Prematurely menopausal women have a doubled lifetime risk of dementia and a 5-fold increased risk of mortality from neurological disorders. However, the molecular mechanisms underlying these enhanced risks remain unknown. Prolonged loss of ovarian-derived 17β-estradiol (E2) is thought to contribute, as low-dose E2 therapy (ET) initiated at the time of premature menopause and continued until the age of 51 normalizes these risks. The central hypothesis of the current study is that following chronic loss of ovarian function, three key changes occur in CA1 hippocampal neurons: 1) elevation of neurodegenerative factors, 2) enhanced stress-induced amyloidogenesis, and 3) a neural E2 signaling deficit, which, collectively, act to sensitize the hippocampus to stressors, such as global cerebral ischemia (GCI), thereby enhancing cell death and worsening cognitive outcome. To test this hypothesis, we used a rat model of surgical menopause (10-week ovariectomy in young, adult females) with ET delayed to the end of the ovariectomy period. One week after continuous, subcutaneous ET, we subjected animals to 10-min GCI to assess cellular damage and E2 neuroprotection status. In support of our hypothesis, the present study revealed basal upregulation of the neurodegenerative Wnt antagonist Dkk1 in CA1 hippocampal neurons of long-term E2-deprived (LTED) female rats, with concurrent dysregulation of pro-survival Wnt/β-Catenin signaling. We also noted a post-ischemic switch to amyloidogenic processing of amyloid precursor protein (APP) and robust induction of β-amyloid in LTED females subjected to GCI. Finally, we saw evidence of a neural E2 signaling deficit, as we observed a 40% decrease in basal
hippocampal expression of the estrogen receptor co-regulator Proline-, Glutamate-, and Leucine-Rich Protein 1 (PELP1) levels after LTED. To further investigate the consequences of decreased hippocampal PELP1 expression, we knocked down PELP1 in vivo with icv anti-sense oligonucleotides in E2-treated rats prior to GCI. Intriguingly, we saw loss of E2 regulation of pro-apoptotic JNK/c-Jun/Dkk1 signaling, loss of E2 regulation of APP processing, and loss of E2 neuroprotection status, similar to events observed in LTED females. These studies partially explain the enhanced risk of dementia and mortality from neurological disorders seen in prematurely menopausal women and support timely initiation of ET to yield maximum neurological benefit.

INDEX WORDS: Estrogen, Menopause, Hippocampus, Neurodegeneration, Alzheimer’s disease, Cerebral Ischemia.
MOLECULAR MECHANISMS UNDERLYING ENHANCED RISK OF
NEUROLOGICAL DISEASE FOLLOWING PREMATURE MENOPAUSE

By

Erin LeeAnn Scott

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of Georgia Regents University in partial fulfillment
of the Requirements of the Degree of
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MOLECULAR MECHANISMS UNDERLYING ENHANCED RISK OF NEUROLOGICAL DISEASE FOLLOWING PREMATURE MENOPAUSE

This dissertation is submitted by Erin LeeAnn Scott and has been examined and approved by an appointed committee of the faculty of The Graduate School of Georgia Regents University.

The signatures, which appear below, verify the fact that all required changes have been incorporated and that the dissertation has received final approval with reference to content, form, and accuracy of presentation.

This dissertation is, therefore, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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This dissertation is dedicated to all women who have experienced or will experience premature menopause, with hope that the knowledge acquired through these studies will help preserve their cognitive health.
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I. INTRODUCTION

1. Statement of the Problem and Specific Aims

More than 1% of women enter menopause prematurely, or before the age of 40 (Shuster et al., 2010), thus incurring a doubled lifetime risk of dementia and a five-fold increased risk of mortality from neurological disorders (Rocca et al., 2007, Rivera et al., 2009). Importantly, however, the molecular mechanisms underlying these enhanced risks remain unknown. Prolonged loss of the neuroprotective ovarian steroid hormone 17β-estradiol (E2 or estrogen) is thought to play a key role, as estrogen therapy (ET) administered at the time of bilateral oophorectomy and continued until the median age of natural menopausal onset normalizes these risks (Rocca et al., 2011). Surprisingly, a large clinical trial for postmenopausal hormone therapy, the Women’s Health Initiative (WHI), was stopped prematurely due to increased incidence of stroke and dementia in the treatment arm (Shumaker et al., 2003, Wassertheil-Smoller et al., 2003, Espeland et al., 2004). However, a major caveat of the negative WHI results was that the average age of the patients enrolled was 63.3, which is more than a decade past the median onset of natural menopause (Harman et al., 2004, Harman et al., 2005b). This led to formation of the “critical period hypothesis,” which suggests that a window of opportunity exists for estrogen to provide neurological benefit after menopause and that delayed
postmenopausal ET may be ineffective or even detrimental to the brain (Brinton, 2005, Maki, 2006, Sherwin, 2007).

