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REVIEW ARTICLE

Oestrogen Signalling and Neuroprotection in Cerebral Ischaemia

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17β-Oestradiol (E_2) is an important hormone signal that regulates multiple tissues and functions in the body. This review focuses on the neuroprotective actions of E_2 in the brain against cerebral ischaemia and the potential underlying mechanisms. A particular focus of the review will be on the role of E_2 to attenuate NADPH oxidase activation, superoxide and reactive oxygen species generation and reduce oxidative stress in the ischaemic brain as a potentially key neuroprotective mechanism. Evidence of a potential novel role of extranuclear oestrogen receptors in mediating E_2 signalling and neuroprotective actions is also discussed. An additional subject is the growing evidence indicating that periods of long-term oestrogen deprivation, such as those occurring after menopause or surgical menopause, may lead to loss or attenuation of E_2 signalling and neuroprotective actions in the brain, as well as enhanced sensitivity of the hippocampus to ischaemic stress damage. These findings have important implications with respect to the 'critical period hypothesis', which proposes that oestrogen replacement must be initiated at perimenopause in humans to exert its beneficial cardiovascular and neural effects. The insights gained from these various studies will prove valuable for guiding future directions in the field.

Key words: stroke, hippocampus, cerebral cortex, menopause, ovariectomy.

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Introduction

Oestradiol and sex differences in stroke risk and outcome

17β-Oestradiol (E_2) is a steroid hormone that is released into the blood where it can exert trophic or regulatory effects on many different target tissues, such as the breast, ovary, uterus, bone and brain (1). The major source of circulating E_2 in the female is the ovary, although other tissues such as adipose and brain have some capacity for E_2 synthesis as a result of expression of the E_2 synthesising enzyme, aromatase (2–4). E_2 levels in the blood fluctuate throughout the cycle in females, with peak circulating levels observed at midcycle in humans, and late dioestrus II to pro-oestrus in rodents (1,5). Interestingly, stroke infarct size has been shown to have an inverse correlation with serum E_2 levels, with smaller infarct size noted upon pro-oestrus in rats, when E_2 levels are highest (6,7). Administration of an oestrogen receptor antagonist, ICI 182 780, to intact female rats has also been shown to result in an increase in infarct size following focal cerebral ischaemia (FCI), suggesting a role for endogenous E_2 and oestrogen receptors in mediating neuroprotection against cerebral ischaemia (8). Sex differences in stroke have been reported in humans, with studies focusing primarily on incidence, age of first stroke, and stroke outcome (9–13). The studies suggest that women are 'protected' against stroke relative to men, at least until the years of menopause, when E_2 levels fall as a result of follicular depletion and stroke incidence increases in women (9,11–13). Intriguingly, stroke outcome in postmenopausal women has been shown to be worse compared to males, with postmenopausal women having a significantly higher disability and fatality rate compared to men (9,10,12,13).

Although the ovary is a significant source of circulating E_2 in women, there is significant evidence that E_2 can be produced in extragonadal tissues as well. Of interest to this review, the enzyme for production of E_2 from androgens, aromatase, has been shown to be expressed in several brain regions, including the hypothalamus, cortex, and hippocampus in male and female rats (2,14), humans (4,15) and monkeys (16). The roles and importance of brain-derived E_2 are currently not fully understood. *In vitro* studies using aromatase inhibitors have suggested that brain-derived E_2 has a role in regulating connectivity/plasticity of neurones (17,18). In addition, *in vivo* studies using aromatase

knockout (KO) mice have shown that infarct volume is significantly increased in the aromatase KO animals following FCI compared to wild-type mice (19,20). Intriguingly, infarct size was reported to be smaller in ovariectomised wild-type mice than in the aromatase KO mice, suggesting that brain-derived E_2 production may have a role in neuroprotection (19). Aromatase expression has also been reported to increase in the peri-infarct region at 24 h after FCI in the rat, with at least part of this increased expression occurring in astrocytes (21). Our laboratory has also observed that E_2 increased aromatase expression in the hippocampal CA1 region at 48 h after global cerebral ischaemia (GCI) (D. Brann and Q.G Zhang, unpublished data). Collectively, the studies suggest that endogenous E_2 production from gonadal and extragonadal sources has a neuroprotective role in the brain against cerebral ischaemia.

Oestrogen receptor (ER)-α mediates E_2 neuroprotection against cerebral ischaemia

Oestradiol is assumed to exert the majority of its biological actions in the body via interaction with two primary oestrogen receptors: ER-α and ER-β. The two receptors exhibit significant homology in their structures, but display differential function, localisation and pattern of expression in the brain (22,23). Both receptors are composed of seven domains, bind E_2 with high affinity, and they both dimerise and utilise the classical oestrogen response elements in a similar fashion. However, several differences do exist between ER-α and ER-β because it has been shown that they contain different ligand-binding domains, and each receptor is encoded by a different gene. The receptors also signal differently at the AF-1 site in the presence of E_2, where E_2 activates transcription at ER-α, whereas it inhibits transcription at ER-β, respectively (24). ER-α and ER-β are primarily localised in the nucleus of cells, although extranuclear localisation has also been demonstrated in the cytoplasm and membrane of cells and neurones (25–29), as is discussed in a subsequent section. Thus, both receptors have been implicated to mediate genomic signalling as well as nongenomic signalling in cells (30–32). Another difference between ER-α and ER-β is that they differ in their tissue distribution, with ER-α being expressed in the breast, ovary, uterus, and brain (33–35), whereas ER-β is expressed in the bone, heart, lungs, kidney, endothelial cells and brain (33,36,37). In the brain, localisation studies have demonstrated that ER-α is localised most densely in the hypothalamus, hippocampus, and preoptic area, with moderate to light density in the cerebral cortex (34,35). Conversely, ER-β localisation has been documented predominantly in the cortex, throughout the hippocampus, in the olfactory bulb, septum, preoptic area, nucleus of striata terminalis, amygdala, paraventricular hypothalamus, thalamus, ventral tegmental area, substantia nigra and cerebellum (33,38,39).

