From the 90's to Now: a Brief Historical Perspective on More Than *Two* Decades of Estrogen Neuroprotection

EB Engler-Chiurazzi[1,2,*], **M Singh**[3], and **JW Simpkins**[1,2]

[1]Center for Basic and Translational Stroke Research, West Virginia University, Morgantown, WV, 26506

[2]Department of Physiology and Pharmacology, West Virginia University, Morgantown, WV, 26506

[3]Department of Pharmacology and Neuroscience, University of North Texas, Fort Worth, TX, 76107

Abstract

We hypothesized that estradiol (E2) serves as a neurotrophomodulatory substance for basal forebrain cholinergic neurons thought to be involved in learning and memory. Learning/memory was assessed using the two-way active avoidance paradigm and the Morris water task. Female Sprague-Dawley rats were either ovariectomized (OVX) or OVX for 3 weeks, followed by s.c. implantation of a Silastic pellet containing 17-ß E2 (E2 pellet), resulting in a replacement of E2 to physiological levels. Ovary-intact (INTACT) animals served as our positive control. Active avoidance behavior and choline acetyltransferase (ChAT) activity in the frontal cortex and hippocampus were assessed at 5 and 28 weeks postovariectomy while performance on the Morris water task and high-affinity choline uptake (HACU) were measured only at the 5-week time point. At the 5-week time point, E2 replacement caused a significant elevation in the level of active avoidance performance relative to OVX animals. At the 28-week time point, OVX animals demonstrated a significantly lower number of avoidances relative to controls (61%) whereas E2-pellet animals not only demonstrated superior performance relative to OVX animals but also showed an accelerated rate of learning. Morris water task performance, on the other hand, was not significantly affected by estrogenic milieu despite a trend towards better performance in the E2-pellet group. Neurochemical analyses revealed that 5 weeks of ovariectomy was sufficient to reduce HACU in both the frontal cortex and hippocampus by 24 and 34%, respectively, while E2 replacement was successful in elevating HACU relative to OVX animals in both regions. ChAT activity was decreased in the hippocampus but not the frontal cortex of 5-week OVX animals. E2 replacement resulted in a reversal of this effect. At the 28-week time period, an unexpected decrease in ChAT activity was observed across all treatment groups. Interestingly, E2-pellet animals demonstrated the least severe decline in ChAT. This phenomenon was most evident in the frontal cortex where ChAT decreased by 61 and 56% in INTACT and OVX animals, respectively,

---

[*]Elizabeth B. Engler-Chiurazzi, Ph.D., Phone: (304)581-1836, elengler@hsc.wvu.edu.
Meharvan Singh, Ph.D., Phone: (817)735-5429, Meharvan.Singh@unthsc.edu
James W. Simpkins, Ph.D., Phone: (304)293-7430, jwsimpkins@hsc.wvu.edu

whereas the decline in E2-pellet animals was only 16% over the same time period, suggesting a previously unreported cytoprotective effect of E2. Taken together, these findings demonstrate important effects of estrogens on cholinergic neurons and support the potential use of estrogen therapy in treatment of dementias in postmenopausal women.

Keywords

Estrogen; cognition; memory; Alzheimer's disease; high-affinity choline uptake; choline acetyltransferase

Prior to the early 1990's, the notion that estrogens exert organizational and activational effects on reproductive tissues and sexual behavior had been well studied (Arnold, 2009; Wilson and Davies, 2007). The site of these effects was the estrogen receptor (ER), first discovered in uterine tissue (Gorski, 1994; Jensen et al., 2010) and cloned in 1986 (Greene et al., 1986). ER-alpha (ERα) was the first nuclear ER that demonstrated binding specificity for estradiol (17βE2) and was thought to be the sole ER with which all estrogens interacted. Although additional receptors would be identified in the coming years, distribution of the then only known nuclear ER was mapped to several tissues including uterus, ovaries, and brain, including hypothalamus; interestingly, ERα also appeared in brain regions associated with cognitive functions such as the cortex and hippocampus (Handa et al., 1994 Miranda and Toran-Allerand, 1992). Realization of ERs in these extrahypothalamic brain regions prompted the investigation of the impact of sex hormone depletion and replacement on substrates that controlled non-reproductive, higher-order cognitive functions.