Animal studies in our lab (and others) support the critical period hypothesis, since low dose ET protects the cerebral cortex and hippocampal CA1 region from cerebral ischemia if administered immediately following the onset of surgical menopause in female rats, but not if a period of long-term E2 deprivation (LTED: e.g. 10-week bilateral ovariectomy or reproductive senescence) elapses prior to administration of ET (Figure 1) (Suzuki et al., 2007, Zhang et al., 2009a, Zhang et al., 2011, Scott et al., 2013, Zhang et al., 2013a). Further studies in our lab have provided a potential clue as to why surgical menopause may lead to an increased risk of dementia and mortality from neurological disorders. Along these lines, recent work has shown that the hippocampus sustains more damage from GCI following LTED; this includes previously unseen neuronal cell death in the hippocampal CA3 region, which is usually highly resistant to GCI, and a worse cognitive outcome following GCI (Zhang et al., 2013a). Intriguingly, additional work demonstrated that the hippocampus may become more susceptible to non-ischemic stressors following LTED as well, since the hippocampus of LTED female rats was also significantly more damaged following exposure to β-Amyloid [1-42], the most neurotoxic form of amyloid (Zhang et al., 2013a). However, the molecular mechanisms underlying this hippocampal hypersensitivity remain unclear.
**Figure 1. Estrogen Neuroprotection is Lost Following Long-Term Estrogen Deprivation.**

Confocal microscopy demonstrates potent 17β-estradiol (E2) neuroprotection in the hippocampal CA1 region of short-term E2-deprived (STED: 1-week ovariectomy) female rats and loss of E2 neuroprotection in the hippocampal CA1 region of long-term E2-deprived (LTED: 10-week ovariectomy) female rats following 10-min GCI. NeuN (left, red) stains neuronal nuclei, and FluoroJadeB (FJB: middle, green) is a marker of neurodegeneration. No neuronal loss is seen in STED (short-term E2-deprived: 1-week ovariectomy) or LTED sham animals. However, it is readily apparent 7 days after GCI in STED Placebo, LTED Placebo, and LTED E2 groups. In contrast, animals that had subcutaneous, low dose E2 treatment initiated immediately following ovariectomy and continued for 1 week prior to GCI (STED E2) had significantly less neuronal cell death compared to the STED placebo group. n = 4 Sham, 5 STED
Placebo, 6 STED E2, 4 LTED Placebo, and 4 LTED E2; Magnification = 40X.
The central hypothesis of the current study is that following the chronic loss of ovarian E2 that occurs in surgical menopause, three key changes occur in hippocampal neurons: 1) elevation of neurodegenerative factors, 2) enhanced stress-induced amyloidogenesis, and 3) a neural E2 signaling deficit; which collectively act to sensitize the hippocampus to stressors (e.g., ischemia, β-amyloid [1-42], oxidative stress), thereby enhancing cell death and worsening cognitive outcome (Figure 2).
Figure 2. Central Hypothesis of the Dissertation. This schematic depicts molecular changes that are proposed to underlie the enhanced risk of dementia and mortality from neurological disorders observed in prematurely menopausal women. Following premature and prolonged loss of ovarian-derived E2, three major events are proposed to occur in hippocampal neurons: 1) elevation of neurodegenerative factors, 2) enhanced stress-induced amyloidogenesis, and 3) a neural E2 signaling deficit. Collectively, these alterations could make the hippocampus more susceptible to stressors and, thus, mechanistically explain the enhanced risk of dementia and mortality from neurological disease observed in prematurely menopausal women.
To test the central hypothesis, the current study used an animal model of premature surgical menopause (10-week ovariectomy in young adult female rats) with delayed ET. Since it is well known that the hippocampal CA1 region is highly vulnerable to GCI, GCI was selected as the stressor for use in our studies. Three specific aims were proposed:

Aim 1. Test the hypothesis that long-term ovarian E2 deprivation (LTED) leads to dysregulation of neurodegenerative Dkk1 and Wnt/Beta-Catenin signaling in the hippocampal CA1 region and to decreased sensitivity of this pathway to E2 regulation.

