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Estradiol is a potent protective, restorative, and trophic factor after brain injury

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Abstract

Estrogens are a group of pleiotropic steroid hormones that exhibit diverse mechanisms of action in multiple physiological systems. Over the past thirty years, biomedical science has begun to appreciate that endogenous estrogens and their receptors display important roles beyond the reproductive system. Our growing appreciation of novel, non-reproductive functions for estrogens has fundamentally contributed to our knowledge of their role in human health and disease. Recent findings from the Women's Health Initiative have caused clinicians and scientists to question whether estrogens are protective factors or risk factors. In light of the dichotomy between basic science and clinical studies, this review will attempt to reconcile differences between them. We will focus on studies from our laboratory and others highlighting the beneficial properties of the most abundant endogenous estrogen, 17 β -estradiol, using *in vivo* and *in vitro* models of cerebral ischemia and neuronal injury. These studies demonstrate that 17 β -estradiol powerfully protects the brain using multiple molecular mechanisms that promote: 1) decreased cell death, 2) increased neurogenesis, 3) an enhancement of neurotrophic support, and 4) the suppression of pro-inflammatory pathways.

Keywords

neuroprotection; estrogen therapy; estradiol; stroke; cerebral ischemia

INTRODUCTION

Estradiol-17 β (E2) is a steroid hormone whose biological effects on the reproductive axis have been appreciated for a long time. More recently we have begun to appreciate that it also exerts important actions far beyond the reproductive system and acts on the adipose/metabolic system, bone and mineral metabolism, the immune system, cardiovascular health, and the central nervous system¹. Numerous studies have shown that E2 exerts powerful neuroprotective actions on multiple brain regions including the cerebral cortex, hippocampus, striatum, basal

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forebrain, and cerebellum. Since estrogens decrease at the time of the menopause, any protective effects they have are lost. This is an important issue for women since the average age of menopause has remained fixed at 51, although our average lifespan is now greater than 80 years of age. Just in the last century, the proportion and total number of women who live over one-third of their lives in a hypoestrogenic state have risen dramatically. As a consequence, postmenopausal women become more vulnerable to a variety of neurodegenerative diseases than women who have not experienced the menopause or are younger. Based on this observation many researchers have hypothesized that replacement of ovarian estrogens via estrogen therapy (ET) may protect women by decreasing the risk for age-related diseases such as stroke, cardiovascular disease, and dementia (reviewed in ^{2,3}).

Results from the Women's Health Initiative (WHI) however, have raised questions regarding whether ET is truly beneficial ⁴. The WHI was a randomized, placebo-controlled clinical trial designed to test whether hormone therapy protected postmenopausal women against cancer, osteoporosis, heart disease, stroke and other neurodegenerative conditions. The results have received wide attention and have prompted the basic scientists and clinicians to question the beneficial properties of ET. Several factors may have contributed to the discrepancies between animal and human data. These include: 1) timing of hormone therapy (i.e. initiating therapy immediately at the menopause (surgical or natural) rather than waiting a prolonged period of time to initiate therapy), 2) dose of hormones (physiological versus pharmacological), 3) hormone formulation and species specificity (naturally occurring hormones found in humans versus horses and/or use of synthetic hormone preparations), and 4) metabolic complications in the WHI population (i.e. many women were obese).

Stroke, the third leading cause of death in the United States occurs more frequently in women than in men: each year, approximately 40,000 more women than men are affected by stroke and studies show that females have worse outcomes and account for a higher proportion of stroke than men⁵. This gender difference may be related to both the longer life expectancy of women since the risk continues to increase with age. Additional studies show that young women are protected against cerebrovascular stroke, compared to age-matched men, but they lose this protection when they become older and postmenopausal, thereby leading to an increased incidence of stroke related disability and death (reviewed in ^{1,3}). In this review, we will examine evidence from basic science supporting a powerful protective role for ET after brain injury and attempt to explain the disparate results between basic science and clinical studies.