At the time of original publication, Alzheimer's disease (AD) was, and still is, a major mental health concern in the United States. With the impending retirement of the first wave of Baby Boomers and the 'greying' of the adult population, concerns regarding costs associated with the medical treatment of a large, and growing, population of at-risk adults were increasing. In the early 1990's, the role of sex and gonadal hormones in risk for developing AD was only beginning to be elucidated. Preliminary case reports and small scale clinical investigations suggested that treatment with estrogen-containing therapies typically given for the management of disruptive menopausal symptoms could reduce AD risk and attenuate disease-associated cognitive deficits (Fillit et al., 1986; Henderson et al., 1994). As well, emerging findings identified that the mechanism by which estrogen imparted its beneficial mnemonic actions in AD patients may be due to its ability to impact the cholinergic system (Luine et al., 1975; Luine, 1985), a system known to be related to learning and memory performance (Flicker et al., 1983). The cholinergic system is associated with sex-specific, age-related functional changes (Luine et al., 1986), is dysregulated in AD (Francis et al., 1999), and is the therapeutic target of the few pharmacological interventions available for the relief of dementia-related symptoms (Giacobini, 1998). However, the memory-enhancing effects of estrogen-containing treatments, the neurochemical basis for these beneficial cognitive impacts, and their therapeutic potential for the treatment of AD-like dementia, were not well characterized at this point.

Using a preclinical animal model to address this important issue (Singh et al., 1994), we administered a subcutaneous silastic capsule containing either cholesterol or 17βE2 to young adult, female rats depleted of endogenous circulating sex hormones via the surgical removal of the ovaries (ovariectomy, OVX). A positive control group of ovary-intact, untreated female rats was included. Behavioral findings indicated that 17βE2 treatment reversed OVX-induced cognitive deficits to at least the level of ovary-intact control rats. Indeed, non-spatial active avoidance learning and memory performance was markedly improved by both short-term (2 weeks) and long-term (25 weeks) estrogen treatment, as 17βE2-treated animals had a higher number of total avoidances and required fewer days to criterion than OVX animals. As well, although findings were not statistically significant, short-term 17βE2 treatment tended to improve spatial memory on the Morris water maze, as evidenced by an increase in time spent in the goal quadrant and a higher number of crossings over the platform area, further supporting the beneficial impact of estrogen on cognitive outcomes.

To relate cognitive impacts of estrogen treatment to neurobiological substrates associated with learning and memory, we assessed high affinity choline uptake (HACU), the process by which neurons take up choline for acetylcholine synthesis, and activity of choline acetyltransferase (ChAT), the enzyme involved in the synthesis of acetylcholine from choline and an acetyl group from acetylcoenzyme A (Jope, 1979; Oda, 1999), in two cognitively-relevant brain regions. The favorable mnemonic impacts of estrogen treatment were associated with modulation of the cholinergic system in a region- and treatment duration-dependent manner. Specifically, surgical hormone removal significantly reduced levels of HACU relative to ovary-intact animals in both frontal cortex and hippocampus. As compared to OVX animals, short-term 17βE2 treatment reversed these reductions in both regions. As well, short-term 17βE2 treatment protected against the OVX-induced reduction in hippocampal ChAT activity. Interestingly, frontal cortex ChAT activity declined to a similar extent in both ovary-intact and OVX groups after 28, but not 5 week time points, possibly representing age-related changes in cholinergic function in this area. Indeed ovary-intact and ovariectomized groups showed declines in ChAT activity of 61% and 56%, respectively. Yet, long-term 17βE2 treatment attenuated the rate of change in ChAT activity in these animals. Thus, data supported the ability of estrogen-containing therapies to alleviate cognitive and cholinergic dysfunction associated with surgical hormone depletion and lay the groundwork for use of estrogen-containing therapies as beneficial therapeutic interventions for the treatment of AD and potentially other brain diseases as well.