Aim 2. Test the hypothesis that LTED leads to enhanced amyloidogenesis after ischemic injury to the hippocampus and to decreased sensitivity of this pathway to E2 regulation.

Aim 3. Test the hypothesis that LTED leads to an E2 signaling defect in the hippocampus, which involves dysregulation of the estrogen receptor (ER) coregulator protein, PELP1.
2. Review of the Literature

2.1 Estrogen As a Neuroprotective Agent

Since Ed Hall and colleagues first discovered that female gerbils were relatively protected from global cerebral ischemia, compared with their male counterparts, in the early 1990s (Hall et al., 1991), gender differences have been a major focus of neurological research. Intriguingly, according to Scott et al. (2012), “gender differences exist in most disorders affecting the central nervous system (CNS), particularly the neurodegenerative conditions, with women typically having a later onset and greater severity of disease (Tang et al., 1996, Brann et al., 2007). Women’s relative protection, later onset, and greater severity of neurodegenerative disorders can be explained, in part, by serum levels of the neuroprotective ovarian hormone, 17β-estradiol (estradiol or E2). From birth to menopause, women’s ovaries produce high circulating levels of estradiol, which correlates with a low incidence of neurodegenerative disease. However, once the menopausal transition occurs, the ovaries cease to [produce] E2, and women’s risk for neurodegenerative diseases, including ischemic stroke and Alzheimer’s disease, increases (Brann et al., 2007). One could dismissively attribute this correlation to senescence. However, women who enter menopause prematurely via bilateral oophorectomy (surgical removal of both ovaries) have a doubled lifetime risk for developing dementia, as well as a significantly increased risk of cognitive decline, Parkinson’s disease, and mortality from neurological disorders (Rocca et al., 2007, Shuster et al., 2010, Rocca et al., 2011). Furthermore, meta-analyses of observational studies demonstrated that postmenopausal women who used oral estrogens had a 29-44% reduced risk of Alzheimer’s disease (Yaffe et al., 1998, Hogervorst et al., 2000, Brann et al., 2007).
In further support of the correlation between high serum levels of estradiol and women’s relatively low risk of neurodegenerative disease, studies in rodents have overwhelmingly demonstrated that E2 is a neuroprotective agent. Female rodents were less susceptible to ischemic stress via experimental stroke procedures, such as middle cerebral artery occlusion (MCAO) and global cerebral ischemia (GCI), than their male counterparts, and ovariectomy prior to stroke induction abolished this gender difference (Brann et al., 2007). Serum E2 levels in intact rodents were also found to be inversely correlated with stroke infarct size (Liao et al., 2001), and pre-treatment with ICI 182,780, a competitive antagonist of both estrogen receptor isoforms alpha (ERα) and beta (ERβ), prior to stroke induction actually enhanced the size of the infarct (Sawada et al., 2000). Additionally, pre-treatment with aromatase inhibitors, which prevent the conversion of androgens to estrogens, exacerbated ischemic injury in rodent brains, and aromatase knockout (KO) mice, which are physically unable to convert testosterone into estradiol, also had larger infarct volumes after MCAO (McCullough et al., 2003). Conversely, pre-treatment with exogenous E2 decreased mortality and infarct size following MCAO and GCI in rodents (Simpkins et al., 1997, Dubal et al., 1998, Shi et al., 1998, Zhang et al., 1998, Rusa et al., 1999, Jover et al., 2002, Shughrue and Merchenthaler, 2003, McCullough et al., 2005, Brann et al., 2007, Zhang et al., 2008, Lebesgue et al., 2009, Zhang et al., 2009a, Yang et al., 2010, Zhang et al., 2011). A systematic review of 161 publications on estradiol and stroke performed by Gibson et al. further confirmed a dose-dependent reduction of stroke lesion volume by E2 in models of transient and permanent cerebral ischemia (Gibson et al., 2006).
Aside from preventing neuronal death, exogenous E2 replacement prior to stroke was shown to attenuate behavioral deficits in ovariectomized female rats subjected to GCI (Plamondon et al., 2006, Brann et al., 2007, Lebesgue et al., 2009). Furthermore, exogenous E2 was shown to facilitate post-stroke recovery in mice by enhancing neurogenesis in the dentate gyrus and subventricular zone after stroke, an effect that was attenuated in estrogen receptor (ER) knockout (KO) mice and aromatase KO mice (Li et al., 2011b). As reviewed previously by our group and others, E2 has also been demonstrated to afford protection in animal models of Parkinson’s disease and Alzheimer’s disease (AD) (McCullough et al., 2003, Brann et al., 2007, Bourque et al., 2009, Pike et al., 2009). Finally, it should be mentioned that there are dissenting studies in the literature, which found that E2 increased ischemic stroke damage in animal models (Harukuni et al., 2001, Carswell et al., 2004a, Bingham et al., 2005, Gordon et al., 2005, Theodorsson and Theodorsson, 2005, Yong et al., 2005). It is not completely clear as to why these studies yielded a different result than the majority of studies in the literature, but a recent review suggested that the difference could be due to use of E2 slow-release pellets that yielded unexpectedly high circulating E2 levels (Strom et al., 2009). Nevertheless, as a whole, research using animal models provides strong evidence for endogenous and exogenous E2 as a neuroprotective agent,” (pp. 85-86) (Scott et al., 2012).

