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Neuroprotective action of acute estrogens: animal models of brain ischemia and clinical implications

Tomoko Inagaki and Anne M. Etgen

Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461

Abstract

The ovarian hormone 17 β -estradiol (E2) exerts profound neuroprotective actions against ischemia-induced brain damage in rodent models of global and focal ischemia. This review focuses on the neuroprotective efficacy of post-ischemic administration of E2 and non-feminizing estrogen analogs in the aging brain, with an emphasis on studies in animals subjected to a long-term loss of circulating E2. Clinical findings from the Women's Health Initiative study as well as data from animal studies that used long-term, physiological levels of E2 treatment are discussed in this context. We summarize major published findings that highlight the effective doses and timing of E2 treatment relative to onset of ischemia. We then discuss recent findings from our laboratory showing that under some conditions the aging hippocampus remains responsive to E2 and some neuroprotective non-feminizing estrogen analogs even after prolonged periods of hormone withdrawal. Possible membrane-initiated signaling mechanisms that may underlie the neuroprotective actions of acutely administered E2 are also discussed. Based on these findings, we suggest that post-ischemic treatment with high doses of E2 or certain non-feminizing estrogen analogs may have great therapeutic potential for treatment of brain damage and neurodegeneration associated with ischemia.

Keywords

Estrogens; Hippocampus; Global ischemia; Stroke; Neuroprotection; GPR 30

Introduction

Focal cerebral ischemia or stroke is a leading cause of death and disability in the United States [1]. Each year, approximately 800,000 Americans, most of them elderly, are affected [2] [3]. Transient global ischemia arises as a consequence of cardiac arrest and results in selective, delayed death of CA1 hippocampal neurons [4]. Sudden cardiac arrest affects nearly 200,000 Americans each year [2]. Each form of ischemia significantly decreases the

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Correspondence should be addressed to: Tomoko Inagaki, Dept. of Neuroscience, Albert Einstein College of Medicine, 1300 Morris Park, Ave. F113, Bronx, NY 10461, tomoko.inagaki@einstein.yu.edu, tomoko8400@gmail.com, TEL: 718-430-3055, FAX: 718-430-2320.

Tomoko Inagaki, Dept. of Neuroscience, Albert Einstein College of Medicine, 1300 Morris Park, Ave. F113, Bronx, NY 10461, tomoko.inagaki@einstein.yu.edu, tomoko8400@gmail.com, TEL: 718-430-3055, FAX: 718-430-2320

Dr. Anne M. Etgen, Dept. of Neuroscience, Albert Einstein College of Medicine, 1300 Morris Park, Ave. F113, Bronx, NY 10461, Anne.Etgen@einstein.yu.edu, TEL: 718-430-3662, FAX: 718-430-8654

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quality of life, and the patients need to cope with severe cognitive, mental and physical impairment associated with neurodegeneration [5]. Brain ischemia remains a major health concerns in the US, but unfortunately few effective and safe treatments are available [2]. Therapeutic hypothermia and thrombolysis with tissue plasminogen activator (tPA) are the only recommended treatments to improve neurological outcomes [6]. However, due to narrow therapeutic time windows (e.g., <3 h for tPA), only few patients can receive treatment [7]. It is thus critical that new molecular and cellular mechanistic information be revealed and brought into the translational arena to ameliorate the deleterious consequences of brain ischemia and to develop novel therapeutic strategies to protect the brain from ischemic cell death.

It is now well documented that the ovarian steroid hormone 17 β -estradiol (E2) exerts profound neuroprotective actions in experimental models of global (4 vessel occlusion) and focal (middle cerebral artery occlusion, MCAO) ischemia (see [8] for review), affording histological protection in young and middle-aged female and male rodents [9–43]. We and others have shown that physiological levels of E2 administered chronically to ovariectomized (OVX) females ameliorate ischemia-induced cell death [20,44,45]. As well, a single dose of E2 given in close proximity to the onset of ischemia promotes cell survival after ischemia [17,23,25,37]. E2 treatment also reduces memory impairment associated with global ischemia [16,37,46] and decreases sensorimotor impairment following focal ischemia [32]. In OVX females, E2 treatment 24 h before MCAO reduces mortality [33]. In ovary intact females, circulating E2 levels are inversely correlated with MCAO-induced infarct size [24]. Furthermore, there is evidence that brain-derived estrogens may act as a natural neuroprotective factor [47–49]. For example, the extent of infarct volume increases in aromatase knockout relative to wild-type females [50]. MCAO also increases brain E2 concentrations, and co-injection of an aromatase inhibitor decreases brain E2 levels and is associated with more damage [51].

