotective effects, remains a top priority

for the field. Some of the deleterious effects resulting from estrogen therapy may be due to activation of ERs in peripheral tissue (e.g., breast and uterus). Use of compounds, such as nonfeminizing estrogens, which exhibit protective actions independent of activation of the known ERs, ERα, ERβ, and GPR30, may allow for an ET strategy with minimal side effects by avoiding activation of ERs in peripheral tissue. Similarly, identifying the parameters, such as duration, route of administration, and cyclic vs. tonic administration, by which ET confers protection is essential to making ET a viable option for neural injury.

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