NEUROPROTECTIVE ACTIONS OF ESTRADIOL IN STROKE

Cerebral ischemia is a process through which blood flow to the brain is impaired and ultimately results in neuronal cell death. The early stages of cerebral ischemic injury are characterized by excitotoxicity and peri-infarct depolarizations. In contrast, inflammatory and apoptotic mechanisms play critical roles in the later stages of injury⁷. Multiple laboratories have shown that physiological and pharmacological concentrations of E2 protect in animal models of both permanent and transient focal cerebral ischemia⁶. Yet the complex array of molecular and cellular mechanisms employed by E2 demonstrates that diverse neuroprotective strategies are employed during the time course of ischemic injury. Our laboratory has investigated the pathophysiology of ischemic stroke using an animal model of stroke in which the middle cerebral artery is permanently occluded. Permanent blockage of the middle cerebral artery at its base leads to severe metabolic impairment in the cerebral cortex and parts of the striatum, causing many neurons to die by necrosis within hours following injury. On the other hand, regions that surround the ischemic core, or penumbra, undergo metabolic impairment and are potentially salvageable by effective therapeutic agents.

Neuroprotection via estrogen receptors

Using the model of permanent middle cerebral artery occlusion (MCAO), our laboratory has employed a paradigm of ovariectomy followed by immediate replacement with low, physiological levels of E2 using a Silastic® capsule. This treatment produces low, physiological levels (15–25 pg/ml) of serum E2 in rat⁸ and mouse⁹. We have found that this low dose of E2 protects the total size of the brain infarct at the later stages of stroke injury, but does not protect at the earlier stages of stroke injury (Fig. 1A–B). Using this model, we have found that E2 protects the cortex in rats⁸, and protects both the cortex and striatum in mice⁹. Further studies have shown that E2 must be administered prior to stroke injury acute E2 administration at the time of injury does not protect during stroke⁸. Finally, the effects of E2 in our model do not involve mechanisms that increase cerebral blood flow or blood glucose levels^{8,9}. These parallel findings in both rats and mice strongly suggest that ET exhibits powerful neuroprotective properties in an animal model of stroke.

The dramatic neuroprotective effects exhibited by low, physiological levels led us to test whether E2-mediated neuroprotection requires involvement of estrogen receptors. The actions of E2 are mediated predominantly by two estrogen receptor (ER) subtypes, ER α and ER β , using both genomic and nongenomic signaling mechanisms¹⁰. Using *in situ* hybridization and reverse-transcriptase PCR (RT-PCR), we observed that ER α mRNA is dramatically upregulated in the peri-infarct region on the ipsilateral side of stroke injury in both ovariectomized and E2-treated rats^{11,12}; however, E2 treatment promotes increased upregulation of ER α at an earlier stage after injury compared to ovariectomized animals (Fig. 1C). These findings were confirmed using immunocytochemistry showing that ER α , but not ER β protein, is dramatically upregulated on the ipsilateral side of the cortex after injury both rats and mice^{11,13,14}. To determine whether ER α expression is restricted to specific cell types, we observed that ER α is expressed in cortical neurons but was not detected in astrocytes or microglia¹¹. Taken together, these findings suggest that ER α expression during injury recapitulates the role of ER α in early neuronal development, when there are increases in ER α protein in specific regions that include the cortex. ER α is necessary in early postnatal development for dendritic outgrowth, synaptogenesis, and neurogenesis, and the reappearance of ER α suggests that E2 serves to initiate repair mechanisms after injury. To test whether ER α indeed plays a critical role in E2-mediated neuroprotection, we conducted MCAO in both ER α and ER β knockout mice. We found that the neuroprotective effects of ET were completely abolished in ER α knockout mice, but not in ER β knockout mice^{9,11}. These results provide definitive evidence that E2 decreases the extent of neuronal cell death through ER α -mediated molecular mechanisms.

Estradiol suppresses apoptotic cell death

While the early stages of ischemic injury are marked by necrotic cell death in the center of the cerebral infarct, the later stages of ischemic injury are characterized by apoptotic cell death in the penumbra. Apoptosis is a process of delayed cell death that has been implicated as a major mechanism of cell death during stroke using both *in vivo* and *in vitro* models¹⁵. The results of our early studies suggested that E2 protects in the later stages of injury. Therefore, we investigated the role of E2 as a regulator of apoptotic cell death. Using RT-PCR, we found that E2 upregulates expression of the proto-oncogene *bcl-2* on the ipsilateral side of ischemic injury but did not influence the expression of any members of the *bcl-2* family (*bax*, *bcl-x_L*, *bcl-x_S*, *bad*, and *bim*)¹². Since *bcl-2* promotes cell survival, these results suggest that E2 protects the ischemic penumbra by decreasing apoptotic cell death. To further explore E2-mediated mechanisms of delayed cell death during MCAO, we employed TUNEL staining, a marker of DNA fragmentation, and found that E2 reduced TUNEL staining in rats¹⁶ (Fig. 2A). In addition, we found that E2 reduced caspase 3 activity as early as 4 hours after injury in rats (Fig. 2B). Our results showing both decreased TUNEL staining and caspase 3 activity