With respect to which receptor is thought to mediate E_2 neuroprotection against cerebral ischaemia, the majority of the literature suggests that ER-α has the primary and critical mediator role for E_2-induced neuroprotection. In support of this contention, E_2 neuroprotection against FCI has been shown to be lost in ER-α KO mice but preserved in ER-β KO mice (40,41). In addition, antisense knockdown studies confirmed a critical role for ER-α, but not ER-β, in mediating E_2 neuroprotection in the hippocampal CA1 region in rats following GCI (42). Furthermore, administration of a selective ER-α agonist, propyl pyrazole triol (PPT) has also been shown to exert neuroprotection in the hippocampal CA1 region following GCI, and rescue the ischaemia-induced deficit in long-term potentiation (43,44). E_2 may achieve its neuroprotective effects through a multitude of effects upon a variety of cell types in the brain, including neurones, astrocytes, microglia and endothelial cells (1). However, emerging evidence suggests that a direct effect of E_2 upon neurones mediated via neuronal ER-α is critical for mediating the neuroprotective effect of E_2 against FCI because E_2 neuroprotection has been shown to be lost in neurone-specific ER-α KO mice, but not in microglia-specific ER-α KO mice (45). The study did not assess E_2 neuroprotective ability in astrocyte- or endothelial-specific ER-α KO mice, and so no definitive conclusion can be inferred about the role of these non-neuronal cell types in E_2 neuroprotection against cerebral ischaemia. There is a significant literature suggesting that E_2 can act on astrocytes to influence release of neuroprotective factors such as growth factors, as reviewed previously (46–48). In addition, E_2 and the ER-α selective agonist, PPT, have been shown to directly enhance the endothelial cell viability *in vitro* of immortalised mouse brain endothelial cells following an ischaemic insult, suggesting that E_2 could act directly on endothelial cells and exert protection of the vasculature following ischaemia (49).

Although the majority of the literature appears to support a critical role for ER-α in mediating E_2 neuroprotective effects against cerebral ischaemia, there are studies suggesting that ER-β may have a neuroprotective role in certain situations. For example, administration of a selective ER-β agonist, WAY 200070-3, has been shown to exert neuroprotection in the rat hippocampal CA1 region following GCI (44), and another study found that the ER-β agonist, DPN, reduced global cerebral ischaemia damage in the mouse hippocampal CA1 region by 55% (50). In addition, the plant phyto-oestrogen, genistein, has also been shown to exert neuroprotection in the hippocampus against global cerebral ischaemia, and this effect was blocked by treatment with an ER-β specific antagonist (51). These studies suggest that exogenous activation of ER-β can exert neuroprotection against cerebral ischaemia. However, evidence of a role for ER-β in mediating endogenous E_2 neuroprotection against cerebral ischaemia is currently lacking because E_2 is fully capable of exerting neuroprotection against cerebral ischaemia in ER-β KO mice (40,41). Nevertheless, there is evidence that ER-β may have a role in basal neuronal survival because it has been reported that there is substantial neuronal loss in the brains of ER-β KO mice at 2 years of age compared to wild-type mice (52).

In addition, a novel, putative third ER, G-Protein-Coupled ER (GPR30, also known as GPER1), has recently been described (53). GPR30 is a seven transmembrane domain G-protein-coupled receptor known to be primarily localised in the plasma membrane and endoplasmic reticulum (53,54) of neurones in the brain and is expressed in several brain regions, including the

islands of calleja, striatum, hypothalamus, area postrema, nucleus of the solitary tract, and hippocampus (54). Evidence supporting the role of GPR30 in neuroprotection was obtained from studies using a purported selective agonist for GPR30, G-1 (55,56). The studies showed that G-1 pretreatment significantly attenuated glutamate-induced neuronal cell death in hippocampal cell cultures (55). G-1 has also been recently shown to exert neuroprotection against FCI in female mice (57). Although these studies are intriguing, they rely on exogenous agonist studies and do not demonstrate a conclusive role for GPR30 in mediating endogenous E_2 neuroprotective actions. More definitive conclusions on the role of GPR30 in mediating E_2 neuroprotection must await the results from studies using GPR30 KO mice, as well as selective GPR30 antagonist and knockdown approaches.

Finally, there is also evidence that nonfeminising oestrogen analogues lacking affinity for oestrogen receptors can also exert neuroprotection in cerebral ischaemia (58–61). As reviewed recently by Yi *et al.* (61), eight different nonfeminising oestrogens have been shown to be neuroprotective against cerebral ischaemia. These findings are very intriguing because nonfeminising oestrogens lacking ER affinity would be predicted to lack negative side effects common to E_2, such as stimulation of the breast and uterus, as well as enhancement of blood clotting. Further work has shown that oestrogen analogues with large bulky groups at the 2 and/or 4 carbon of the phenolic A ring eliminate ER binding but enhance neuroprotective potency in cell culture screening models (61). It is not known whether the nonfeminising oestrogens bind to GPR30 to mediate their effects. Further studies are needed to address this interesting question. Further studies are also needed to determine the mechanism of action underlying the neuroprotective effects of nonfeminising oestrogens and to establish whether they might have efficacy for postmenopausal hormone therapy.

Oestrogen regulation of reactive oxygen species and oxidative stress

Reactive oxygen species (ROS), particularly superoxide, have been implicated to play a key role in neuronal cell death following cerebral ischaemia (62–66). The superoxide anion radical (O_2^-) is the product of a one electron reduction of oxygen and it is the precursor of most ROS, including the highly toxic and damaging hydroxyl ion and peroxynitrite (67,68). Although ROS are suggested to mediate physiological processes at low concentrations, when they are over-produced in pathological situations, they can be highly injurious to adjacent structures in cells and neurones, including lipid membranes, DNA and proteins (63). It is well known that, following the onset of either permanent or transient FCI, ROS increase significantly in the cerebral cortex and other brain regions (1,62–66). Along these lines, it has been shown that there is a marked steady elevation of ROS in the penumbra (infarct border) of the parietal cortex during a 3-h measurement period post ischaemia in permanent cerebral ischaemia (64). Similarly, studies using a marker of O_2^- production, hydroethidine (HEt), have yielded a similar pattern of increased O_2^- oduction in the cortex of male mice and ovariectomised female rats within 1–3 h of permanent cerebral ischaemia (1,65,66). In addition, as shown in Fig. 1(A), work by our laboratory has shown that O_2^- production increases rapidly in the hippocampal CA1 region following GCI in both male and female rats, with an elevation occurring as early as 30 min after reperfusion and peak levels observed at 3 h after reperfusion (42,69). As also shown in Fig. 1(A), E_2 treatment strongly attenuated the elevation of O_2^- levels in the hippocampal CA1 region following cerebral ischaemia, which correlated with its neuroprotective effect (42). Further studies showed that the E_2 attenuation of O_2^- levels was associated with a dramatic attenuation of oxidative stress damage in the hippocampal CA1 region at 24 h after cerebral ischaemia, as determined by