Despite the overwhelming evidence of beneficial effects of E2 against ischemia-induced brain injury, E2 has limited translational potential. First, in apparent contrast to beneficial E2 effects reported in basic research, the results of large clinical trials such as the Women Estrogen Stroke Trial (WEST) and the Women's Health Initiative (WHI) indicated that hormone therapies (HT) in women after menopause may increase risk of cardiovascular disease, stroke and dementia [52,53]. Similarly, E2 administered chronically at physiological levels (experimentally mimicking HT) in long-term OVX female rodents provides little or no benefit in focal or global ischemia [35,42]. Second, because E2 is a potent feminizing hormone, it cannot be administered in aging men. Therefore, one possible alternative therapeutic strategy to natural estrogens is development of synthetic estrogenic compounds that can be administered after an ischemic event, and that can be used for both men and women.

This review discusses neuroprotective actions of E2 as a potential candidate for a new therapeutic approach. Following a brief overview of the current controversies about HT and long-term E2 pretreatment in animal models, the review focuses on the efficacy of post-ischemic treatment E2 and non-feminizing estrogen analogs in the aging brain, with an emphasis on studies in animals subjected to a prolonged period of hormone withdrawal.

Critical period hypothesis and pretreatment with physiological levels of E2: Overview

Early basic research provided solid evidence that pretreatment (days to weeks) with E2 at physiological levels affords robust protection against ischemia-induced cell death in male and OVX female rodents (see [8,54] for review). As well, early clinical observational studies

demonstrated that estrogen therapy in postmenopausal women may enhance certain types of cognitive function [55,56] and reduce neurodegeneration associated with stroke and Alzheimer's disease [57]. Therefore, it was surprising that large clinical trials such as the WEST and WHI reported that HT did not reduce the incidence of cardiovascular and neurodegenerative diseases or cognitive impairment in elderly women [58–60], but may slightly increase the risk of stroke and dementia in postmenopausal women who initiated treatment long after menopause [52,53]. The average age of the women who enrolled in WHI clinical trials was 63.3 year olds, and the majority were postmenopausal for 13 years on average prior to the initiation of the hormone treatment [61]. Thus, the “critical period hypothesis” was proposed to explain the unexpected results. It proposes that there is an opportune time window for E2 to retain its beneficial actions following ovarian hormone withdrawal; if HT is not initiated within this time window, E2 may no longer protect the brain against various neurodegenerative insults [62]. Support for this hypothesis comes from both human [53,59,63] and animal studies [42,64–67]. In humans, recent updated clinical data from WHI trials in women aged 50–79 years with a hysterectomy history [68], and the 2012 Hormone Therapy Position Statement from The North American Menopause Society [69] further support the concept of “critical period hypothesis”. In animals, data on E2 effects on cognitive functions also support the hypothesis (see [70] for review).

With respect to ischemia, the outcomes of animal studies are mixed. We searched PubMed to identify animal studies that tested impact of long-term hormone deprivation on neuroprotective action of E2 with the following criteria: 1) studies should be *in vivo* and should use rodent models of focal or global ischemia; 2) treatment should be physiological concentrations of E2 given chronically for 1–2 weeks before injury; 3) subjects should be OVX or reproductively senescent females; 4) quantitative evaluation of neuronal survival or lesion volume/size should be reported. We identified nine such studies that evaluated “the critical period hypothesis” with chronically administered, physiological levels of E2 (10–80 pg/ml) in long-term OVX or reproductively senescent females [9,32,35,36,42–44,67,71]. This treatment regimen experimentally mimics HT in postmenopausal women. Five of the nine studies supported the critical period hypothesis, demonstrating loss of E2 neuroprotection when treatment was delayed for 10 weeks and four reported beneficial effects of E2 are not lost. For example, one week pretreatment that maintains low physiological levels of E2 (10–25 pg/ml) protects young mice [35] following focal ischemia, and young and middle-aged rats [43] after global ischemia when initiated immediately, but not when initiated 10 weeks after OVX. Similarly, one week treatment with low physiological levels of E2 (10–25 pg/ml) initiated at the time of OVX is protective after global ischemia in young and middle-aged rats, but not in 24 month-old females, which are likely to have ceased normal estrous cycles for many months [42]. Moreover, E2 pellets that maintain high physiological hormone levels (60–80 pg/ml) exacerbate focal ischemia-induced infarcts in reproductively senescent, middle-aged female rats [32,67].