2.2 Mechanisms of Estrogen Signaling and Neuroprotection

Furthermore, according to Scott et al. (2012), “In addition to providing support for estradiol as a neuroprotective hormone, the aforementioned animal studies have also
yielded mechanistic insight into how E2 protects the brain from ischemia. **Figure 3** provides a schematic overview of the major mechanisms of E2 neuroprotection from cerebral ischemia. As shown in **Figure 3**, E2 is proposed to mediate neuroprotection via a multimodal mechanism that involves: 1) genomic signaling, 2) extranuclear signaling, 3) antioxidant actions, and 4) regulation of mitochondrial bioenergetics. The majority of these effects are suggested to involve mediation by ERs, in particular ERα. In the sections below, we will discuss evidence of a principal role for ERα in mediating E2 neuroprotection, and subsequently, we will discuss the evidence supporting each proposed mechanism of E2 neuroprotection depicted in **Figure 3**.
Figure 3. Mechanisms of Estradiol Neuroprotection. This figure summarizes four major mechanisms through which 17β-estradiol (E2) protects neurons: 1) classical genomic signaling, 2) rapid extranuclear signaling, 3) antioxidant scavenging, and 4) mitochondrial bioenergetics. Adapted from (Scott et al., 2012).
ERα is a Key Mediator of E2 Neuroprotection in Cerebral Ischemia. Initial evidence suggesting ER mediation of E2 neuroprotection in cerebral ischemia came from the observation that the ERα and ERβ antagonist ICI182,780 prevented E2’s beneficial effects when it was given simultaneously with estradiol prior to GCI (Miller et al., 2005). Furthermore, development of genetically modified mice led to the suggestion of ERα as the major mediator of E2 neuroprotection in experimental stroke. Ovariectomized ERα KO mice displayed a complete loss of E2 neuroprotection upon pre-treatment with E2 prior to cerebral ischemia, but ovariectomized ERβ KO mice maintained E2 neuroprotection when treated with E2 prior to stroke (Dubal et al., 2001, Merchenthaler et al., 2003)” (p. 86) (Scott et al., 2012). In addition, our laboratory provided solid evidence that ERα, but not ERβ, is the key mediator of E2 neuroprotection against GCI through separate antisense (AS) oligonucleotide knockdown studies of ERα and ERβ, which showed a loss of E2 neuroprotection in ERα AS knockdown animals only (Zhang et al., 2009a).

Scott et al. (2012) also stated, “A recent study also suggested that E2 may act directly on neurons to exert neuroprotection, as E2 neuroprotection was lost in neuron-specific ERα knockout mice, but not in microglia-specific ERα knockout mice (Elzer et al., 2010). However, while this study suggests that estradiol can act directly on neurons to exert neuroprotection in cerebral ischemia, it does not completely rule out that ERs in other cell types, such as endothelial cells or astrocytes, [could] also contribute, in part, to mediation of E2’s neuroprotective effects. Along these lines, there is a significant body of evidence demonstrating that E2 can act on astrocytes to influence release of neuroprotective compounds, such as growth factors, which could provide an indirect
mechanism of E2 neuroprotection (Dhandapani and Brann, 2007, Arevalo et al., 2010, Azcoitia et al., 2010). Additionally, a recent study showed that ERα-specific knockout in astrocytes, but not neurons, resulted in the loss of E2’s neuroprotective ability in an animal model of experimental autoimmune encephalomyelitis (Spence et al., 2011). Therefore, while E2 has been implicated to act directly upon neurons to exert neuroprotection against cerebral ischemia, it is important to examine astrocyte- and endothelial-specific ER knockout animals, so as to better clarify the contribution of these non-neural cell types to E2 neuroprotection in cerebral ischemia.