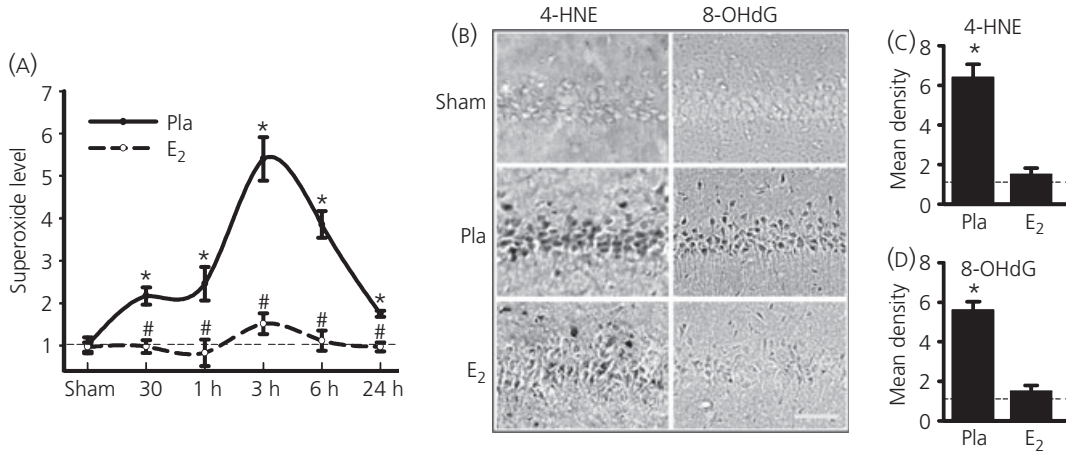

Fig. 1. E_2 attenuates superoxide production and oxidative damage in hippocampal CA1 after global cerebral ischaemia. Adult ovariectomised rats were treated with 17β-oestradiol (E_2) for 1 week prior to 10-min global cerebral ischaemia (GCI) and killed at various times after reperfusion. The E_2 minipumps produced serum levels of 10–15 pg/ml. (A) Superoxide production in the hippocampal CA1 region from sham, placebo (Pla) and E_2-treated rats was measured using a luminol-based photoemissions assay. (B–D) The effect of E_2 on oxidative damage markers for lipid peroxidation (4-HNE) and DNA damage (8-OHdG) 1 day after ischaemia. Note that E_2 strongly decreased 4-HNE and 8-OHdG staining. Values are the mean ± SE of four or five rats in each group and are expressed as the fold change versus sham + Pla group. *$P < 0.05$ versus sham; $^{\#}P < 0.05$ versus Pla at the same time point. Reproduced with permission (42).

measurement of oxidative damage markers for lipid peroxidation (4-HNE) and DNA damage (8-OHdG) (Fig. 1B,C) (42). A similar E_2 suppression of O_2^- production was demonstrated in the cerebral cortex following FCI (1). Below, we discuss how E_2 may regulate ROS generation in cerebral ischaemia with a particular focus on an emerging key enzyme for O_2^- production, NADPH oxidase.

E_2 attenuates NADPH oxidase activation following global cerebral ischaemia

In vitro studies have suggested that there may be three distinct mechanisms for generating ROS in hippocampal and cortical neurones during hypoxia/reoxygenation (70). The studies provided evidence that the mitochondria generates the initial ROS burst during hypoxia, followed by xanthine oxidase during the delayed phase, and ending with NADPH oxidase-generated ROS production in reperfusion. It is well known that E_2 can have beneficial effects upon mitochondria to preserve mitochondrial function. These effects include regulation/preservation of ATP generation, ROS production, mitochondrial apoptotic factors and antioxidant mechanisms. Several excellent reviews provide additional information on the effects of E_2 upon mitochondria (71,72). New emerging evidence suggests that the membrane, via NADPH oxidase, may play an additional critical role in ROS generation in neurones following cerebral ischaemia. The NADPH oxidase enzyme is composed of key subunits from the NOX family, whose primary job is to transport electrons across biological membranes to reduce molecular oxygen to O_2^- (73–76). The NOX family is composed of five isoforms (NOX1–NOX5). Despite their similar structure and enzymatic function, NOX family isoforms differ in their mechanism of activation. NOX1 activity requires the subunits p22phox, NOXO1 and NOXA1, and is Ras-Related C3 Botulinum Toxin Substrate 1 (Rac1)-dependent, whereas NOX 3 requires similar subunits for its activation, but is Rac1-independent. NOX4 and NOX5 isoforms do not appear to require many subunits for their activation because they are considered to be constitutively active and Rac1-independent (73). The activation of NOX2, the most studied and best characterised NOX isoform and a major focus of our studies, involves interaction with the subunits p22phox, p67phox, p40phox and p47phox subunits. In addition, the GTPase, Rac1 has been shown to be critical for NOX2 activation (69,73,75). NOX2 and p22phox are found primarily on the membrane, in resting cells, existing in close association and stabilising one another. Upon cell activation/stress, there is an exchange of GDP for GTP on Rac1, a Rho GTPase, leading to its activation and translocation to the membrane. Simultaneously, phosphorylation of cytosolic p47phox allows for its binding with other membrane subunits (p67phox and p40phox), leading to conformational changes that allow interaction with p22phox on the membrane. This activates the NOX2 enzyme complex, which transports electrons from cytoplasmic NADPH to oxygen and generates O_2^- (73).

Localisation of the NOX family isoforms has been studied extensively in many tissues throughout the body. In 2001, Lambeth and his group documented strong NOX2 mRNA expression and faint reverse transcriptase-polymerase chain reaction bands of NOX4 and NOX5 in the brain (77). Moreover, further studies by our group and others revealed NOX2 (42,69,78) and NOX4 (79) expression in the hippocampus, as well as NOX2 localisation in the cerebral cortex (78). Of the different NOX enzyme isoforms, the greatest evidence to date implicates a critical role for NOX2 in ROS generation following cerebral ischaemia and the resultant oxidative stress damage. In support of this contention, infarct volume was shown to be significantly reduced in NOX2 KO mice compared to their wild-type litter mates (80,81). Furthermore, the administration of the NADPH oxidase inhibitor, apocynin was shown to reduce infarct size after FCI (82) and significantly reduced neurological deficit score in mice, thus achieving an improved behavioral cognitive outcome (80–82). The ability of apocynin to reduce infarct volume, neurological impairment and mortality was lost when it was administered in NOX2 KO mice, which strongly suggests that its beneficial neuroprotective effects are specifically a result of inhibition of NOX2 NADPH oxidase (81). Apocynin neuroprotection against cerebral ischaemia was associated with reduced levels of apoptotic factors and markers, such as Bax, Bcl-2 and terminal deoxynucleotidyl transferase dUTP nick end labelling staining (83), suggesting that NADPH oxidase activation plays a key role in the induction of apoptosis following cerebral ischaemia. Additional work by our laboratory showed that administration of a specific competitive NOX2 inhibitor, gp91ds-tat, significantly attenuated elevation of NADPH oxidase activity and O_2^- levels in the hippocampal CA1 region following GCI, and was strongly neuroprotective (42). This suggests that NOX2 NADPH oxidase plays a significant role in the elevation of O_2^- and resultant neuronal damage in the hippocampus following cerebral ischaemia. Further work by our laboratory and others demonstrated that NOX2 is not only predominantly localised in neurones in the hippocampus following cerebral ischaemia (42), but also appears in microglia at later time-points after cerebral ischaemia (84). *In situ* O_2^- determination using the hydroethidine method also revealed O_2^- elevation in neurones, with some occurring in microglia/macrophages, and little in endothelial cells in the cortex and hippocampus at early time-points after cerebral ischaemia (42,85). There is also some evidence that NOX2-derived OO_2^- from circulating lymphocytes that infiltrate the infract area may also contribute to O_2^- elevation at the infarct site (86).