demonstrate that E2 also attenuates neuronal cell death through caspase-dependent pathways. Induction of immediate early genes (IEG) after injury also influences cell death. We found that stroke increased the expression of several immediate IEGs including, *c-fos*, *fosB*, *c-jun*, and *junB* 24h after stroke injury, and that ET significantly attenuated induction of c-fos protein 16–24 h following ischemic injury¹⁷ (Fig. 2C). The combined up-regulation of *bcl-2* and downregulation of caspase 3 and c-fos demonstrate that E2 plays pivotal roles in multiple pathways which all converge on the attenuation of neuronal cell death after brain injury to protect the brain.

NEUROGENIC ACTION OF ESTRADIOL

One of the most remarkable discoveries in modern neuroscience is that the adult brain continues to generate new neurons under both normal and neurodegenerative conditions. Adult neurogenesis is thought to be restricted to two major forebrain regions; the dentate gyrus of the hippocampus and the subventricular zone (SVZ) lining the lateral ventricle^{18,19}. Under normal conditions, neural progenitor cells of the SVZ differentiate into neuroblasts, and migrate predominantly through the rostral migratory stream to the olfactory bulb where they become functional interneurons²⁰. However, numerous pathological processes induced by cortical aspiration, traumatic brain injury, and cerebral ischemia redirect the SVZ neuroblasts from their normal migratory route towards the sites of injury. Notably, a proportion of newly generated neurons of the SVZ migrate to the ischemic regions including the cerebral cortex and striatum^{21–23}, two forebrain regions most severely affected by MCAO-induced stroke injury. Thus, the SVZ has tremendous potential as a target for neuronal replacement therapy against stroke injury. However, the number of neuroblasts that migrate to the ischemic regions appears to be insufficient for proper functional recovery of the injured animals^{21,23}. Therefore, we investigated whether estradiol enhances the generation of new neurons in the SVZ under ischemic conditions, to potentially facilitate repair and restoration of the ischemic brain. Our data show that low physiological levels of E2 treatment, which exert no effect in the uninjured state, significantly increase the number of newborn neurons co-labeled with a proliferating cell marker 5-bromo-2'-deoxyuridine (BrdU) and an immature neuronal marker doublecortin (Dcx) following stroke injury (Fig. 3A–C; 11). This effect of E2 was limited to the dorsal region of the SVZ from which neuroblasts begin to migrate towards the injured regions, and was absent from the ventral portion of the SVZ. These neural precursor cells did not express a mature neuronal marker NeuN, indicating that they have not fully differentiated into mature neurons at this time point (Fig. 3D). In addition, we have shown that the proliferative actions of E2 are confined to neuronal precursors, and do not induce unwanted or possibly detrimental gliosis in the SVZ following stroke injury (Fig. 3E).

These findings clearly demonstrate that E2 stimulates neurogenesis in the SVZ of adult ischemic brain, and suggest that E2 may facilitate the brain to undergo repair and remodeling, by increasing the number of newborn neurons that can migrate to the ischemic regions to potentially replace damaged neurons. Future studies to examine underlying mechanisms of E2's neurogenic action may promote the design of effective neuronal replacement therapy against cerebrovascular stroke, as well as other neurodegenerative conditions that often accompany significant neuronal loss.

EFFICACY OF ESTROGEN THERAPY AGAINST STROKE

Over the past two decades, numerous observational and retrospective studies demonstrated that E2 given to postmenopausal women provides notable benefits to cardio- and cerebrovascular diseases (reviewed in^{1,3,24}). However, recent clinical trials including the WHI reported that hormone therapy under certain circumstances increases the risk for stroke, and that it does not exhibit any beneficial actions on long-term stroke outcomes^{25–28}. It is important