In contrast, pretreatment with intermediate/high physiological levels of E2 protects the brain from ischemic cell death in middle-aged female rats OVX for 8 weeks [36,44] and in reproductively senescent females [9,71]. In one study from our laboratory, we initiated E2 treatment immediately, 1 wk or 8 wk after OVX to test whether the duration of hormone withdrawal affects E2 mediated CA1 cell survival and memory performance in middle-aged rats using a clinically relevant model of transient global ischemia [44]. This E2 treatment yielded high physiological levels of E2 and afforded histological neuroprotection at all OVX durations, suggesting that sensitivity to E2 neuroprotection is retained after long-term E2 withdrawal. Interestingly, the increased survival of CA1 hippocampal neurons was not associated with improved cognitive performance on either the visual or spatial memory test. This may reflect the poor performance of middle-aged rats on these tasks prior to ischemia. Because only nine studies examined this issue and the experimental conditions differed

widely (e.g., age at the time of OVX and the onset of ischemia, E2 concentration, duration and route of hormone administration, type of ischemia), it is difficult to explain the dichotomy in results. Any of these factors might have affected the outcomes. Indeed, there is evidence that type of menopause (natural vs. surgical, experimentally equivalent to reproductively senescent vs. animals OVX at a young age) may affect responsiveness to E2 after long-term hormone deprivation [72]. Strom and colleagues reviewed 66 studies on neuroprotective effects of E2 in animal models of ischemia and concluded dose is an important factor that may reduce or exacerbate damage [73].

Efficacy of post-ischemic treatment with E2 in young adult animals: Therapeutic time window and dose-response relationships

Accumulating evidence indicates that high-dose treatment with E2 effectively reduces cell death and lesion volume when administered acutely after onset of ischemia. Acute treatment is more clinically relevant than long-term pretreatment and has greater therapeutic potential, because agents can be administered after the onset of ischemic symptoms. We and others have investigated the efficacy of this treatment regimen to protect brains from ischemic injury and have found that a single dose of E2 given in close proximity to the onset of ischemia affords robust neuroprotection in both global and focal ischemia in young [16,19,23,74,75], middle-aged [23] and old [17] female OVX rats as well as in male rats [27,76,77], even when treatment is delayed up to 6 h after the onset of ischemia [25,74]. The range of effective E2 concentrations in promoting neuronal survival after ischemic insults is very large, from 10 µg/kg [77] to 4000 µg/kg [37], depending on the timing of treatment relative to the onset of ischemia [25,40] and model of ischemia used. Although these doses are supraphysiological, they do not elicit toxic effects [25]. No neuroprotection is afforded with physiological concentrations (10–60 pg/ml) when E2 pellets are implanted at the time of MCAO in young OVX females [78]. Interestingly, a recent study reports that therapeutic hypothermia alone or acute post-ischemic E2 (10 µg/kg) treatment alone immediately after global ischemia affords the same level of neuroprotection in adult male rats, but when the two treatments are combined (hypothermia+acute E2), surviving CA1 neurons are markedly increased [77]. The investigators suggest that acute post-ischemic E2 and hypothermia may work synergistically to exert neuroprotection. Moreover, consistent with beneficial effects of acute pre-ischemic E2 treatment observed in some memory tasks [37], our laboratory shows that a high dose E2 (50µg) given acutely after ischemia ameliorates ischemia-induced impairment of memory performance in object recognition, but not spatial memory, tests in young adult rats [16]. Behavioral significance of acute post-E2 treatment in older animals is currently unclear and remains to be evaluated. Table 1 summarizes findings reported in acute post-ischemic E2 studies.

In seminal work, Simpkins and his group examined the therapeutic time window and dose-response relationships for E2 at selected time points after ischemia in MCAO [25,40,41]. In the initial experiment, subcutaneous injection of 100 µg/kg of E2 administered 30 min, 1, 2, 3, 4 h after the onset of ischemia was neuroprotective up to 3 h after the onset of ischemia (Fig. 1A) [40,41]. They later reported the therapeutic time window could be prolonged up to 6 h if animals are treated with a higher dose (1000 µg/kg) of E2 [25]. In a dose-response study with 100, 500, 1000, 2000 and 5000 µg/kg administered 6 h after the onset of ischemia, dose-dependent reduction in lesion volume is observed up to 1000 µg/kg E2 treatment. Thus, the dose-response relationship for E2 treatment at 6 h post-MCAO appears to be an inverted-U with the maximum neuroprotective effects at 1000 µg/kg and no effects at the lowest and two higher doses (Fig 1B) [40]. This non-monotonic response pattern is not surprising as inverted-U dose-response curves are relatively common features for acute effects of some hormones, drugs, or neurotransmitters on CNS mediated function [79–81], including E2 modulation of hippocampus-dependent memory function [82–85].