Having demonstrated cognitive enhancements of exogenous estrogen treatment, we next began an important series of studies to elucidate the mechanism by which estrogen imparts these beneficial neurologic impacts. To this end, we assessed the modulatory impact of hormone depletion and estrogen treatment on brain-derived neurotrophic factor (BDNF) messenger ribonucleic acid (mRNA) expression in cognitive brain regions. Relative to ovary-intact controls, 17βE2 treatment rescued OVX-induced reductions in BDNF mRNA in a region-dependent manner, with beneficial effects observed in the CA3, CA4, dentate gyrus (Singh et al., 1995). In parallel, we also investigated the cytoprotective actions of estrogen *in vitro*. In glial (C6 glioma) and neuronal (SK-N-SH), 17βE2 prevented cell death following serum-deprivation (Bishop and Simpkins, 1994). Spurred by these exciting findings, we next

identified estrogenic neuroprotection in several other *in vivo* and *in vitro* models of neurological challenge, including stroke (Simpkins et al., 1997b; Yang et al., 2000; Yang et al., 2001; Zhang et al., 1998) and AD (Green et al., 1996; Simpkins et al., 1997a). Collectively, these findings identified mechanistic underpinnings for 17βE2-induced memory enhancements and further supported the notion that exogenous estrogens could be used as an effective treatment for brain disease in post-menopausal women.

As enthusiasm for estrogenic neuroprotection increased, an important question still remained: what about the estrogen molecule was responsible for its beneficial actions in brain? We discovered that estrogenic protection was not entirely dependent on interaction with the ER as co-administration of tamoxifen only partially inhibited the cytoprotecive actions of 17βE2 in SK-N-SH cells (Green et al., 1996). Further, we (Green et al., 1997) and others (Behl et al., 1997) were the first to demonstrate that the phenolic structure of the estrogen molecule (specifically the preservation of an intact phenolic A-ring and three rings of the steroid nucleus) is essential for the realization of protection against oxidative stress and serum deprivation. This discovery of the neuroprotective molecular characteristics of 17βE2 revealed an important potential strategy in the optimization of current and future estrogen-containing hormone therapy options. To this end, we developed a chemical library of estrogen-like molecules and tested more than 70 for neuroprotective potential in several *in vitro* models of cellular challenge. In general, A-ring modifications such as the addition of bulky alkyl groups at the 2- and 4-carbon positions enhanced neuroprotection following a variety of *in vitro* insults as well as following cerebral ischemia *in vivo* without stimulation of peripheral tissues (Perez et al., 2005a; Perez et al., 2005b; Simpkins et al., 2004). Conversely, 3-O-conjugation of the phenolic A-ring abolished neuroprotective ability.

In the broader context of estrogen and neuroprotection, the mounting number of investigations documenting beneficial impacts in several domains of neurological function (Chakrabarti et al., 2014) facilitated the conduct of large-scale investigations such as Women's Health Initiative series to evaluate estrogen-containing menopausal therapies for the prevention of a variety of age-related issues, including stroke, coronary heart disease, hip fractures, and cognitive decline (Manson et al., 2013). Unfortunately, the null or even detrimental outcomes of this and other clinical trials led to hesitation by both clinicians and patients in the use of exogenous estrogenic treatments for nervous system outcomes (Gleason et al., 2015; Maki and Henderson, 2012). A number of hypotheses, including the critical window for estrogenic intervention (Resnick and Henderson, 2002), have been posed to explain these surprising and disheartening findings and it is now generally accepted that estrogen acts as a conditional neuroprotectant with a complex pattern of biological actions that are modulated by several interacting factors (Engler-Chiurazzi et al., in press).

