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Age-Related Changes in Neuroprotection

Is Estrogen Pro-inflammatory for the Reproductive Senescent Brain?

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Estrogen replacement therapy (ERT) is widely prescribed to postmenopausal women for relief from the adverse vasomotor effects of menopause, to reduce bone loss, to improve cardiovascular health, and to protect against metabolic disorders. However, there is now greater awareness of the increased risk to benefit ratio from the recently concluded Women's Health Initiative Memory Study (WHIMS), which reported that ERT increased the risk of cognitive impairment and dementia in elderly women. Studies from the experimental literature indicate that while estrogen is neuroprotective in many instances, estrogen replacement can be deleterious in some cases. These differences may be partly due to the age and species of the experimental model. The majority of the experimental data comes from studies where the age or endocrine status of the animal model is not comparable to that of menopausal or postmenopausal women, such as those in the WHIMS study. In this review, we will focus on age-related changes in estrogen's neuroprotective effects and evidence that reproductive senescence-related changes in the blood–brain barrier and the immune system may result in deleterious consequences for ERT.

Key Words: Reproductive senescent; blood–brain barrier; neuroinflammation; microglia; hormone replacement therapy.

Estrogen Replacement Therapy and Cognition

Steroid hormones, primarily secreted by the ovaries, regulate numerous vital functions of brain in addition to their role in reproduction. Menopause, whether occurring naturally or induced surgically, is accompanied by declining titers of circulating estrogen, thereby affecting the functional capacity of certain brain regions involved in learning, memory, and cognitive ability. Estrogen replacement therapy (ERT), which is typically recommended for menopause-associated symptoms such as sleeplessness, hot flashes, vaginal dryness, also has the added advantage of improving cognitive function and memory. Animal studies have shown that estrogen improves learning and memory in rodents *(32,61)* and cognitive function in aged monkeys *(57,79,99)*. Estrogen also improves cognitive function and decreases depression and psychosis in postmenopausal women *(29, 48,84,108,112,113)*. ERT has also been shown to decrease the risk/delay the onset of various neurodegenerative diseases *(53,77,94)*. However, estrogen failed to improve cognition in women with mild to moderate Alzheimer's disease *(66)*. Moreover, the recent Women's Health Initiative Memory Study (WHIMS) concluded that hormone replacement therapy (estrogen + progesterone) increased the risk for dementia *(85)* and estrogen alone increased global cognitive impairment *(28,85)*. This WHIMS cohort consisted of asymptomatic women who were 65 yr or older, and therefore significantly beyond the onset of menopause. ERT, on the other hand, is typically given to pre- and perimenopausal women for specific symptoms. Hence it may be argued that estrogen replacement is only likely to be effective when initiated in the perimenopausal period, and may not be neuroprotective when given to significantly postmenopausal women, as in the WHIMS. Some support for this idea comes from the Cache county prospective study where it was noted that ERT improved cognition in women who used this therapy immediately at menopause, but had adverse effects in women who initiated the therapy during the postmenopausal period *(109)*. These findings underscore the importance of the timing of estrogen treatment in relation to the menopause. In the experimental literature, this issue has not been well studied, primarily due to the scarcity of animal models that mimic the menopause, and the study of appropriate downstream measures to determine the protective/deleterious effects of estrogen. In this review we will first focus on studies from reproductive aging models, and then review the evidence for estrogen's effects on neural inflammation.

Neuroprotective Actions of Estrogen in Reproductive Aging

While females of all mammalian species undergo reproductive aging, the pattern of hormonal loss can differ, making cross-species comparisons challenging. In humans, ovar-

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ian aging is accompanied by a gradual decline of estrogen with a concomitant increase in follicle stimulating hormone (FSH) and decrease in inhibin B *(40)*. In monkeys, a similar pattern occurs; however, unlike the human female, where longevity far exceeds reproductive aging, monkeys may continue to cycle regularly until fairly aged *(39)*. In the case of rats, the most common experimental model for studying the neuroprotective effects of estrogen, reproductive aging is accompanied by a lengthening of the estrous cycle with a period of persistent estrus characterized by moderate levels of estrogen followed by constant diestrus where hormone levels are low *(30,35,47,58)*. However, like humans, reproductive aging in the rat occurs at middle age.