Recent evidence suggests that the therapeutic time window for acute post-ischemic E2 treatment may be extended more than 6 h in global ischemia [74]. The effects of icv infusion of 20 μ g of E2 or the phytoestrogen coumestrol were examined at 0, 3, 6, or 24 h after transient global ischemia in adult OVX rats. Robust neuroprotective effects of coumestrol were observed even when treatment was delayed 24 h after the onset of ischemia. The therapeutic time window for this dose of E2 was shorter, with protection being lost at some point between 6 h and 24 h after ischemia [74]. CA1 cell survival rates in ischemic rats treated with 20 μ g of E2 immediately or 6 h after induction of ischemia are not significantly different. These results raise the possibility that E2 and other estrogenic compounds could be potential therapeutic agents following stroke and cardiac arrest.

Post-ischemic E2 treatment in the aging brain after long-term hormone deprivation

Because all experiments discussed above examined post-ischemic E2 effects in young adult animals (< 3–4 mo old) with short OVX intervals (1–2 wk), they do not address the efficacy of this form of E2 treatment in old animals or after long-term hormone deprivation. As stroke and cardiac arrest occur more frequently in elderly individuals, and the risk increases in women after menopause [86,87], it is critically important to evaluate the efficacy of neuroprotective strategies in age-appropriate, long-term hormone-deprived animals. Therefore, we investigated whether the aging hippocampus remains responsive to neuroprotective actions of post-ischemic E2 after a prolonged period of hormone withdrawal. Middle-aged retired breeders were OVX at 9–11 mo and subjected to global ischemia 2 or 6 mo after OVX. A single icv infusion of 2.25 μ g of E2 or sc injection of 100 μ g/kg of E2 immediately after reperfusion significantly promotes CA1 cell survival in females OVX for 2 mo (Fig. 2) [23]. This dose of E2 administered immediately after ischemia is also neuroprotective in old (15–18 mo) rats that were OVX 6 mo before experimentation (Fig. 3) [17]. CA1 pyramidal cell survival rates in E2-treated ischemic animals OVX for 2 and 6 mo were not statistically different. Thus, these data suggest that post-ischemic treatment with a high dose of E2 provides similar levels of neuroprotection regardless age and OVX duration.

E2 is known to increase synaptic transmission rapidly in the CA1 of the hippocampus via non-genomic actions [88], enhancing both NMDA receptor dependent [89–94] and non-NMDA receptor dependent [95–99] excitatory synaptic currents. To determine if the aging hippocampus retains the ability to respond to E2 after a prolonged period of hypoestrogenicity, we recorded field excitatory postsynaptic potentials (fEPSP) at CA1 synapses in response to Shaffer collateral stimulation in acute hippocampal slices derived from elderly, non-ischemic female rats OVX for 6 months. As shown in Fig 4A, bath application of E2 (1 nM) rapidly facilitates fEPSPs, and this action of E2 is indistinguishable in latency and magnitude to that recorded in slices from young rats. Furthermore, slices from old, hormone-withdrawn females exhibit normal LTP in response to high frequency stimulation (Fig. 4B). The ability of E2 to enhance synaptic transmission and plasticity in the hippocampus of long-term hormone-deprived rats is also reported in middle-aged rats 6 mo after OVX [97] and in old rats OVX for up to 15 mo [91]. As whole, this work provides solid evidence that the aging hippocampus retains synaptic responsiveness to E2 under long-term hypoestrogenicity, and that post-ischemic treatment with E2 immediately after ischemia is neuroprotective in middle-aged and old females. If delayed post-ischemic treatment is also found to be neuroprotective in old, long-term hormone-deprived animals, E2 therapy may have clinically important implications for treatment of brain ischemia in post-menopausal women.

Non-feminizing estrogens as neuroprotective agents

There is also evidence that some non-feminizing estrogens and synthetic estrogen analogues that have little or no affinity for estrogen receptors (ER) can exert neuroprotective effects via ER independent pathways [100–103]. For instance, the stereoisomer 17 α -E2 [104], the enantiomer of 17 β -E2 (*ent*-E2, mirror image of 17 β -E2) [105], and several synthetic non-ER binding estrogen analogs that contain the phenolic A ring of steroids [106] are neuroprotective in both *in vitro* screening assays and *in vivo* MCAO studies with adult rodents [100]. These findings are encouraging because the negative side effects of long-term E2 treatment result from activation of peripheral nuclear ERs. Neuroprotective efficacy of estrogenic compounds that do not interact with classical ERs raises the possibility that non-feminizing estrogens can be potential therapeutic agents that may be useful for both aging men and women to protect brain from ischemic insults.