As shown in Fig. 2, work by our laboratory showed that NADPH oxidase activity increases rapidly in the hippocampal CA1 region following GCI in ovariectomised female rats, with peak levels observed at 3 h after reperfusion (42). Note that the pattern of NADPH oxidase activation following cerebral ischaemia is similar to that we observed for O_2^- elevation. As also shown in Fig. 2, E_2 treatment strongly attenuated the elevation of NADPH oxidase activity in the hippocampal CA1 region following cerebral ischaemia, which correlated with its suppression of O_2^- levels and its neuroprotective effect (42). As shown in Fig. 3, the ability of E_2 to exert neuroprotection and attenuate the elevation of NADPH oxidase activity and O_2^- in the hippocampal CA1 region after global cerebral ischaemia was lost in animals in which ER-α was knocked by antisense oligonucleotides, but was preserved in ER-β antisense knockdown animals (Fig. 3) (42). This suggests that the neuroprotective and antioxidant effects of E_2 in global cerebral ischaemia are primarily mediated by ER-α. We further showed that E_2 inhibited activation of the GTPase, Rac1, in an Akt-dependent manner

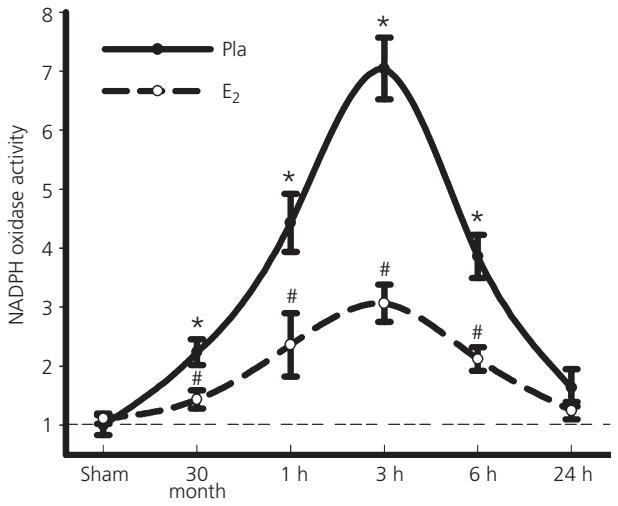

Fig. 2. 17β-Oestradiol (E_2) attenuates NADPH oxidase activity in hippocampal CA1 after global cerebral ischaemia. Adult ovariectomised rats were treated with E_2 for 1 week prior to 10-min global cerebral ischaemia (GCI) and killed at various times after reperfusion. The E_2 minipumps produced serum levels of 10–15 pg/ml. NADPH oxidase activity in the hippocampal CA1 region from sham, placebo (Pla) and E_2-treated rats was measured using a lucigenin-based photoemissions assay. Values are the mean ± SE of four or five rats in each group and are expressed as the fold change versus sham + Pla group. *$P < 0.05$ versus sham; $^{\#}P < 0.05$ versus Pla at the same time point. Reproduced with permission (42).

following cerebral ischaemia, which is critical for NOX2 NADPH oxidase activation (42). Additional work showed that administration of a Rac1 inhibitor markedly attenuated NADPH oxidase and superoxide generation in the hippocampal CA1 region following cerebral ischaemia and was neuroprotective and preserved cognitive function (69).

Oestrogen extranuclear receptor signalling and E_2 neuroprotection

It has been predominantly considered that E_2 neuroprotection in the brain is mediated principally by the 'classical' nuclear ER-mediated genomic signalling pathway, which involves E_2 interaction with nuclear ER and regulation of the transcription of various genes that mediate neuroprotection. For example, E_2 has been shown to increase the expression of the anti-apoptotic gene, *bcl-2*, in the ischaemic penumbra following FCI and GCI (87). E_2 also increases *bcl-2 in vitro* in rat hippocampal neurones and human NT2 neurones (88,89), whereas it inhibits expression of pro-apoptotic BAD (*bcl-2*-antagonist of cell death) (87–90). Additionally, E_2 enhances expression of the anti-apoptotic pro-survival factor, survivin in the hippocampus CA1 following GCI, which facilitates neuronal survival (91). E_2 has also been shown to enhance expression of brain-derived neurotrophic factor (BDNF) in the brain, which has been implicated as a neuroprotective factor and to be important for synaptic plasticity, learning and memory (92,93).

In addition to genomic signalling, there is increasing evidence that rapid nongenomic signalling via membrane localised extranuclear ER may also play a role in mediating E_2 neuroprotective effects in the brain (30,94,95). Along these lines, several studies have shown that the rapid activation of extracellular signal-regulated kinases 1,2 (ERKs) by E_2 is critical for its neuroprotective effects because the administration of a mitogen-activated protein kinase kinase (MEK) inhibitor blocks E_2 neuroprotection in neurones *in vitro* (94–96). Furthermore, E_2-induced ERK activation in the CA1 region after GCI, which is critical for its neuroprotective effects because treatment with a MEK inhibitor blocked E_2-induced ERK activation and E_2 neuroprotection in the hippocampus (97). Similarly, a role for the pro-survival serine kinase Akt in E_2 neuroprotection has been implicated because E_2 rapidly up-regulates Akt activation in cortical neurones *in vitro* (98) and in the hippocampus CA1 *in vivo* following GCI (99), whereas treatment with a phosphoinositide 3-kinase inhibitor attenuates the neuroprotective effects of E_2 both *in vitro* and *in vivo* (98,99). In addition, we recently demonstrated that E_2 attenuates the rapid activation of the pro-apoptotic signalling kinase, c-Jun N-terminal kinase in the hippocampal CA1 region after GCI (91). As a whole, these findings suggest that E_2-induced rapid nongenomic signalling may play a critical role in E_2 neuroprotection.