to remember that, in the WHI, the mean age of the subjects was 63 years, and the vast majority of subjects were on average 12 years postmenopause prior to the initiation of hormone treatment^{1,29}. In striking contrast, observational studies that reported cardio- and cerebrovascular benefits of ET examined women averaging 51 years of age, many of whom initiated hormone therapy in their perimenopausal period^{30–32}. Thus, to decipher the circumstances under which ET provides benefits against cerebral stroke, we have recently investigated whether a prolonged period of hypoestrogenicity disrupts the ability of E2 to protect the brain against stroke injury. One prominent feature that follows stroke injury is massive inflammatory reactions, and evidence now suggests that ischemia-induced inflammation strongly contributes to the extent of ischemic injury. We have shown that E2 exerts profound neuroprotective action when administered immediately upon ovariectomy, but not when administered after 10 weeks of hypoestrogenicity¹³. Consistently, E2 treatment given immediately at the time of ovariectomy attenuated both central and peripheral pro-inflammatory cytokine production following ischemic stroke. In contrast, E2 did not suppress ischemia-induced production of pro-inflammatory molecules when administered after 10 weeks post-ovariectomy. These findings demonstrate that an extended period of hypoestrogenicity both prevents E2 from protecting the brain against ischemia, and simultaneously suppresses its anti-inflammatory actions. Our data may help to explain the results of the WHI that reported no beneficial effect of ET against stroke, because the majority of the subjects initiated ET after an extended period of hypoestrogenicity. In addition, the discrepancies between previous observational studies and recent clinical trials over the efficacy of ET may have resulted from the differences in the timing of hormone administration relative to the perimenopausal transition.

Importantly, a recent secondary analysis of the WHI reported differential effects of hormone therapy on a risk of cardiovascular disease based on the age and years since menopause. In this study, the use of postmenopausal conjugated equine estrogens (CEE) was associated with a reduced risk for coronary heart disease (a hazard ratio of 0.63 [95% confidence interval], 0.36–1.09) in a group of women aged 50–59 years³³. In addition, this re-evaluation of the WHI reported a hazard ratio of 0.89 ([95% CI], 0.47–1.69) for stroke incidence in the CEE trial within the same age group, supporting the concept that the timing of initiation of hormone therapy influences its efficacy against stroke injury. Together, our findings, in combination with the results of the WHI and its recent re-evaluation emphasize the tremendous importance of strengthening the collaboration between basic science and clinical researchers to take the maximum advantage of empirical and mechanism-based information and approaches.

ESTRADIOL REGULATION OF NEUROINFLAMMATION

Estrogens display novel, nonreproductive functions within the immune system. The dynamic and complex communication among the endocrine, nervous, and immune systems influences the brain's immune response, and ultimately the extent of inflammation during brain injury as well as neurodegenerative disease^{1,34}. Significant experimental evidence shows that E2 may directly or indirectly regulate three distinct components of the inflammatory response: 1) microglial activation³⁴, 2) activation of the enzyme inducible nitric oxide synthase (iNOS)³⁵, and 3) the activation of cytokines/chemokines³⁶. Each component represents a distinct feature of inflammation that is not necessarily mutually exclusive. *In vivo* and *in vitro* studies also pinpoint role(s) for both ER α ^{37,38} and ER β ^{37,39,40} as regulators of the brain's inflammatory response.

Enhanced inflammation may mediate both toxic and reparative mechanisms during brain injury, particularly during stroke. The inflammatory response associated with stroke is complex, and the molecular mechanisms employed by E2 to regulate this response are unclear. To examine the putative anti-inflammatory mechanisms employed by E2 during stroke, we explored the interactions between E2 and inducible nitric oxide synthase (iNOS) in both

wildtype (WT) and iNOS knockout (iNOSKO) female mice following permanent MCAO ⁴¹. In contrast to previous reports, we found that female iNOSKO mice are protected during MCAO but they are not protected further in the presence of E2 (Fig. 4A). We also found that E2 attenuated *NOS2* mRNA, the gene product that codes for iNOS protein, in the cortex of both sham and injured WT mice, suggesting that estradiol protects by downregulating a critical gene in the stroke-associated inflammatory response. To determine whether E2 can suppress other components of the immune response we measured peripheral cytokines and found that estradiol suppresses production of the chemokine macrophage inflammatory protein 1- α (MIP1 α /CCL3) in WT mice, but does not suppress MIP1 α /CCL3 levels further in the iNOSKO mouse (Fig 4B). Suppression of the pro-inflammatory chemokine MIP1 α /CCL3 by both estradiol and the absence of iNOS demonstrates that MIP1 α /CCL3: 1) exacerbates brain injury during stroke, 2) contributes to the gender-based pathophysiology of stroke, and 3) like E2, serves a regulatory role only when iNOS is present. These findings demonstrate that estradiol and iNOS play complementary roles during stroke, and that E2 treatment is efficacious only when iNOS protein retains its functionality. Collectively, our results suggest that a relationship exists between E2 and iNOS with critical repercussions for our understanding of gender-based molecular mechanisms underlying stroke-related injury.