Four different non-feminizing estrogens (estrone or E1, *ent*-E2, and two synthetic estrogen analogs) are neuroprotective against MCAO ischemia administered acutely 2 h before the onset of ischemia [100]. In global ischemia, we tested two estrogen analogs that do not bind classical ERs, the GPR 30 (G protein-coupled estrogen receptor, also known as GPER) agonist G1 [107] and the Gq-coupled membrane-associated ER (Gq-mER) agonist STX [108,109]. STX is a diphenylacrylamide compound that elicits a potent non-genomic estrogen response in the absence of ER- α or ER- β [110,111]. STX mimics E2 modulation of ion channels [110,111] and calcium dynamics [109] in hypothalamic neurons, and activates a phospholipase C–protein kinase C–protein kinase A pathway through a novel G-protein coupled receptor that has not been identified [112–114]. G1 is a specific agonist for GPR 30, which belongs to the family of seven-transmembrane G protein-coupled receptors [107,115]. GPR 30 mediates a variety of rapid biological responses to E2 including transcription-dependent and independent actions [107,115–124].

To test if these non-feminizing estrogen analogs attenuate ischemia-induced cell death when administered immediately after ischemia, middle-aged female rats OVX for 8 wk were subjected to global ischemia or sham surgery and E2, G1, STX or vehicle was injected icv immediately after reperfusion. E2-, G1- and STX-treated ischemic animals had higher numbers of surviving CA1 pyramidal neurons than the vehicle-treated controls [23], promoting survival of approximately 50% of CA1 pyramidal neurons (Fig. 2). Because G1 and STX do not activate classical ERs, these results suggest that neuroprotective effects of acute E2 treatment may be mediated by extranuclear estrogen receptors. The ability of E2 to facilitate basal synaptic transmission within a few minutes of hormone exposure through non-genomic action is well demonstrated in many studies [89–94]. Thus, we measured fEPSPs to determine if E2 and these neuroprotective estrogen analogs have similar synaptic effects at CA1 synapses in hippocampal slices from non-ischemic OVX rats. Consistent with previous studies, E2 rapidly increased fEPSPs at CA1 synapses (Fig 5A), and G1 mimicked the E2 effect with a similar short latency (Fig. 5B). Furthermore, bath application of STX also facilitated fEPSP (unpublished observation). These observations suggest that E2-dependent increases in synaptic transmission in CA1 pyramidal neurons may involve the activation of membrane associated estrogen receptors unrelated to classical ERs and that GPR 30 or Gq-mER may mediate the neuroprotective actions of E2 when administered acutely after ischemia.

Interestingly, both STX [120] and G1 [125,126] can activate two well known pro-survival signaling pathways, mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) in cancer cell lines, which suggests that STX and G1 may exert their neuroprotective actions via the same G-protein coupled receptor system and the subsequent phosphorylation of MAPK and/or PI3K signaling cascades. However, in normal cells, rapid

inhibition of inwardly rectifying K⁺ currents in hypothalamic GABAergic neurons by E2 and STX are not abolished in GPR 30 knock-out mice [110]. Likewise, E2 and STX modulation of [Ca²⁺]_i oscillations and peptide release in primate GnRH neurons are GPR 30 independent [109]. Thus, it is likely that G1 and STX exert their neuroprotective effects through the activation of two distinct types of membrane bounded G protein-coupled receptors.

Indeed, a recent study provided the first direct evidence that membrane initiated rapid signaling via GPR 30 may play an important role in the neuroprotective actions of E2 [121]. Using *in vivo* MCAO in mice and *in vitro* cultures of cortical neurons, Liu et al. showed that E2 exerts neuroprotection from NMDA excitotoxicity by suppressing NR2B-containing NMDA receptor-mediated currents through activation of GPR 30 and the extra-cellular-regulated kinase (ERK), resulting in inhibition of the dephosphorylation of death-associated protein kinase 1 (DAPK1). Because the classical ER antagonist ICI 182780 did not block G1 activity, but knockdown of GPR30 by shRNA significantly reduced the E2-induced neuroprotection, the investigators concluded that this neuroprotection is dependent on GPR30 initiation of membrane signaling, ERK activation and p-DARK 1 inhibition, but is independent of transcription and translation mediated by classical ERs [121]. It is interesting to note that E2 increases synaptic transmission by enhancing NR2B-containing NMDA receptor mediated currents in healthy hippocampus [90,92].