However, because the above studies principally used E_2, which can activate both extranuclear and nuclear oestrogen receptors, it has been difficult to distinguish the importance and contribution of extranuclear receptor-mediated signalling in E_2 neuroprotective effects. To address this issue, we employed two E_2 conjugates, E_2-bovine serum albumin (BSA) conjugate (100–102) and the newer E_2 dendrimer conjugate (EDC) (103), which, as a result of their size and charge, cannot enter the cell nucleus. EDC and E_2-BSA retain their ability to induce rapid extranuclear-mediated nongenomic signalling, but lack significant nuclear ER-mediated genomic signalling ability as a result of their inability to enter the cell nucleus and interact with nuclear ER (102,103). Using FITC-labelled EDC and E_2-BSA, we demonstrated that following i.c.v. injection in the lateral ventricle, the compounds are heavily localised in the hippocampal CA1 region and display a membrane/cytoplasmic localisation without any appearance of nuclear localisation (104). The results of the study further revealed that EDC and E_2-BSA administered i.c.v. rapidly activates ERK, Akt and CREB signalling pathways in the hippocampus, enhances levels of the CREB transcriptional target, BDNF, strongly protects the hippocampal CA1 region against neuronal cell death, and significantly improves hippocampal-dependent cognitive function in the Morris water maze following GCI (104). The effects required oestrogen receptor mediation because they were blocked by administration of the oestrogen receptor antagonist, ICI182,780. In addition, further studies showed that EDC attenuated Rac1 and NADPH oxidase activation and elevation of O_2^- in the hippocampal CA1 region after cerebral ischaemia, and that its effects involved activation of the pro-survival kinase, Akt (42). The results of these studies thus provides important new evidence supporting an important role for extranuclear oestrogen receptor activation in oestrogen-induced neuroprotection and improved functional cognitive outcome following GCI, and suggests that ERK-Akt-CREB-BDNF signalling is an important component mediating extranuclear oestrogen receptor beneficial neural effects. It should be noted that, in addition to the proposed neuroprotective role of ERK1/2 activation

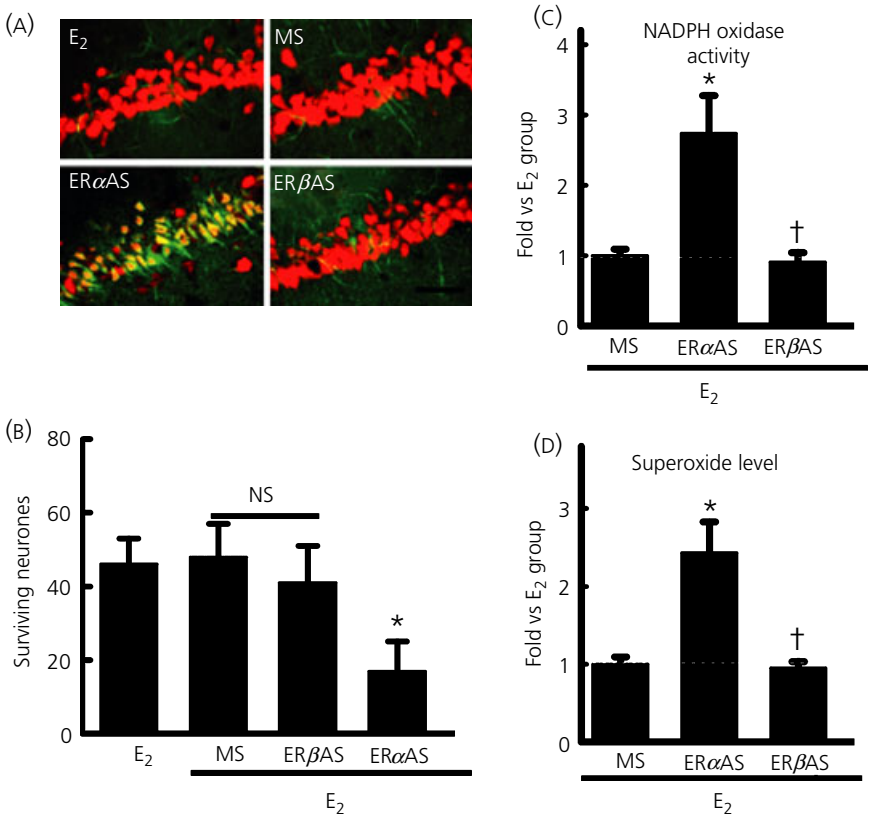

Fig. 3. Evidence that oestrogen receptor (ER)-α mediates 17β-oestradiol (E_2) antioxidant and neuroprotective effects in the hippocampal CA1 region following cerebral ischaemia. Ovariectomised rats were treated with E_2 for 1 week prior to 10-min global cerebral ischaemia (GCI). The E_2 minipumps produced serum levels of 10–15 pg/ml. (A) Missense (MS) oligodeoxynucleotides, ER-α or ER-β antisense (AS) oligodeoxynucleotides (10 nmol) were injected bilaterally i.c.v. every 24 h for 4 days prior to GCI reperfusion. Hippocampal CA1 sections were collected at 7 days after reperfusion and assessed for neuroprotection by immunohistochemistry for NeuN (neuronal marker – red) and staining for FluoroJadeB (neuronal degeneration marker – green). E_2 neuroprotection was imaged and visualised using confocal microscopy. Note that E_2 neuroprotection was abolished only in the ER-α AS treated animals. Values are the mean ± SE of six or seven animals. (B) Quantification of surviving neurones by counting NeuN positive and FluoroJade B negative cells. *P < 0.05 versus E_2 + MS group. Scale bar = 50 μm; × 40. NS, not significant. (C, D) NADPH oxidase activation (C) and superoxide production (D) was assessed at 3 h reperfusion using a lucigenin and luminol-based photoemission assay, respectively. Note that E_2 attenuation of NADPH oxidase activity and superoxide elevation was abolished in ER-α AS treated animals but not ER-β treated rats *P < 0.05 versus E_2 + MS, †P > 0.05 versus E_2 + ER-α-AS. Reproduced with permission (42).

in cerebral ischaemia, there is also evidence for a pro-death role of ERK activation. For example, administration of MEK inhibitors has been shown to significantly reduce ischaemic damage to the brain following GCI or FCI (105–107), which suggests a neurodegenerative role for ERK activation after cerebral ischaemia. It has been postulated that enhanced ERK1/2 activation may send a neuroprotective signal that involves the eventual down-regulation of its own activation, thereby preventing a prolonged elevation of ERK. However, in our studies *in vivo* in the GCI model, we found that ERK activation in the vehicle-treated rat is biphasic, with an early elevation at 10 and 30 min after reperfusion, a fall to control levels at 3 and 6 h after reperfusion, followed by a secondary elevation at 24 h after reperfusion (104). Interestingly, acute EDC treatment significantly elevated ERK activation at 10 min, 30 min, and 3 and 6 h post-reperfusion compared to the vehicle-treated group, although it did not enhance the secondary elevation that occurred at 24 h after reperfusion. Hence, in our studies, acute oestrogen analogue treatment enhanced and prolonged ERK activation *in vivo* in the hippocampal CA1 region following GCI. Thus, our studies did not show an oestrogen-induced reduction of ERK activation that would fit the proposed model of ERK activation leading to its own inactivation. However, our study only examined up to 24 h after GCI, and thus studies at more prolonged timepoints after GCI may be needed to determine whether there is a subsequent down-regulation of ERK at later timepoints. The apparently contradictory 'good role' versus 'bad role' of ERK activation in cerebral ischaemia could depend on many factors, including (i) cell type of induction (neurone, glia or endothelial cell); (ii) pattern/duration of induction (acute, biphasic, chronic); and (iii) subcellular localisation of ERK (nucleus versus cytoplasm). For an elegant discussion and treatment of this complex subject, an excellent review is provided by Sawe *et al.* (108) on the dual role of ERK activation in cerebral ischaemia.