The most typical model employed in studies of estrogen's neuroprotective actions is the ovariectomized rat, where the loss of ovarian hormones is sudden, and the estrogen replacement is typically (but not always) immediate. In this model, ovariectomy has been shown to impair performance on a variety of cognitive, mainly spatial, tasks, and this impairment can be attenuated by estrogen replacement *(36, 37,60,87)*. However, while the surgical ovariectomy model mimics the loss of endogenous gonadal hormones, it is not physiologically similar to the menopause. This model more appropriately resembles the clinical population of young women who have undergone oophorectomy for benign disease and are subsequently replaced with estrogen or estrogen + androgens. In this population, women replaced with either estrogen or estrogen + androgen after the surgery maintained their performance on a verbal memory test as compared to placebo *(84)*, and this was further confirmed in a more extensive battery of neuropsychological tests *(76)*.

The surgical ovariectomy model has also been used to demonstrate the neuroprotective effects of estrogen in a variety of injury paradigms. A commonly used strategy for neural injury is the occlusion of a major cerebral artery, typically the middle cerebral artery (MCA), to mimic a stroke injury. Ischemia resulting from MCA occlusion causes cell death, and estrogen replacement to ovariectomized animals has been shown to attenuate the size of the infarct resulting from transient *(5,86,110)* or permanent occlusion of the MCA *(25)*. However, several recent studies indicate that neither acute nor chronic estrogen replacement is effective in decreasing infarct volume *(105)* and in some cases, actually increased infarct volume at 24 h *(13,44)* and 3 d *(98)* after MCA occlusion. These studies suggest the need for a careful re-evaluation of estrogen's role as a neuroprotective agent in the injured forebrain.

Estrogen therapy in humans is most widely used at menopause, where levels of endogenous hormones decrease gradually, unlike the abrupt loss that occurs with surgical menopause. Recently, some laboratories have begun to investigate the effects of estrogen replacement in an animal model where the cyclic pattern of endogenous gonadal hormone synthesis and release has deteriorated. In rats this is referred to as the reproductive senescence, and is in many ways physiologically similar to the menopause. Interestingly, in the reproductive senescent animal, estrogen replacement is not always neuroprotective. In an ischemia model, older animals had a much larger infarct resulting from the injury when compared to the younger females. However, estrogen was able to reduce infarct size in both young and middle-aged animals *(5)*. Similarly, work from Wise's laboratory shows that estrogen replacement to young and middle-aged animals decreased ischemic injury by almost 50% *(26)*. While estrogen replacement to surgically menopausal rats has been shown to increase neurotrophic support by increasing growth factors and their receptors *(49,50)* and increasing the transport of growth factors in a forebrain cholinergic circuit *(114)*, estrogen replacement to reproductive senescent females decreases trophic support in the forebrain as seen by decreasing growth factor proteins in the olfactory bulb *(49)* and growth factor receptor mRNA in the basal forebrain *(38)*. In the case of cognitive tasks, estrogen replacement improves cognitive tasks when given to young adult females; however, estrogen replacement to older females has mixed effects. For example, long-term estrogen replacement to aged Sprague–Dawley female rats improves performance on spatial tasks *(31,38,61)*, but long-term estrogen replacement to Fisher 344 female rats did not improve performance on the water maze task *(62)*. Recent studies on carefully characterized reproductive senescent females suggest that estrogen's effects on cognitive tasks may be sensitive to subtle physiological changes in the reproductive aging process. For example, the muscarinic receptor antagonist scopolamine severely impairs acquisition of a T-maze avoidance task in middle-aged female rats in constant diestrus (where estrogen levels are chronically low) as compared to young adult rats and older females in constant estrus (where mid-range estrogen levels are available). Furthermore, estrogen replacement fails to protect against this impairment in senescent females that are in constant diestrus, although hormone replacement is effective in senescent females that are in constant estrus *(81)*.