It should be noted that there are a few studies, which used ERβ-specific agonists to suggest a role for ERβ in neuroprotection in cerebral ischemia (Carswell et al., 2004b, Miller et al., 2005). However, when using agonists, one cannot totally exclude potential ‘off target’ effects of the agonists that could explain the observed effects. In addition, exogenous agonist treatment studies alone do not prove a physiological role of the receptor in mediating E2 neuroprotection. As such, conclusions regarding the role of a receptor must be based on a variety of approaches, including the use of receptor knockdown and receptor knockout mice. As mentioned previously, the results of ERβ KO mouse studies did not support a major role for ERβ in mediating E2 neuroprotection in cerebral ischemia. Rather, the available evidence to date argues that ERα is the principal ER that mediates E2 neuroprotection in cerebral ischemia. Nevertheless, it should be mentioned that ERβ KO mice have been reported to show significant neurodegeneration in the cerebral cortex, beginning at six months of age and peaking at two-years of age (Wang et al., 2001). This intriguing observation suggests that ERβ may have a role in mediating basal neuronal survival in the cerebral cortex.
GPR30 – A Putative New ER? A third putative ER called G-Protein-Coupled ER (GPR30, also known as GPER1) has been recently identified as a potential membrane ER (Funakoshi et al., 2006). GPR30 is a seven transmembrane domain G-protein coupled receptor that is expressed in the hippocampus, cortex, striatum, and other brain regions (Funakoshi et al., 2006, Matsuda et al., 2008, Hammond and Gibbs, 2011). Intriguingly, co-localization studies showed that the majority of cholinergic neurons in the forebrain co-localize with GPR30 immunoreactivity (Hammond and Gibbs, 2011, Hammond et al., 2011), suggesting that it may have a regulatory role in cholinergic neurons. Evidence of a neuroprotective role for GPR30 has been primarily derived from studies using a purported selective agonist for GPR30, G-1 (Bologa et al., 2006, Gingerich et al., 2010). G-1 pre-treatment has been reported to significantly attenuate glutamate-induced or oxidative stress-induced neuronal cell death in neuronal cell cultures (Gingerich et al., 2010, Liu et al., 2011). *In vivo* studies using the MCAO model have also shown that G-1 exerts neuroprotection against cerebral ischemia in female mice (Zhang et al., 2010). A caveat of these studies is that they only show an effect of an *exogenous* GPR30 agonist and do not prove a role for GPR30 in mediating neuroprotection by either endogenous or exogenous E2. Further work is needed to address the *physiological* role of GPR30 in mediating E2 neuroprotection through use of various approaches, including the use of mutant GPR30 mice and GPR30 knockdown,” (pp.86-87) (Scott et al., 2012).

Genomic Signaling and E2 Neuroprotection. E2 signals through both classical genomic and rapid extranuclear signaling mechanisms. Furthermore, both types of signaling have been implicated in mediation of E2 neuroprotection. With respect to classical genomic, or nuclear, signaling, Scott et al. (2012) state, “E2, as a lipophilic
molecule, diffuses through the cell’s membrane, binds to ERs in the cytosol, and translocates to the nucleus in order to regulate the transcription of genes in a matter of hours. In support of the importance of genomic signaling in E2 neuroprotection, E2 has been shown to increase transcription of pro-survival bcl-2 \textit{in vivo} after MCAO and prevent transcription of pro-apoptotic bad (Dubal et al., 1999). E2 also increases bcl-2 in human NT2 neurons and rat hippocampal neurons \textit{in vitro} (Dubal et al., 1999, Wu et al., 2005). Interestingly, E2 has been shown to regulate expression of other bcl-2 family members, including enhancing expression of anti-apoptotic bcl-w while attenuating expression of pro-apoptotic bim in cortical neurons, effects shown to be important for E2 neuroprotection against beta-amyloid-induced neuronal death (Yao et al., 2007). Furthermore, E2 has been demonstrated to prevent the translocation of cytochrome c, activation of caspase-3, and fragmentation of DNA, all events which occur during programmed cell death (Rau et al., 2003, Choi et al., 2004).