Currently, it is unclear which extranuclear oestrogen receptor mediates the rapid effects of E_2 or E_2 conjugates in neurones. Previous work has shown that ER-α and ER-β can exist as dimers in the plasma membrane of cells (32,109), and that COS-7 cells engi-

neered to express ER-α and ER-β display localisation of approximately 2–5% of ER-α and ER-β protein to the plasma membrane (102). These studies suggest that classical ERs can be targeted to the plasma membrane. Key mechanisms for targeting ER-α and ER-β to the plasma membrane include palmitoylation of ER-α and ER-β, and interaction of ERs with the scaffold protein, caveolin-1 (110,111). Although these studies were conducted in non-neuronal cells, numerous studies have confirmed the presence of both ER-α and ER-β at the plasma membrane of neurones in various brain regions including the hippocampus, and at other extranuclear sites, such as in dendrites and spines (25,28,112–116). Furthermore, membrane localisation of ER-α and ER-β has been demonstrated in glia cells in different brain regions (113,115,117,118), and glia cells have also been implicated as potentially participating in mediating oestrogen neuroprotection via the release of growth factors and neuroactive steroids (48,119,120).

Finally, there is evidence that oestrogen extranuclear receptor-induced nongenomic signalling can cross-talk to the nucleus to effect genomic signalling. Along these lines, Madak-Erdogan *et al.* (121) have demonstrated that EDC can regulate gene expression in cells *in vitro* and that the effect does not involve interaction with or activation of nuclear ER genomic signalling. Rather, EDC effected changes in gene expression via its activation of rapid ERK and Src kinase signalling, which can regulate phosphorylation of transcription factors, histones and other factors, and thereby modulate gene transcription. The study further showed that EDC was incapable of recruiting nuclear ER-α to oestrogen responsive regions of genes, whereas ER-α recruitment by E_2 was very effective. Thus, EDC nongenomic signalling can induce genomic signalling that is independent of nuclear ER. Intriguingly, previous studies have also demonstrated that nongenomic signalling by E_2 in the hypothalamus can actually potentiate E_2 genomic actions to induce lordorsis behavior (122,123), suggesting that rapid effects of E_2 may also modulate genomic effects of E_2. Interestingly, our own findings revealed that EDC and E_2-BSA enhanced phosphorylation of the transcription factor, CREB, in a rapid fashion following reperfusion, and that this effect is ERK- and Akt-dependent. Among the best known CREB transcriptional targets is the growth factor, BDNF, and, intriguingly, our study also demonstrated it to be elevated by EDC. This finding raises the possibility that EDC activation of extranuclear oestrogen receptors may involve a nongenomic to genomic signalling cascade via kinase-induced activation of the transcription factor, CREB. As a whole, the studies suggest that both extranuclear and nuclear receptor signalling mediates E_2 neuroprotective actions and that there may be cross-talk between the two signalling pathways.

Long-term E_2 deprivation alters the sensitivity of the brain to E_2

Basic science and clinical observation studies have provided evidence of a beneficial effect of E_2 upon cardiovascular disease, neuroprotection and neurodegenerative diseases such as stroke and Alzheimer's disease (1,124–128). However, the Women's Health Initiative (WHI) surprisingly failed to observe a protective effect of hormone replacement therapy upon the cardiovascular and neural system and, in fact, reported a small, but significant increase in risk for stroke and dementia (129–131). The average age of subjects in the WHI study was 63 years, which is far past the onset of menopause. It has been suggested that there may be a 'critical period' for beneficial protective effect of E_2 upon the brain, and that oestrogen may need to be administered at peri-menopause or earlier to observe a beneficial effect upon the cardiovascular and neural system (132–134).

In support of a 'critical period' hypothesis for E_2 beneficial effects in the brain, a significant body of work has emerged which has shown in animal and human studies that long-term E_2 deprivation (LTED) (long-term ovariectomy) leads to a loss of many E_2 effects in the brain, such as neuroprotection, synaptic plasticity and cognitive function, and enhances the risk of neurological diseases and mortality. As shown in Table 1, LTED has also been shown to lead to a loss of the ability of E_2 to enhance long-term potentiation, spine density, attention processes and working memory, as well as exert vascular protective actions in rodents (135–138). In addition,

Table 1. Neural and Cardiovascular Effects of Long-Term Ovariectomy.

Group	Species	Tissue	Effect
Rocca *et al.* 2007 (141)	Human	Brain	↑ Risk cognitive impairment and dementia
Rocca *et al.* 2008b (142)	Human	Brain	↑ Risk Parkinson's disease
Rocca *et al.* 2008a (140)	Human	Brain	↑ Risk depression and anxiety
Rocca *et al.* 2009 (139)	Human	Brain	↑ Mortality for neurological and mental diseases
Suzuki *et al.* 2007 (144)	Rat	Cortex	Loss of E_2 neuroprotective effect
Zhang *et al.* 2009 (42)	Rat	Hippocampus	Loss of E_2 neuroprotection; ↓ ERα; ↑ ischaemic damage to hippocampal CA3 region
Daniel *et al.* 2006 (137)	Rat	Cortex and hippocampus	Loss of E_2 enhancement of working memory
Bohacek & Daniel 2010 (138)	Rat	Cortex and hippocampus	Loss of E_2 enhancement of attention processes
Smith *et al.* 2010 (136)	Rat	Hippocampus	Loss of E_2 enhancement of spine density and long-term potentiation
Wu *et al.* 2011 (135)	Rat	Hippocampus	↓ Intrinsic excitability and loss of E_2 sensitivity
Pinna *et al.* 2008 (146)	Rat	Aorta	↓ ERα and loss of E_2 protective vascular actions
Jesmin *et al.* 2003 (145)	Rat	Cerebral vessels	↓ ERα and ERβ; ↓ cerebral capillary density

E_2, 17β-oestradiol; ER, oestrogen receptor.

surgical menopause (long-term ovariectomy) in humans has been shown to increase cognitive decline, dementia, Parkinson's disease, depression and mortality as a result of neurological and mental diseases (139–142) (Table 1). Intriguingly, E_2 replacement has been shown to reverse these effects in surgical menopausal subjects, indicating it is the loss of E_2 that leads to these increased risks and negative outcomes (124,143). Recent work by our group and other has shown that E_2 neuroprotection in animal models of FCI and GCI is lost following LTED (42,144). Along these lines, Fig. 4(A) shows that E_2 treatment administrated after a 10-week period of E_2 deprivation (ovariectomy) was no longer able to exert neuroprotection against GCI. Interestingly, the uterus was still responsive to E_2, as demonstrated by a robust uterotrophic response to E_2 in the LTED animals (Fig. 4B). Thus, there was a tissue-dependent loss of sensitivity to E_2 in the LTED animals. We thus examined whether the loss of E_2 sensitivity in the hippocampal CA1 region could be the result of an alteration in oestrogen receptor levels. As shown in Fig. 4(C,D), western blot analysis revealed a dramatic attenuation of ER-α, but not ER-β protein levels in the hippocampal CA1 region of LTED animals weeks later compared to animals who received imme-