Membrane initiated E2 signaling and E2 neuroprotection

Molecular and cellular mechanisms underlying neuroprotective action of E2 administered after the onset of ischemia remain to be elucidated. However, it is likely that distinct modes of actions are involved in mediating the effects of acute high doses E2 effects and chronically administered physiological levels of E2. Classical ER- α and - β are ligand-activated transcription factors [127], and play a crucial role in mediating the neuroprotective actions of low physiological levels of E2 [128]. Slow genomic actions of E2 involve transcription and regulation of various pro- and anti-apoptotic genes [129,130], and activation of multiple signaling cascades such as ERK/ MAPK [20] and PI3K/Akt [131] to promote survival of CA1 neurons. The importance of classical ERs, in particular ER- α , for neuroprotection from ischemia is well documented for global and focal ischemia [43,45,132,133].

In contrast, acute E2 effects are mediated via rapid, non-genomic, membrane-initiated E2 signaling pathways [108,121,134]. Membrane localization of ER- α and ER- β has been demonstrated in CA1 pyramidal neurons and in glia cells [135,136]. However, the robust neuroprotective actions of some non-feminizing estrogenic compounds that do not activate ERs [100], such as STX and G1 [23,137], support the hypothesis that neuroprotective actions of high E2 doses given in proximity to the onset of ischemia may involve membrane-associated estrogen binding sites that are independent of ER- α and ER- β and that engage signaling cascades associated with plasma membrane receptors.

Rapid activation of PI3K/Akt signaling cascades may be an important neuroprotective mechanism when E2 is administered acutely after induction of ischemia. We administered E2 (50 μ g) to young OVX rats, either alone or in combination with the PI3K inhibitor LY294002 icv immediately after induction of ischemia. A second injection of LY294002 was given 12 h after surgery [19]. LY294002 alone did not have any effects, but it completely abolished the neuroprotective effects of E2 (Fig. 6). In addition to providing histological protection, post-ischemic administration of E2 rapidly inhibited global ischemia-induced changes in Akt phosphorylation, dephosphorylation of the Akt targets, GSK3 β and FOXO3A, and activation of caspase-3 shortly (1–3 h) after ischemia.

Interestingly, these rapid neuroprotective effects of E2 were associated with elevation of pCREB levels shortly after ischemia [8], and high pCREB levels were maintained in E2 treated ischemic rats. These findings suggest that E2 administered in proximity to induction of ischemia can maintain pCREB levels and PI3K/Akt signaling in the early post-ischemic period and that these pathways may be important for neuronal survival.

Data from Brann and colleagues also suggest that PI3K/Akt-CREB signaling plays an important role in E2 neuroprotection [39]. They administered two membrane-impermeable E2 conjugates, EDC and E2-BSA, to young OVX rats 1 h before induction of transient global ischemia. Both EDC and E2-BSA significantly promoted CA1 cell survival and improved spatial memory, which was accompanied by rapid enhancement of the activation of the pro-survival kinases ERK and Akt [130], inhibition of pro-apoptotic kinase JNK [138], and elevation of pCREB and BDNF 10 min–6 h after ischemia. Enhanced levels of pCREB were observed within 10 min after EDC injection, and the effects were persistent at 3, 6, even 24 h after ischemia. These effects were blocked by the classical ER antagonist ICI 182780 and a PI3K or MEK inhibitor. Thus, the investigators concluded that ERK-Akt-CREB-BDNF signaling mediates E2-induced neuroprotection following global ischemia and that extranuclear ERs play an important role in this form of E2 neuroprotection.

Conclusion/clinical implications

The beneficial effects of a single injection of E2 administered after ischemic insult on neuronal survival, and the ability of non-feminizing estrogen analogs that do not activate classical estrogen receptors to ameliorate brain damage, suggest that acute E2 treatment, as well as development of non-feminizing estrogens, has great clinical potential as a therapeutic strategy to protect the brain from neuronal damage associated with ischemia. As stroke and cardiac arrest occur more frequently in elderly individuals, and the risk increases in women after menopause, it is critically important to evaluate the efficacy of neuroprotective strategies in age-appropriate, long-term hormone-deprived animals. Thus, findings that E2, G1 and STX retain their efficacy when administered after ischemia in old females after long-term hypoestrogenicity are highly clinically relevant. Especially promising for future translational studies is the finding that a single dose of hormone can be neuroprotective when administered up to 6 h after the onset of ischemia. At this time, however, it is not known whether delayed post-ischemic administration of E2 is neuroprotective in older animals. In addition, no study has examined functional consequences of acute post-ischemic E2 treatments in old, long-term hormone deprived females. Thus, in future research, it will be essential to evaluate the therapeutic time window of post-ischemic E2 treatment both on hippocampal cell survival and on cognitive performance in old male and female rodents.