Additionally, work by our laboratory showed that E2 strongly enhances expression of the anti-apoptotic, pro-survival factor, survivin in the CA1 hippocampal region 24-48h following GCI, which facilitates neuronal survival (Zhang et al., 2008). Recent work has also shown that E2 induces survivin expression in the peri-contusional area of the cerebral cortex following traumatic brain injury (TBI), which is thought to contribute to its neuroprotective effects in TBI (Bao et al., 2011). Estradiol is also able to enhance expression of growth factors in the brain, such as transforming growth factor-β1 (TGFβ1) and brain-derived neurotrophic factor (BDNF) (Galbiati et al., 2001, Dhandapani et al., 2005, Mahesh et al., 2006), as well as growth factor receptors, such as the insulin-like growth factor 1 (IGF-1) receptor (Cardona-Gomez et al., 2001, Garcia-
Segura et al., 2010). Importantly, the growth factor BDNF is well known to exert neuroprotective and neurotrophic effects in the cortex and hippocampus and to be important for synaptic plasticity, learning, and memory (Singer et al., 1999, Singh et al., 1999, Bekinschtein et al., 2008a, Bekinschtein et al., 2008b, Yang et al., 2010). Finally, work by the Garcia-Segura laboratory has shown that there is significant cross-talk between the E2 and IGF-1 receptor signaling pathways and further suggested that this cross-talk plays a role in mediating E2’s neuroprotective effects (Cardona-Gomez et al., 2001, Garcia-Segura et al., 2010),” (p.88) (Scott et al., 2012).

Rapid Extranuclear Signaling and E2 Neuroprotection. Recent work has also identified a role for rapid extranuclear ER signaling in mediating E2 neuroprotection. According to Scott et al. (2012), “in addition to nuclear localization of ERs, both ERα and ERβ have been demonstrated to be at the plasma membrane of neurons in various brain regions, including the cortex and hippocampus, and at other neuronal extranuclear sites, such as dendrites and dendritic spines (Milner et al., 2001, Kow and Pfaff, 2004, Dhandapani et al., 2005, Garcia-Ovejero et al., 2005, Kalita et al., 2005, Vasudevan and Pfaff, 2007, Madak-Erdogan et al., 2008, Roepke et al., 2011, Wu et al., 2011a). Extranuclear ERs are thought to play a key role in mediating rapid signaling effects of E2 in neurons to regulate kinase signaling pathways, calcium signaling, and activation/inactivation of key cellular proteins (Wu et al., 2011a),” (p.88) (Scott et al., 2012). This rapid extranuclear signaling by E2 may also exert genomic regulation through nuclear cross-talk, which will be discussed later. Scott et al. (2012) also state, “currently, it is not entirely clear how ERα and ERβ are targeted to the membrane and to other extranuclear sites. Palmitoylation of ERs, and ER interaction with the scaffold
protein, caveolin-1 have been suggested to play a role in trafficking of ERs to the membrane (Chaban et al., 2004, Bondar et al., 2009). A recent study also provided evidence that heat shock protein-27 (HSP27) can bind to ERα, thereby promoting its palmitoylation and delivery to the cell membrane (Razandi et al., 2010). The majority of the studies on mechanisms of ER trafficking to the membrane were conducted in non-neuronal cells; however, it is assumed that the same mechanisms apply to neurons as well.

A large body of evidence supports a role for E2 and extranuclear ERs in the rapid activation/phosphorylation of neuronal extracellular signal-related kinases (ERK) and the phosphotidylinositol-3-kinase (PI3K)-Akt pathway in neurons (Watters et al., 1997, Singh et al., 1999, Honda et al., 2000, Kuroki et al., 2000, Honda et al., 2001, Manthey et al., 2001, Wilson et al., 2002, Bryant et al., 2005, Setalo et al., 2005, Wu et al., 2005, Mannella and Brinton, 2006, Dominguez et al., 2007). Once activated, ERK can phosphorylate/regulate over 100 downstream targets, including transcription factors, cytoskeleton proteins and kinases, and nuclear kinases (Ramos, 2008). As such, ERK activation has been implicated in the regulation of many key cellular functions such as survival, adhesion, metabolism and proliferation (Ramos, 2008). Akt, also known as protein kinase B, is a kinase that can promote cell survival by inhibiting the pro-apoptotic JNK-c-jun signaling pathway and by inhibiting the activity of pro-apoptotic bad and glycogen synthase kinase-3β (GSK-3β) (Wang et al., 2006). Since JNK and GSK-3β are kinases known to phosphorylate the microtubule associated protein tau, and hyperphosphorylated tau is one of the hallmarks of Alzheimer’s disease neuropathology,
this may be one of the mechanisms through which E2 can protect the brain from AD (Goodenough et al., 2005).