Estrogen's Effects on Neural Inflammation

An important aspect of the neuroprotective role of estrogen is its effect on the inflammatory response. Inflammation contributes to the etiology of neurodegenerative diseases like Alzheimer's disease (AD) *(3,4)*, Parkinson's disease (PD) *(96)*, and multiple sclerosis (MS) *(71)*. Inflammation is characterized by increased levels of circulating cytokines, the intracellular signaling polypeptides secreted by the patrolling immune cells (monocytes, macrophages, and other immune cells). Peripheral inflammation is triggered by tissue damage, entry of microorganisms/foreign substance, and autoimmune disorders. Specific types of cytokines are also readily transferred to the brain via the active transporters in

the blood–brain barrier and present a major challenge to the normal functioning of CNS (reviewed in refs. *9* and *115*). Once in the brain, these molecules are capable of increasing lipid peroxidation and reactive oxygen species. The cytokine IL-1β, for example, is believed to be an important mediator of neurodegeneration induced by cerebral ischemia *(100)* or traumatic brain injury *(80)*. A recent study showed that metabolic disease syndrome, which results in chronic increases in circulating inflammatory cytokines, is associated with cognitive impairment in the elderly *(107)*. Hence, understanding estrogen action on neural inflammation is an important consideration in determining whether estrogen replacement is neuroprotective or neurodegenerative.

Unlike glucocorticoids, estrogen's effects on the inflammatory system are more ambiguous. In the case of anterior uveitis *(64)*, carrageenan-induced pleurisy in the lungs *(20, 21)* and adjuvant-induced arthritis *(6)*, estrogen appears to decrease inflammation. On the other hand, estrogen promotes prostatitis *(67)*, stimulates edema *(95)*, and increases vascular permeability and influx of macrophages in the uterus *(22,52)*. In humans, estrogen and progestin replacement to patients with systemic lupus erythematosus (SLE) increases the occurrence of mild to moderate (but not severe) symptoms *(12)*. Estrogen's actions on neural inflammation have typically been investigated on primary cultures of microglia or glial cell lines. In in vitro models, estrogen pretreatment attenuates LPS-induced superoxide release, phagocytic activity and an increase in inducible nitric oxide synthase (iNOS) *(11,24,103,104)*. In vivo intracerebral LPS injections that activate local microglia and peripheral monocytes are reduced by estrogen treatment *(102)*. In cerebral vessels, estrogen significantly inhibits the pro-inflammatory COX-2 pathway *(72)*. However, estrogen's actions are not always benign. Recent studies using LPS as an inflammagen show that 17β-estradiol fails to suppress cytokine release in activated N9 microglial cells in vitro *(93)*, and stimulates cytokine mRNA in the brain in animals injected with LPS systemically *(90)*. Furthermore, long-term treatment of ovariectomized animals with estrogen (8 wk) failed to attenuate microglial activation in vivo when animals were infused with LPS into the ventricles *(62)*. Interestingly, intact females had an attenuated microglial response as compared to ovariectomized animals *(62)*, suggesting that some combination of endogenous gonadal hormones may regulate the activation of microglia.

In our studies, we find that estrogen's effects on the inflammatory response are best predicted by the reproductive age of the individual. Using an excitotoxic forebrain injury model, which results in microglial activation and inflammatory cytokine synthesis, we noted that estrogen replacement to ovariectomized young adult animals reduced the expression of the pro-inflammatory cytokine IL-1β. On the other hand, estrogen replacement to reproductive senescent females with forebrain injury increased the expression of this cytokine *(69)*. Furthermore, while estrogen replacement or forebrain injury did not alter levels of the anti-inflammatory cytokine IL-10, there was significantly greater expression of this cytokine in the reproductive senescent animals as compared to young adult females. A constitutive increase in IL-10 is often considered to be a sign of chronic immune activation. These data suggest that reproductive aging alters the basal and constitutive expression of inflammatory mediators. The reproductive aging process may therefore be the earliest indicator of other aging-related changes in the immune system. It is fairly well known that chronological aging alters cytokine expression in mice *(97)* and microglial activation in rodents *(70)*, nonhuman primates *(82)*, and humans *(83)*. Furthermore, inflammatory responses to forebrain injury are altered in chronologically aged animals *(56)* as are the effects of certain NSAIDs *(45)*.