IN VITRO MODELS FOR NEUROTROPHIC AND NEUROPROTECTIVE ACTIONS OF ESTRADIOL

Neurotrophic actions of estradiol in vitro

Over three decades ago, Toran-Allerand reported that E2 elicits a striking enhancement of the growth and arborization of neuronal processes (neurites) in organotypic cultures of fetal and neonatal hypothalamus, preoptic area and cerebral cortex ^{42,43}. *In vitro* studies bolster the pioneering *in vivo* studies performed by Woolley ^{44,45} demonstrating that E2 promotes the growth of synapses and spines in high-density cultures, increases the appearance of a variety of structural changes in the spine and synapse morphology, and promotes the differentiation of multiple synaptic boutons ^{46,47}. In addition, estrogens are trophic *in vitro* and capable of stimulating neuronal outgrowth and a rise in spine density in dispersed cell cultures of several brain regions: amygdala, hypothalamus, hippocampus, and cerebral cortex ^{48,49}. *In vitro* studies have elucidated numerous pathways by which E2 exerts neurotrophic effects, which are ultimately thought to enhance LTP/LTD and memory. These studies have also established a family of factors that are broad acting and impact multiple interdependent downstream events. We now know that a variety of signaling mechanisms are activated in response to estrogen stimulation, such as membrane estrogen receptor (ER) coupling to G-proteins, activation of adenylyl cyclase, protein kinase A, protein kinase C, src, phosphorylation of cyclic AMP response element binding protein, and mitogen-activated protein kinases (reviewed in ²).

Estrogen-induced dendritic spine formation and synaptogenesis *in vitro* clearly depend on postsynaptic NMDA receptor transmission. These effects of E2 are NMDA-mediated ^{47,50}, working through intracellular calcium pathways in neuronal cultures ⁴⁶ and spines of CA1 pyramidal neurons in hippocampal slices ⁵¹. To further determine the role E2 plays in presynaptic protein (vesicular glutamate transporter) and postsynaptic protein (NMDA receptor, NMDAR) localization during synaptogenesis, our lab used dispersion cultures from the rat hippocampus ⁵². Our results corroborate the findings that E2 stimulates glutamatergic synapse formation *in vitro*. In the developing hippocampus, E2 caused an ER α -mediated increase in synapse density, as detected by NR1 and vGlut1 colocalization, along dendrites of neurons cultured in steroid-stripped media (Fig. 5A–G). E2 enriched the NR1 subunit in synaptic clusters providing more evidence that E2 can act directly at the synapse ⁵⁰. In addition, punctate staining of extranuclear ERs along dendrites of neurons expressing NR1 was observed. E2 increased the density of both ER α and ER β protein clusters along dendrites.

To test whether ERs play an important functional role in the E2-induced synaptogenesis, we used the ER antagonist (ICI 182,780) and the ER α and ER β specific agonists (PPT and DPN, respectively) (Fig. 5H). ICI 182,780 blocked the increase in synapse density suggesting an ER-dependent process. Treatment with the ER α specific agonist PPT, but not ER β specific agonist DPN, induced a significant increase in synapse density that mimicked treatment with E2. These studies are the first to demonstrate a critical role of ER α in synapse formation along dendrites and provide further evidence that local signaling may occur between NMDARs and ERs to drive synaptogenesis. While the local signaling mechanism between NMDARs and ERs is presently unknown, the results of *in vitro* studies provide evidence for trophic effects within minutes of adding E2 or estrogen-like compounds^{10,53}. The presence of ER in dendritic spines and axons of hippocampal neurons suggests a role for the ER as a transducer of rapid, non-genomic E2-mediated events during dendritic spine and synapse formation.