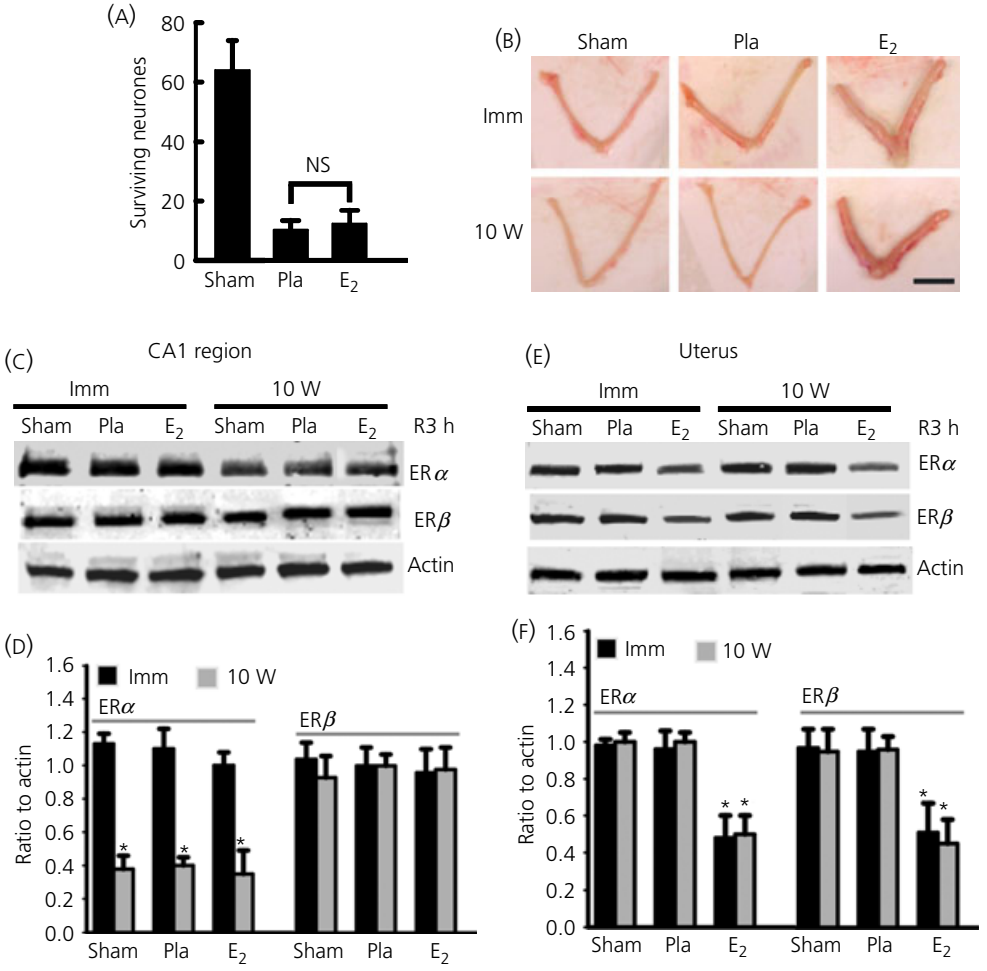

Fig. 4. Attenuation of hippocampal CA1 region oestrogen receptor (ER)-α levels and loss of 17β-oestradiol (E_2) neuroprotective ability against global cerebral ischaemia (GCI) following long-term E_2 deprivation (LTED). (A) Adult female rats were ovariectomised and 10 weeks later treated with placebo (Pla) or E_2 for 1 week and then subjected to 10 min GCI. Sham animals were included as controls and were subjected to the surgeries but no cerebral ischaemia. The animals were killed at 7 days after reperfusion and the number of surviving neurones (NeuN positive and FluoroJadeB negative) in the hippocampal CA1 region was counted. Note that E_2 does not protect against GCI in the LTED animals. NS, not significant. (B) Rats were ovariectomised and treated either immediately (Imm) or 10 weeks later (10W) with placebo (Pla) or E_2. One week after Pla or E_2 treatment, the animals underwent 10-min GCI and, 7 days after reperfusion, the animals were killed and uterus examined for uterotrophic effect of E_2. Note that E_2 exerted a robust uterotrophic effect in both Imm and 10W (LTED). Scale bar = 1 cm; × 1. (C) Western blot analysis for ER-α and ER-β protein levels in the hippocampal CA1 region of Imm versus 10W (LTED) animals at 3 h of reperfusion show a profound reduction of ER-α, but not ER-β, levels in the hippocampal CA1 region of 10W (LTED) animals compared to the Imm animals. (D) Semi-quantitative analysis of data from western blot analysis in (C). Data are expressed as the mean ± SE (n = 4–6 rats per group) and as a ratio to actin. *P < 0.05 versus Imm group. (E) By contrast, western blot analysis of uterine samples reveal that 10W (LTED) animals have the same pattern and levels of ER-α and ER-β levels as Imm animals (e.g. no decrease of either ER-α or ER-β levels by LTED). Note that E_2 exerts a significant down-regulation of both ER-α and ER-β in the uterus in both Imm and 10W (LTED) rats, further indicating that the uterus maintains sensitivity to E_2 following LTED. (F) Semi-quantitative analysis of data from wblot analysis in (E). Data are expressed as the mean ± SE per group (n = 4–6) and expressed as ratio to actin *P < 0.05 versus Pla group. Reproduced with permission (42).