Injury related increases in IL-1β in the brain can result from activation of local immune cells or from increased traffic of circulating immune cells into the brain. In order to determine whether estrogen's actions occurred at the level of brain resident immune cells, we separately cultured microglia from young adult and reproductive senescent females. Microglia from both young and senescent animals responded to LPS with an increase in IL-1β; however, estrogen failed to attenuate cytokine expression at either age *(51)*. This is similar to the effect reported in a recent paper that compared the actions of 17β-estradiol and SERMS *(93)*. We also noted that estrogen's actions varied depending on the inflammatory mediators assayed. Hence, estrogen decreased NO in young adult microglial cultures (irrespective of LPS treatment) but did not in the senescent-derived cultures. On the other hand, estrogen increased MMP-9, the matrix-remodeling protein, in microglial cultures derived from senescent females (irrespective of LPS treatment), but failed to regulate this protein in microglial cultures derived from young adult animals *(51)*. Hence, it appears that estrogen's effects on inflammatory response is a complex interaction between age of the tissue donor and the type of inflammatory mediator.

Because estrogen effects on the injured brain did not mirror estrogen effects on microglia, we proposed that estrogen may suppress neural inflammation by either acting on circulating immune cells or by its actions on the blood–brain barrier. Our recent evidence indicates that estrogen may act on both targets, and, furthermore, that estrogen action on these targets depends on the reproductive age of the animal. In whole blood cultures obtained from young adult females, estrogen was able to attenuate LPS-induced increases in the inflammatory cytokine TNF-alpha. However, in whole blood cultures obtained from reproductive senescent animals, estrogen actually increased expression of this cytokine *(51)*.

This pattern of estrogen regulation of an inflammatory cytokine was similar to the one we observed in the in vivo injury model *(69)*, and suggests that estrogen's effects on

neural inflammation may be secondary to its effects on the circulating immune system. Under most conditions, the brain is impermeable to circulating immune cells due to the presence of the blood–brain barrier. Hence, it becomes critical to understand how the barrier function changes with age and, furthermore, how estrogen affects barrier function.

Estrogen and Blood–Brain Barrier

The blood–brain barrier is a physical barrier that is formed by a complex of specialized endothelial cells, pericytes of the basal membrane, and astrocytic end feet. The endothelial cells of the blood–brain barrier differ from other endothelial cells in possessing distinct properties such as tight junctions, high transendothelial resistance, lack of fenestrae, and fewer pinocytic vesicles. The presence of tight junctions restricts the passage of blood-borne pathogens, complements, and inflammatory cytokines in the periphery from entering the brain (for review see refs. *1* and *19*).

Pathologic conditions increase the permeability of the blood–brain barrier through a number of factors, such as the products of arachidonic acid (eicosanoids), free radicals, and histamines *(42)*. Blood–brain barrier breakdown/dysfunction is associated with several neurodegenerative diseases such as AD *(101)* and MS *(75)*. Entry of peripheral cytokines into the brain is believed to be the major step in the development and progression of neurodegenerative diseases *(9)*.