In conclusion, the findings from our seminal paper (Singh et al., 1994) demonstrating cognitive benefits and cholinergic impacts with exogenous estrogen treatment in a rodent model of surgical hormone depletion provided initial support for use of estrogen-containing therapies as a treatment for age-related brain disorders. After more than two decades of intense investigation, the beneficial action of 17βE2 has been well-verified. Currently, understanding of estrogen's therapeutic potential has been vastly improved and clinical recommendations have been modified appropriately. Still, effective treatments for a number

of age-related neurological conditions, including AD, do not exist. As the population ages and increasing numbers of aging women face spending nearly a third of their lives in a hormone-deprived state associated with memory problems, the development of interventions that optimize cognitive aging will be of critical importance. Based on the multitude of supportive evidence, estrogen, or an estrogen-like analogue, that imparts the desired beneficial peripheral and neuroprotective outcomes while simultaneously avoiding detrimental stimulatory actions may still be one of these interventions. It is our hope that continued investigations will yield clarification on this important clinical need.

Acknowledgements

References

Arnold AP. The organizational-activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues. Horm Behav. 2009; 55:570–8. [PubMed: 19446073]

Behl C, et al. Neuroprotection against oxidative stress by estrogens: structure-activity relationship. Mol Pharmacol. 1997; 51:535–41. [PubMed: 9106616]

Bishop J, Simpkins JW. Estradiol treatment increases viability of glioma and neuroblastoma cells in vitro. Mol Cell Neurosci. 1994; 5:303–8. [PubMed: 7804599]

Chakrabarti M, et al. Estrogen receptor agonists for attenuation of neuroinflammation and neurodegeneration. Brain Res Bull. 2014; 109:22–31. [PubMed: 25245209]

Engler-Chiurazzi EB, et al. Estrogens and neuroprotectants: estrogenic actions in the context of cognitive aging and brain injury. Prog Neurobiol. 2016

Fillit H, et al. Observations in a preliminary open trial of estradiol therapy for senile dementia-Alzheimer's type. Psychoneuroendocrinology. 1986; 11:337–45. [PubMed: 3786638]

Flicker C, et al. Behavioral and neurochemical effects following neurotoxic lesions of a major cholinergic input to the cerebral cortex in the rat. Pharmacol Biochem Behav. 1983; 18:973–81. [PubMed: 6889421]

Francis PT, et al. The cholinergic hypothesis of Alzheimer's disease: a review of progress. J Neurol Neurosurg Psychiatry. 1999; 66:137–47. [PubMed: 10071091]

Giacobini E. Invited review: Cholinesterase inhibitors for Alzheimer's disease therapy: from tacrine to future applications. Neurochem Int. 1998; 32:413–9. [PubMed: 9676739]

Gleason CE, et al. Effects of Hormone Therapy on Cognition and Mood in Recently Postmenopausal Women: Findings from the Randomized, Controlled KEEPS-Cognitive and Affective Study. PLoS Med. 2015; 12:e1001833. discussion e1001833. [PubMed: 26035291]

Gorski J. A hindsight view of early studies on the estrogen receptor: a personal history. Steroids. 1994; 59:240–3. [PubMed: 8079377]

Green PS, Gridley KE, Simpkins JW. Estradiol protects against beta-amyloid (25-35)-induced toxicity in SK-N-SH human neuroblastoma cells. Neurosci Lett. 1996; 218:165–8. [PubMed: 8945754]

Green PS, Gordon K, Simpkins JW. Phenolic A ring requirement for the neuroprotective effects of steroids. J Steroid Biochem Mol Biol. 1997; 63:229–35. [PubMed: 9459189]

Greene GL, et al. Sequence and expression of human estrogen receptor complementary DNA. Science. 1986; 231:1150–4. [PubMed: 3753802]

Handa RJ, et al. Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. Horm Behav. 1994; 28:464–76. [PubMed: 7729815]