diate E_2 replacement after ovariectomy. Note that the reduction in ER-α protein levels occurred in all groups, including sham controls, suggesting that LTED leads to lower ER-α levels regardless of treatment and that E_2 and ischaemia cannot reverse the suppression of ER-α protein levels (42). This decrease in ER-α and E_2 sensitivity was tissue-specific because ER-α did not decrease in the uterus following LTED (Fig. 4E,F). It should be noted that LTED has been shown to lead to a significant decrease of ER-α in the vasculature as well, which was correlated with a loss of E_2 vascular protective actions (145,146). Additional work by our group has shown that the hippocampal CA3 region, which is resistant and not normally damaged following global cerebral ischaemia, becomes heavily damaged in LTED rats following global cerebral ischaemia (42). There is also a dramatic induction of Alzheimer's disease-related proteins such as β-amyloid, amyloid precursor protein, and phospho-tau in the hippocampal CA3 region of LTED rats following GCI (147). It is speculated that the hypersensitivity of the hippocampal CA3 region to ischaemic stress damage and Alzheimer's disease-related protein induction observed in our study could help explain the increased risk for cognitive decline and dementia observed in women following surgical menopause. Finally, a new 10-year re-evaluation of a component of the WHI study has provided important support for the critical period hypothesis (148). The study examined 11 000 women aged 50–79 years who had hysterectomies and were treated with either placebo or oestrogen alone. The WHI study was stopped in 2004 as a result of increased stroke risk and the women stopped taking oestrogen at that time. The 10-year follow-up study found significant beneficial cardiovascular effects of oestrogen in women in their 50 s, neutral effects for those in their 60s, and increasingly negative effects in women in their 70s. Women who were treated with oestrogen in their 50s had a 41% lower coronary disease risk, a 46% lower heart attack risk, significantly decreased invasive breast cancer risk, and a significant decrease in overall mortality. By contrast, women who began oestrogen treatment in their 70s had an increased risk of cardiovascular disease, colorectal cancer and mortality. The study shows that age has an important effect on outcome of oestrogen replacement therapy in humans, and that oestrogen replacement in women in their 50s exerts many beneficial effects that are lost if E_2 treatment is delayed to later in life (e.g. age 70 years or greater). These findings are consistent with the 'critical period' hypothesis suggesting that oestrogen replacement, to be beneficial, must be given prior to a long-term period of oestrogen deprivation such as occurs after the menopause. It should be noted that there are several other large clinical trials ongoing on oestrogen replacement therapy benefits in humans, and it will be interesting to see the outcomes of these studies.

Conclusions

Based on the literature summarised in this review, there is abundant evidence that E_2 has a significant neuroprotective effect against cerebral ischaemia. Figure 5 provides a summary pathway for the mechanisms of E_2 neuroprotection. As shown in Fig. 5, E_2 neuroprotection is suggested to be mediated by both extranuclear and nuclear oestrogen receptor-signalling pathways. Based on knockout and knockdown studies, as well as selective agonist studies, the predominant view is that E_2 neuroprotection against cerebral ischaemia is mediated by ER-α. Exogenous agonist studies suggest that activation of GPR30 and ER-β exogenously may also exert neuroprotection against cerebral ischaemia, although studies showing these receptors mediate endogenous E_2 neuroprotection against cerebral ischaemia are lacking. As further shown in Fig. 5, E_2 activation of nuclear ER leads to genomic signalling in which the expression of pro-survival and anti-apoptotic genes are

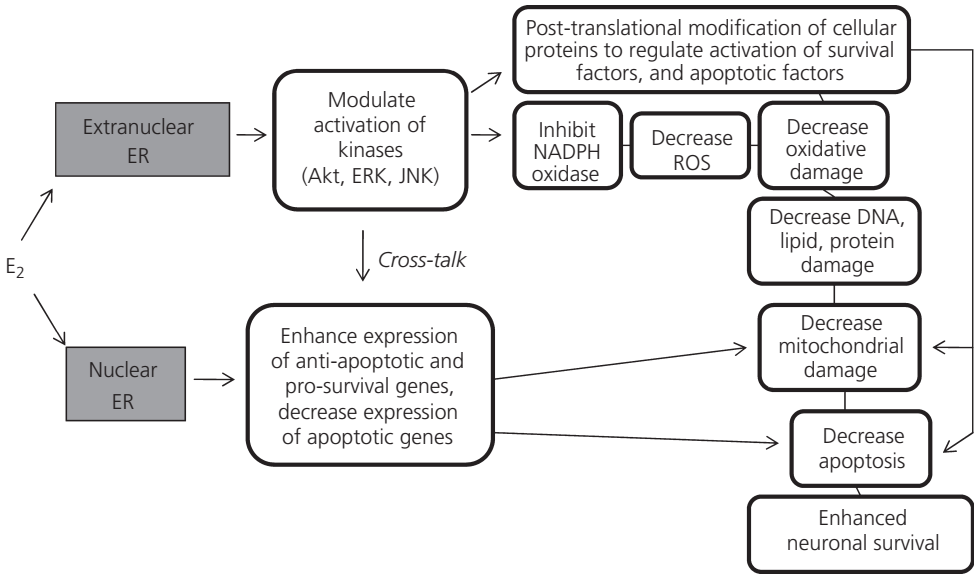

Fig. 5. Summary diagram depicting the neuroprotective mechanisms of 17β-oestradiol (E_2) via nuclear and extranuclear signalling pathways. For additional discussion, see text. ER, oestrogen receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; ROS, reactive oxygen species.

up-regulated and pro-death/apoptotic genes are down-regulated. By contrast, E_2 activation of extranuclear ER is proposed to modulate activation of kinases that can post-translationally modify the activity of other key cellular proteins to exert neuroprotection. For example, our studies showed that extranuclear signalling by E_2 can activate the pro-survival kinase, Akt, which phosphorylates Rac1 and inhibits its activation. The inhibition of Rac1 activation is proposed to lead to a profound inhibition of NADPH oxidase activation, and a resultant attenuation of cerebral ischaemia-induced O_2^- elevation, and oxidative stress damage, as well as decreased mitochondrial damage and apoptosis. Although not shown, there is also abundant evidence that E_2 can act directly on mitochondria, as well preserve ATP production, decrease ROS generation and inhibit apoptotic signalling. Finally, the extranuclear nongenomic signalling pathway may cross-talk to the genomic signalling pathway because E_2 activation of kinases can lead to their translocation to the nucleus, where they can regulate gene expression by post-translationally modifying the transcription factors and thus changing their activity. It should be noted that this summary is obviously not 'all inclusive' of the many possible signalling roles and actions of E_2. Nevertheless, it highlights some important signalling pathways that have been elaborated recently and are considered to play a key role in E_2 neuroprotection in cerebral ischaemia. Finally, LTED can lead to a loss of E_2 neuroprotection and other key neural effects in the brain. For the hippocampus, the loss of E_2 neuroprotective effect following LTED was shown to be correlated with a significant decrease of ER-α levels in the hippocampal CA1 region. LTED was also shown to lead to hypersensitivity of the hippocampal CA3 region to ischaemic stress. As a whole, the findings of decreased sensitivity of certain brain regions to E_2 provide support for the 'critical period' hypothesis that oestrogen replacement therapy may need to be administered at peri-menopause to observe many of its beneficial neural effects. In support of this contention, new results from the WHI 10-year evaluation on oestrogen alone replacement in women with prior hysterectomy provides support for the 'critical period' hypothesis by demonstrating that the beneficial effects of oestrogen alone on cardiovascular disease, heart attack, invasive breast cancer and mortality were observed when administered to subjects in their 50s, but not when administered to subjects in their 70s (148). Finally, the studies by our group and others on LTED may also provide insights as to why surgical menopausal patients have increased risks for cognitive decline and dementia, as well as increased mortality for neurological diseases.

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