Neuroprotective actions of E2 *in vitro*

In vitro models also provide convincing evidence that estrogen is neuroprotective. A variety of studies have reported that E2 protects cultured neurons from neurotoxic insults used to mimic Alzheimer's disease, Parkinson's disease, hypoxia and oxidative stress, excitotoxicity, and physical injury. To understand the protective effect of E2 in the face of neuronal injury, we used varying doses of ultraviolet (UV) irradiation, shown to induce apoptosis *in vitro*⁵⁴ to damage cultured hippocampal and cortical neuronal cultures. This work is the first to report that E2 significantly protects cultured cortical and hippocampal neurons against UV irradiation-induced cell death and may serve as a model to study the mechanisms whereby E2 can protect neurons from apoptosis. E2 (10 nM and 100 nM) was added to cultured cortical (Fig. 6A) and hippocampal (Fig. 6B) neurons 48 hours prior to toxic UV irradiation. We found that E2 significantly protected neurons from death at UV doses that decreased neuronal viability by 50%, suggesting that populations of neurons that can be rescued by E2 are those susceptible to moderate levels of UV irradiation.

CONCLUSIONS

The studies reviewed here provide unequivocal evidence for a neuroprotective role for E2 after brain injury. In spite of overwhelming evidence from basic science studies that ET is protective, differences between preclinical and clinical studies must be reconciled. There are several important factors to consider. First, the diverse and complex mechanisms of hormone and hormone receptor signaling pathways can impact estrogenic properties in different tissues. Second, genetics also plays a critical role. Differences in genetic background are easily controlled in animal studies, while the genetic diversity in human populations introduces multiple variables into clinical studies. Finally, experimental designs between animal studies and clinical studies are very different. While animal studies are designed to assess a cellular/molecular outcome between hormone treatment and the induction of ischemic stroke, clinical studies are designed to test whether hormone therapy alters the risk and/or mortality of stroke in the study population. All of these factors must be considered when extrapolating from animal studies to human clinical studies. An ideal solution for this dilemma would be for basic scientists to use animal models subjected to identical experimental paradigms that most closely mimic human menopause and whose endpoints assess both cellular/molecular correlates as well as ischemic stroke risk. In addition, the ideal human studies would utilize populations who 1) initiate hormone therapy shortly after menopause, 2) use physiological doses and formulations of hormones, and 3) have controlled metabolic conditions. Studies such as these will, ultimately, be the gold standard to determine the efficacy of ET in both preclinical animal models and in clinical studies.

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Abbreviations

BrdU	5-bromo-2'-deoxyuridine
CEE	conjugated equine estrogens
Dcx	Doublecortin
E2	17 β -estradiol
ER	estrogen receptor
ET	estrogen therapy
IEGs	immediate early genes
iNOS	inducible nitric oxide synthase
NMDAR	NMDA receptor
SVZ	subventricular zone
UV	ultraviolet
WHI	Women's Health Initiative
WT	wildtype

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Figure 1. Physiological levels of estradiol protect against ischemic brain damage through ER α (A) Representative coronal brain sections from oil- (Oil) and estradiol-(E) treated rat brains were collected at 1h, 4h, 24h over the evolution of injury and stained with hematoxylin and eosin. E2 exhibits substantial neuroprotection in the cerebral cortex compared to oil-treated controls by 24h. (B) Quantification of infarct volume over 24h reveals that E2 fails to protect at early stages of injury (1h, 4h, 8h) but provides significant neuroprotection against delayed cell death at the later stages of injury (16h, 24h) compared to oil-treated controls (*, $p < 0.001$, $n = 7-11$ /experimental group at each time point). (C) Injury induces *ER α* mRNA in oil and E2 treated rat brains, as measured by in situ hybridization ($p < 0.004$, $n = 3-5$ /group at each time point), but E2 amplifies the induction of *ER α* mRNA at earlier stages of injury (*, $p < 0.03$) compared to oil-treated rats. Values represent mean $\pm$ SEM. (Reprinted with permission from: Dubal DB, Rau SW, Shughrue PJ, et al. Differential modulation of estrogen receptors (ERs) in ischemic brain injury: a role for *ER α* in estradiol-mediated protection against delayed cell death. *Endocrinology* 2006;147:3076–3084.)