Estrogen effects on the blood–brain barrier are typically shown to decrease permeability. Blood–brain barrier disruption due to VEGF *(16)*, cerebral ischemia *(17,18)*, and bicculline-induced seizures *(73)* is attenuated by estrogen in young adult rats as compared to ovariectomized animals. Similarly, edema and extravasation of IgG resulting from systemic injections of 3-NPA (nitroproprionic acid) were attenuated in estrogen-replaced animals as compared to ovariectomized controls *(68)*. Chronic treatment with ethinyl estradiol however, increased the permeability of the blood–brain barrier to sucrose and inulin *(111)*. Our studies on the permeability of the blood–brain barrier show that barrier function, as well as estrogen's effects on the barrier, is crucially dependent on the reproductive age of the animal. Permeability of the cerebral vasculature was assessed by extravasation of Evan's blue from the blood to brain tissue. Dye extravasation was almost two to three times greater in the olfactory bulb and hippocampus of senescent animals as compared to their young counterparts. Furthermore, estrogen treatment decreased dye extravasation in both regions in the young adult groups, but not the reproductive senescent groups. In fact, estrogen treatment significantly increased dye extravasation in the hippocampus of senescent animals, as compared to placebo-treated age-matched controls *(7)*. These findings confirm our earlier observations that hormonal changes occurring during, or leading up to, the reproductive senescence make the brain vulnerable to inflammation, and further alter the responsiveness of several cellular targets to estrogen.

Age-dependent changes in blood–brain barrier permeability may be affected by changes in the endothelial cells of the barrier. Endothelial cells are at the interface between the peripheral pool of cytokines and brain parenchyma, and thus play an important role in vascular recruitment and transmigration of immune cells like leukocytes. Transport across the blood–brain barrier is effected through two major routes, transcellular (active transporters, pinocytosis) and paracellular (via tight junctions). Endothelial cells are responsive to estrogen *(23)*, and estrogen regulates NO expression in these cells through activation and expression of endothelial (e)NOS *(91)*. Inhibition of NO is a significant event because it further inhibits intracellular adhesion molecule (ICAM), vascular adhesion molecule (VCAM), and selectins, proteins that mediate leukocyte transmigration into the adjacent tissues. Similarly, 17β-estradiol has been shown to directly attenuate IL-1β–induced expression of ICAM-1 and NF kappa B stimulation in brain endothelial cultures *(33)*.

In our Evan's blue dye experiments, it remains to be resolved whether reproductive aging enhances transcellular or paracellular passage of dye. Evan's blue binds albumin, and albumin transport may be mediated by either means *(78)*. Paracellular transport is modulated by the presence of tight junctions, which restricts the passage of blood-borne pathogens, complements, and inflammatory cytokines in the periphery from entering the brain. Our preliminary studies indicate that tight junctions may be affected by reproductive aging (Bake and Sohrabji, unpublished observations). Tight junctions are composed of multiple transmembrane and cytoplasmic proteins (for review see ref. *43*) and some of these proteins are altered under pathological conditions. The transmembrane proteins include members of the claudin family and occludins, which are tethered to the actin cytoskeleton by the cytoplasmic proteins of the zona occludens family (ZO-1; ZO-2) and together they maintain the integrity of endothelium *(8)*. Claudin molecules on adjacent endothelial cells bind to form a seal, while occludin and claudin may potentially form fluctuating channels, which allow for selective diffusion of molecules *(63)*. Inflammation-induced changes in blood–brain barrier permeability are accompanied by loss of claudin-3 expression in experimental autoimmune encephalitis in mice *(106)*. Similarly, loss of the 55-kDa occludin protein is observed in cases of blood–brain barrier breakdown in brain tumors *(74)* and carageenan-induced inflammatory pain *(48)* in vivo. In vitro, occludin has been shown to relocate from the cell membrane to the cytoplasm under hypoxic conditions *(10)*. Similarly, in case-control studies of MS, abnormalities in tight junction protein ZO-1 result in fibrinogen leakage in vessels of MS patients (41%) compared to neurological controls (8%) *(116)*. Interestingly, occludin also decreases significantly with chronological age in 24-mo-old rats compared to 12-mo-old rats *(65)*. In our pilot studies we find that

the pattern of claudin-5 expression is reduced and more discontinuous in microvessels obtained from reproductive senescent animals as compared to young adult animals (Bake and Sohrabji, unpublished observations).