There is significant evidence that ERK activation is important for E2 neuroprotection. For instance, in vitro studies have shown that administration of a MEK inhibitor, which prevents E2-induced ERK activation, blocks the neuroprotective effects of E2 in neurons (Kuroki et al., 2001, Guerra et al., 2004, Wu et al., 2005, Numakawa et al., 2007). In addition, E2 enhances ERK activation in the hippocampal CA1 region following GCI, and E2’s neuroprotective effects in GCI are blocked by administration of a MEK inhibitor (Jover-Mengual et al., 2007). However, it is important to note that while E2’s activation of ERK1/2 has been proposed to be neuroprotective in cerebral ischemia, evidence also suggests that ERK activation may play a pro-apoptotic role. Along these lines, MEK inhibitors have been shown to attenuate ischemic damage following GCI and MCAO, which suggests that ERK activation may actually contribute to neurodegeneration following cerebral ischemia (Namura et al., 2001, Wang et al., 2003, Henriksson et al., 2007). In contrast, it has also been hypothesized that enhanced ERK1/2 activation may initiate a neuroprotective signaling cascade, which eventually culminates in the down-regulation of ERK itself, thereby preventing prolonged ERK activation. The apparent paradox of ERK activation having both positive and negative effects in cerebral ischemia could be feasibly resolved by considering several factors, such as the neural cell type in which ERK is activated (neuron, glia, or endothelial cell), the pattern/duration of ERK activation (acute, biphasic, or chronic), and/or the subcellular localization of activated ERK (nucleus versus cytoplasm). For a more authoritative and thorough discussion of this complex subject, the reader is referred to Sawe et al.’s review.
concerning activated ERK’s dual role in cerebral ischemia (Sawe et al., 2008),” (pp. 88-89) (Scott et al., 2012).

In further support for a role of extranuclear ERs in E2’s neuroprotection, the Brann lab used two E2 conjugates (E2 dendrimer conjugate [EDC] and E2-BSA), both of which are unable to enter the nucleus and can only interact with extranuclear ERs to exert rapid nongenomic signaling (Harrington et al., 2006), to show that, similar to E2, both EDC and E2-BSA afforded neuroprotection against GCI and improved cognitive outcomes (Yang et al., 2010). Importantly, this effect was ER-dependent, as concomitant exposure to the ER antagonist ICI182,780 completely abolished the neuroprotective effects of both compounds. According to Scott et al. (2012), additional work in the same study “demonstrated that EDC and E2-BSA rapidly enhanced ERK and Akt activation in the hippocampal CA1 region after cerebral ischemia, and that inhibition of either ERK or Akt activation by specific inhibitors abolished the neuroprotective effects of the E2 conjugates (Yang et al., 2010). While we observed that acute E2 conjugate treatment in vivo both enhanced and prolonged ERK activation in the hippocampal CA1 region following GCI, this finding does not support a role for E2 in the aforementioned proposed model of prolonged ERK activation facilitating its own inactivation. However, it is important to note that our study was limited to 24 hours after GCI. As such, examination of more distant time points post ischemia-reperfusion may be required to determine if prolonged ERK activation by E2 leads to subsequent down-regulation of ERK more than one day following GCI. Interestingly, our studies also revealed that EDC (and E2-BSA) enhanced phosphorylation of the transcription factor, CREB in a rapid fashion following reperfusion, and that this effect was both ERK- and Akt-dependent
Among the best known CREB transcriptional targets is the growth factor, BDNF, and subsequent studies by our group demonstrated that EDC strongly increased BDNF expression in the hippocampal CA1 region following GCI (Yang et al., 2010),” (p.89) (Scott et al., 2012). As such, these findings suggest that extranuclear ER-mediated signaling may cross-talk with the nucleus through kinase-induced activation of transcription factors, which could subsequently modulate expression of pro-survival and/or pro-death genes.