Figure 2. Differential regulation of cell death pathways by estradiol after MCAO injury
(A) E2 treatment significantly decreases the number of TUNEL-positive cells during the early (1–8h, # $p < 0.05$) and late (16–24h, * $p < 0.05$) stages of injury compared to oil-treated controls ($n = 8–10$ /group at each time point). **(B)** Estradiol reduces the caspase –mediated (120kDa) spectrin breakdown product at 4h after MCAO injury in the ischemic penumbra (* $p < 0.05$, $n = 3–4$ /group at each time point). **(C)** Estradiol attenuates c-Fos immunoreactive (IR) cells in ischemic cortex during the secondary rise in number of c-Fos IR cells at 16–24h in comparison with oil treated counterparts (* $p < 0.05$, $n = 6–10$ /group at each time point). Values represent mean $\pm$ SEM. (Reprinted with permission from: Rau SW, Dubal DB, Bottner M, Gerhold LM, Wise PM. Estradiol attenuates programmed cell death after stroke-like injury. *Journal of*

Neuroscience 2003;23:11420–11426 and Rau S, Dubal D, Bottner M, Wise P. Estradiol differentially regulates c-Fos after focal cerebral ischemia. J Neurosci 2003;23(33):10487–10494)

Figure 3. Estradiol increases the number of newborn neurons in the subventricular zone (SVZ) after MCAO injury

(A, B) Confocal photomicrographs of BrdU+ cells (green) double-labeled with an early neuronal marker doublecortin (Dcx, red) in the ipsilateral SVZ of oil- (A) vs. E2- (B) treated mice at 96 h after MCAO injury. (C) E2 pretreatment significantly increased the number of BrdU/Dcx dual-labeled newborn neurons (* $P = 0.0008$, $n = 6-7$). (D, E) BrdU+ cells (green) did not co-label with markers for mature neurons (NeuN, red; D) or astrocyte (GFAP, red; E). CC, corpus callosum; STR, striatum. Values represent mean $\pm$ SEM. (Reprinted with permission from: Suzuki S, Gerhold LM, Bottner M, et al. Estradiol enhances neurogenesis following ischemic stroke through estrogen receptors alpha and beta. *J Comp Neurol* 2005;500:1064–1075.)

Figure 4. Estradiol and inducible nitric oxide synthase are both neuroprotective after brain injury (A) Estradiol reduces total infarct volume in WT mice (*, $p < 0.05$), but does not further suppress infarct size in iNOSKO mice. An effect of genotype was observed in iNOSKO oil-treated mice which were also protected during stroke compared to WT oil-treated mice (#, $p < 0.05$, $n = 8-14$ mice/treatment/genotype). (B) MIP1 α /CCL3 was significantly suppressed by E2-treatment after MCAO injury in WT mice (*, $p < 0.05$), while E2 provided no further suppression in iNOSKO mice compared to oil-treated controls. As with infarct volume, an effect of genotype was observed in iNOSKO oil-treated mice which were also protected during stroke compared to WT oil-treated mice (#, $p < 0.05$, $n = 7-12$ mice/genotype/treatment). Values represent mean $\pm$ SEM. (Figure 4A reprinted with permission from: Brown CM, Dela Cruz CD, Yang E, Wise PM. Inducible nitric oxide synthase and estradiol exhibit complementary neuroprotective roles after ischemic brain injury. *Exp Neurol* 2008;210:782-787)

Figure 5. Estradiol treatment increases synaptic cluster density and cluster size through ER α (A–F) Double-label immunocytochemistry for NR1 (green) and vGlut1 (red); yellow in the superimposed images indicates colocalization (G) Estradiol treatment significantly increased the density of colocalized NR1 and vGlut1 clusters (48h, $*p < 0.0005$, $n = 34$ and 6d, $*p < 0.003$, $n = 23$). (H) Dual immunocytochemistry and confocal imaging revealed that exposure for 48h to E2 (10 nM; $*p < 0.027$, $n = 22$) and the ER α specific agonist, PPT (10 nM, $*P < 0.034$, $n = 20$), significantly increased colocalization of NR1 and vGlut1, thereby increasing the density of synaptic clusters. Values represent mean $\pm$ SEM. (Reprinted with permission from: Jelks KB, Wylie R, Floyd CL, McAllister AK, Wise P. Estradiol targets synaptic proteins to induce glutamatergic synapse formation in cultured hippocampal neurons: critical role of estrogen receptor- α . *J Neurosci* 2007;27:6903–6913)

Figure 6. Estradiol protects UV-irradiated neurons from neuronal cell death
 (A) Cortical and (B) hippocampal neurons pretreated with 10 nM and 100 nM E2 were significantly protected against UV irradiation compared to control cultures that were not exposed to E2. (* $p < 0.05$, ** $p < 0.01$, $n = 14-20$ wells/treatment). Values represent mean $\pm$ SEM.