Henderson VW, et al. Estrogen replacement therapy in older women. Comparisons between Alzheimer's disease cases and nondemented control subjects. Arch Neurol. 1994; 51:896–900. [PubMed: 8080389]

Jensen EV, et al. Estrogen action: A historic perspective on the implications of considering alternative approaches. Physiology & Behavior. 2010; 99:151–162. [PubMed: 19737574]

Jope RS. High affinity choline transport and acetylCoA production in brain and their roles in the regulation of acetylcholine synthesis. Brain Res. 1979; 180:313–44. [PubMed: 394816]

Luine VN, Khylchevskaya RI, McEwen BS. Effect of gonadal steroids on activities of monoamine oxidase and choline acetylase in rat brain. Brain Res. 1975; 86:293–306. [PubMed: 1115999]

Luine VN. Estradiol increases choline acetyltransferase activity in specific basal forebrain nuclei and projection areas of female rats. Exp Neurol. 1985; 89:484–90. [PubMed: 2990988]

Luine VN, et al. Age and sex-dependent decreases in ChAT in basal forebrain nuclei. Neurobiol Aging. 1986; 7:193–8. [PubMed: 3724953]

Maki PM, Henderson VW. Hormone therapy, dementia, and cognition: the Women's Health Initiative 10 years on. Climacteric. 2012; 15:256–62. [PubMed: 22612612]

Manson JE, et al. Menopausal hormone therapy and health outcomes during the intervention and extended poststopping phases of the Women's Health Initiative randomized trials. JAMA. 2013; 310:1353–68. [PubMed: 24084921]

Miranda RC, Toran-Allerand CD. Developmental expression of estrogen receptor mRNA in the rat cerebral cortex: a nonisotopic in situ hybridization histochemistry study. Cereb Cortex. 1992; 2:1–15. [PubMed: 1633405]

Oda Y. Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system. Pathol Int. 1999; 49:921–37. [PubMed: 10594838]

Perez E, et al. Neuroprotective effects of estratriene analogs: structure-activity relationships and molecular optimization. Drug Development Research. 2005a; 66:78–92.

Perez E, et al. Neuroprotective effects of an estratriene analog are estrogen receptor independent in vitro and in vivo. Brain Res. 2005b; 1038:216–22. [PubMed: 15757637]

Resnick SM, Henderson VW. Hormone therapy and risk of Alzheimer disease: a critical time. JAMA. 2002; 288:2170–2. [PubMed: 12413378]

Simpkins JW, et al. Role of estrogen replacement therapy in memory enhancement and the prevention of neuronal loss associated with Alzheimer's disease. Am J Med. 1997a; 103:19S–25S. [PubMed: 9344403]

Simpkins JW, et al. Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. J Neurosurg. 1997b; 87:724–30. [PubMed: 9347981]

Simpkins JW, et al. Estrogen-like compounds for ischemic neuroprotection. Stroke. 2004; 35:2648–51. [PubMed: 15472107]

Singh M, et al. Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. Brain Res. 1994; 644:305–12. [PubMed: 8050041]

Singh M, Meyer EM, Simpkins JW. The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. Endocrinology. 1995; 136:2320–4. [PubMed: 7720680]

Wilson CA, Davies DC. The control of sexual differentiation of the reproductive system and brain. Reproduction. 2007; 133:331–59. [PubMed: 17307903]

Yang SH, et al. Estradiol exerts neuroprotective effects when administered after ischemic insult. Stroke. 2000; 31:745–9. discussion 749-50. [PubMed: 10700514]

Yang SH, et al. 17-beta estradiol can reduce secondary ischemic damage and mortality of subarachnoid hemorrhage. J Cereb Blood Flow Metab. 2001; 21:174–81. [PubMed: 11176283]

Zhang YQ, et al. Effects of gender and estradiol treatment on focal brain ischemia. Brain Res. 1998; 784:321–4. [PubMed: 9518671]