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A single high dose of estradiol (E2) given acutely after ischemia protects brain.
Aging hippocampus remains responsive to acute E2 effects.
Post-treatment is effective even after long-term hypoestrogenicity.
Some non-feminizing estrogen analogs also promote cell survival.
Acute E2 effects are mediated by membrane initiated E2 signaling.

Figure 1. Neuroprotective effects of acute post-ischemic E2 treatment are time and dose dependent

Therapeutic time window for 100 μg/kg of E2 administered after ischemia and dose-response relationships at 6 h after MCAO treatment were examined in adult female rats. Two weeks after bilateral ovariectomy (OVX), rats in the experimental groups were subjected to MCAO. Panel A: Therapeutic time window for post-ischemic treatment with 100 μg/kg of E2. E2 was administered to OVX females at 0.5 (n=8), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) h after MCAO. Rats in the control groups, OVX (n=12) and intact females (INT, n=6), received saline and empty pellets at the same time points following MCAO. Data are means ± SEM. *, $p < 0.05$ versus OVX and INT. Data are adapted from Yang et al. (2003) [40] with permission. Panel B: Dose-response relationships for 6 h delay treatment. E2 was administered subcutaneously 6 h after MCAO at doses of 100 (n=9), 500 (n=10), 1000 (n=10), 2000 (n=9), or 5000 μg/kg (n=9). Data are means ± SEM. INT, intact rats; *, $p < 0.05$ versus OVX and intact rats (INT). Data are adapted from Yang et al. (2003) [40] with permission.

Figure 2. E2, G1 and STX afford neuroprotection in middle-aged females 8 weeks after hormone deprivation

Middle-aged female rats (11–13 mo) were subjected to sham surgery or global ischemia (Isch) 8 weeks after OVX. Top panel: Representative photomicrographs of hippocampal neurons in the dorsal CA1 subfield 7 days after sham surgery or global ischemia in animals injected ICV immediately after ischemia with either E2 (2.25 μ g), G1 (50 μ g), STX (50 μ g) or vehicle (Veh). A separate group of animals was injected sc with E2 (100 μ g/kg). Scale bars: low magnification, 400 μ m; higher magnification, 60 μ m. Bottom panel: Surviving CA1 neurons 7 days after ischemia were counted in 3 sectors (lateral, middle and medial, 250 μ m $\times$ 250 μ m each) of 4 sections of the dorsal hippocampus. Data are means $\pm$ SEM. *, $p < 0.001$ versus sham; **, $p < 0.001$ versus ischemia/vehicle. Data are adapted from Lebesque et al. (2010) [23] with permission.

Figure 3. E2 affords neuroprotection in aged females 6 months after hormone deprivation

Female rats aged 15–18 mo were subjected to sham surgery or global ischemia 6 months after OVX. Left panels: Representative photomicrographs of neurons in dorsal CA1 7 d after sham or ischemia (Isch) surgery in rats infused immediately upon reperfusion with vehicle (veh) or 2.25 μ g of E2. Scale bars, 60 μ m. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Right panel: Quantification of surviving CA1 pyramidal neurons was performed in 3 sectors (lateral, middle and medial, 250 μ m $\times$ 250 μ m each) of 4 sections of the dorsal hippocampus 7 d after ischemia. Because there were no significant differences in cell counts in vehicle (black circles) and E2 (gray circles) treated sham rats, data were combined and shown as a single sham group. Data are means $\pm$ SEM. ***, $p < 0.001$ versus ischemia; **, $p < 0.01$ versus ischemia/vehicle. Data are adapted from Inagaki et al. (2012) [17] with permission.

Figure 4. CA1 neurons in the aging hippocampus retain responsiveness to E2 and high frequency stimulation after a long period of hormone deprivation

Evoked field excitatory postsynaptic potentials (fEPSPs) were recorded in hippocampal slices from aged (15–18 mo old, 6 mo after OVX, white circles, n=7 slices from 6 rats) and young (2 mo old, 7–10 days after OVX, black circles, n=5 slices from 4 rats) females. Panel A left: E2 (1 nM) was bath applied after 15 min stable baseline. Panel A right: the averaged responses for 5 min baseline recording (last 5 min before E2 infusion), and the averaged responses during 25–30 min after the start of E2 application, respectively, were compared by ANOVA. Panel B left: High frequency stimulation (HFS, 2 trains of 100 stimulations at 100 Hz) was applied in acute slices from young (n=7 slices from 6 rats) and aged (n=4 slices from 4 rats) OVX rats after 20 min baseline recording in the presence of E2. Panel B right: the averaged responses for 5 min baseline (last 5 min before LTP induction), and the averaged responses during 25–30 min after delivery of tetanus, respectively, were compared

by ANOVA. Data are means $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$. Data are adapted from Inagaki et al. (2012) [17] with permission.