Taken together with our findings on the elevated levels of inflammatory cytokines and the leakiness of the barrier, it seems clear that the process of hormonal loss, culminating in reproductive senescence, makes the aging brain highly vulnerable to inflammation, and, consequently, to neurodegeneration.

Mechanism of Action of Estrogen

Although we have documented several constitutive and estrogen-mediated differences in the forebrains of young and senescent females, the mechanisms underlying these dimorphisms are poorly understood. In an early paper documenting neurotrophin changes in the senescent brain, we reported that estrogen-receptor systems were altered in the senescent brain *(49)*. Specifically, estrogen receptor (ER)–alpha was abnormally overexpressed in the olfactory bulbs of senescent females with a concomitant decrease in the steroid receptor coactivator SRC-1 *(49)*. Estrogen's actions are mediated by estrogen receptors located in either the nucleus or the cell membrane of target cells. The classical estrogen receptor is a member of the family of ligand-associated transcription factors that initiates or enhances transcription of genes containing specific hormone-response elements. Currently, two such ligand-activated transcription factors mediate estrogen's effects, ER-alpha *(41)* and ER-beta *(55)*. Although both types of estrogen receptors are present in the brain, studies from null mice indicate that ER-alpha plays a profound role in neuroprotection. For example, 17β-estradiol mediated neuroprotection was completely abolished in ER-alpha knockout female mice in an ischemic injury model *(27)*, as well as in an inflammation model *(34,104)*. In light of these studies, it would seem paradoxical that ER-alpha is overexpressed in animals where estrogen treatment is actually proinflammatory *(69)*. We have therefore proposed that there is a homeostatic dose of ER-alpha that is neuroprotective and that exceeding this optimal dose is likely to result in harmful effects for the cell *(88)*. We are currently testing this hypothesis in a cell line engineered to express graded levels of ER-alpha and our preliminary data indicate that growth factor regulation by estrogen peaks at low levels of ER-alpha, while at high levels of the receptor, estrogen fails to stimulate growth factor synthesis/release (Bake and Sohrabji, unpublished observations). This receptor–dose-dependent regulation of growth factor is reminiscent of the pattern of brain derived neurotrophic factor (BDNF) regulation noted in the young and senescent females, where estrogen increased BDNF in the olfactory bulb/basal forebrain circuit of the low-ER-alpha expressing young adults, while suppressing growth factor in the high-ER-alpha expressing senescent animal *(49)*.

Several recent studies have noted that aging causes an increase or alteration in the pattern of ER-alpha expression. ER-alpha positive cells are significantly increased in specific hypothalamic nuclei of the aged female rat *(14,15)*, as compared to the young adult or middle aged rat, while ER-beta expression decreases with advancing age. In humans, the pattern of ER-alpha expression reportedly changes from nuclear localization in young females to a more cytoplasmic localization in older females, who are free of AD pathology. Remarkably, in both male and female AD patients, there is a stronger nuclear localization of ER-alpha *(46)*. In an animal model of AD, however, nuclear localization of ER-alpha decreases in the forebrain neurons of 12-mo-old double transgenic APP-presenilin mice compared to 6-mo-old mice *(117)*. In the rhesus monkey brain, where ER-alpha is localized to select spines in hippocampal pyramidal neurons, aging results in a decline in the number of spines that express ER-alpha *(2)*. Because both neurons and neuronal support cells such as glia and endothelial cells are all estrogen sensitive, an important next step is to determine which one of these cells or combination of cells is altered with aging. Based on our recent studies, components of the blood–brain barrier appear to be specifically altered by reproductive aging. An informative approach would be to study, separately and in co-culture, the components of the barrier, such as endothelial cells, astrocytes, and microglia to determine (a) age-dependent changes in ER-alpha expression, (b) the regulatory controls on estrogen receptor expression, and (c) the dysregulation of estrogen-sensitive genes and signaling pathways in these cell types resulting from reproductive senescence.

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