Figure 5. G1 mimics E2 potentiation of synaptic transmission in CA1

Changes in fEPSPs recorded in CA1 in the presence of E2 or G1 were examined in acute hippocampal slices prepared from OVX young adult female rats. Evoked fEPSPs were recorded in response to stimulation of the Schaffer collaterals. After a stable 15 min baseline was established, vehicle solution containing 0.01% DMSO was introduced. Fifteen min later, 10 nM E2 (A, n=9 slices from 6 rats) or 10 nM G1 (B, n=14 slices from 9 rats) was bath applied. Data are adapted from Lebesque et al. (2010) [23] with permission.

Figure 6. PI3K inhibitor LY294002 attenuates E2 neuroprotection

Young OVX rats were subjected to global ischemia (white and black bars) or sham surgery (grey bars). E2 (50 μg) or vehicle was administered immediately after ischemia. Some rats also received the PI3K inhibitor LY294002 or vehicle at 0 and 12 h after sham or ischemia surgery (n=3–12). Surviving CA1 neurons were counted 7 days later (a–p). SO, stratum oriens; SP, stratum pyramidale; Sr, stratum radiatum. ***, $p < 0.001$ vs. all sham groups; ##, $p < 0.01$ ischemia+estradiol vs. ischemia and ###, $p < 0.001$ vs. ischemia+estradiol +LY294002. Data are means $\pm$ SEM and are adapted from Jover-Mengual et al. (2010) [19] with permission.

Table 1

Summary of post-ischemic estrogen treatment studies

Study	Year	sex	ovx	age/weight	species	ISX	Effective Time (relative to the onset)	preparation	concentration	route	Ref.
Simpkins et al.	1997	F	ovx	young adult	SDR	MCAO	40 min	E2	1 mg/kg	sc	33
Zhang et al.	1998	F	ovx	200–250 g	SDR	MCAO	40 min	E2	1 mg/kg	iv	75
Yang et al.	2000	F	ovx	adult	SDR	MCAO	0.5, 1, 2, 3 h	E2	100 µg/kg	sc	41
McCullough et al.	2001	M	n/a	adult	WR	MCAO	10 min	Premarin ¹	1 mg/kg	sc	76
Yang et al.	2003	F	ovx	adult	SDR	MCAO	0.5 h	E2	100–5000 µg/kg	sc	40
Gulinello et al.	2005	F	ovx	100–130 g	SDR	TGI	0 h	E2	50 µg	icv	16
Lie et al.	2007	F	ovx	3 mo	SDR	MCAO	6 h	E2	0.5, 1 mg/kg	sc	25
Liu et al.	2007	F	ovx	3 mo	SDR	MCAO	30 min	E2	100 µg/kg	sc	25
Jover-Mengual et al.	2010	F	ovx	young (100–150 g)	SDR	TGI	0 h	E2	50 µg	icv	19
Lesbeque et al.	2010	F	ovx	11–13 mo	SDR	TGI	0 h	E2, G1 ² , STX ³	2.25 µg (E2), 50 µg (G1, STX)	icv	23
Lesbeque et al.	2010	F	ovx	11–13 mo	SDR	TGI	0 h	E2	100 µg/kg	sc	23
Castro et al.	2012	F	ovx	170–210g (3 mo)	WR	TGI	0, 3, 6 h	E2	20 µg	icv	74
Castro et al.	2012	F	ovx	170–210g (3 mo)	WR	TGI	0, 3, 6, 24 h	Coumestrol ⁴	20 µg	icv	74
Inagaki et al.	2012	F	ovx	15–18 mo	SDR	TGI	0 h	E2	2.25 µg	icv	17
Oh et al.	2012	M	n/a	280–320 g	WR	TGI	0 h	E2	10 µg/kg	ip	77
Perez-Alvarez et al.	2012	M	n/a	adult	SDR	MCAO	6+24+ 48h	E2	40 µg/kg	sc	27

F, female; M, male; ovx, ovariectomy; SDR, Sprague-Dawley rats; WR, Wistar rats; ISX, ischemia; MCAO, middle cerebral artery occlusion; TGI, transient global ischemia; sc, subcutaneous injection; iv, intravenous injection; icv, intracerebroventricular injection; ip, intraperitoneal injection

¹ conjugated estrogens;

² GPR 30 agonist;

³ Gq-mER agonist;

⁴ phytoestrogen