An additional example of extranuclear E2 signaling exhibiting cross-talk with the nucleus is E2’s regulation of JNK/c-Jun signaling. Scott et al. (2012) state, “along these lines, work by our lab has shown that E2 and EDC exert a prolonged attenuation of phosphorylation of JNK at Thr183/Tyr185 in the hippocampal CA1 region after cerebral ischemia, phosphorylation sites known to be critical for JNK activation (Zhang et al., 2009a, Yang et al., 2010). JNK is known to phosphorylate many cellular proteins, including several implicated in apoptosis, and it can translocate to the nucleus to activate c-Jun and AP-1-mediated gene transcription, which leads to upregulation of pro-death genes (Dhandapani et al., 2005). Previous work by our group and others has shown that administration of a JNK inhibitor or knockout of JNK results in profound protection of the brain against cerebral ischemia (Garcia-Ovejero et al., 2005, Zhang et al., 2008, Elzer et al., 2010), further demonstrating a key pro-apoptotic role of JNK in ischemic neuronal cell death. Thus, the ability of E2/EDC to markedly attenuate JNK activation after cerebral ischemia likely contributes significantly to its neuroprotective actions,” (p.89) (Scott et al., 2012). Collectively, these studies suggest that aside from the classical nuclear ERs, extranuclear ERs are also critical for E2 neuroprotection and preservation of
cognitive function after cerebral ischemia. Furthermore, they suggest that extranuclear ER-initiated E2 signaling can regulate gene transcription through rapid modulation of kinase signaling pathways and subsequent cross-talk with nuclear transcription factors.

Oxidative Stress, Mitochondrial Bioenergetics and E2 Neuroprotection. Another important mechanistic layer of the neuroprotective effect of E2 is its ability to regulate oxidative stress and mitochondrial bioenergetics. According to Scott et al. (2012), “oxidative stress and mitochondrial dysfunction have been implicated to play a key role in promoting neuronal damage and demise in cerebral ischemia and other neurological disorders, such as Alzheimer’s disease (Niizuma et al., 2009, Chen et al., 2011, Facecchia et al., 2011, Readnower et al., 2011). With respect to mitochondrial dysfunction, a growing body of evidence indicates that E2 can have beneficial effects upon mitochondria and preserve mitochondrial function. These effects include regulation/preservation of ATP generation, [reactive oxygen species] (ROS) production, mitochondrial apoptotic factors, and antioxidant mechanisms. With respect to mitochondrial bioenergetics, E2 has been demonstrated to protect the brain by enhancing regional cerebral blood flow in vivo and facilitating the utilization of glucose as the primary source of energy for the brain by up-regulating glucose transporters in neurons (Brinton, 2008b, Yao et al., 2010). Furthermore, E2 has been shown to promote oxidative phosphorylation of energy substrates by enhancing activity of electron transport chain complexes I and II in order to maintain sufficient energy supply for neurons (Hamilton et al., 2011, Yao et al., 2011). It is intriguing that these aforementioned mechanisms of E2 neuroprotection also implicate E2 with protection of the brain from Alzheimer’s disease, due to theories of neuronal free radical damage and cerebral hypometabolism as critical
precursors to AD onset (Sun et al., 2006, Yao et al., 2009, Yao et al., 2010, Yao et al., 2011). E2’s effects on mitochondria have been extensively reviewed previously, and the reader is referred to these excellent reviews (Brinton, 2008b, Suh et al., 2008, Sorce and Krause, 2009).

In addition to mitochondria-generated ROS, recent evidence suggests that the plasma membrane, via NADPH oxidase, may play an additional critical role in ROS generation in neurons following cerebral ischemia. The NADPH oxidase enzyme is composed of key subunits from the NOX family, whose primary job is to transport electrons across biological membranes in order to reduce molecular oxygen to superoxide (O$_2^-$). O$_2^-$ is a highly reactive free radical anion that is the precursor of most ROS, including the highly toxic and damaging hydroxyl ion and peroxynitrite (Arnold and Beyer, 2009, Simpkins et al., 2010). The NOX family is composed of five isoforms (NOX1-NOX5). The activation of NOX2, the most studied and best characterized NOX isoform, involves interaction between the subunits p22phox, p67phox, p40phox and p47phox subunits (Cheng et al., 2001, Vallet et al., 2005, Bedard and Krause, 2007). In addition, the GTPase, Rac1 has been shown to be critical for NOX2 activation (Cheng et al., 2001, Bedard and Krause, 2007). Work by our laboratory has shown that NADPH oxidase activation and 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 minutes after reperfusion and peak levels observed at 3 hours after reperfusion (Zhang et al., 2009a). Further work demonstrated that NOX2 is predominantly localized to neurons in the hippocampus following GCI (Zhang et al., 2009a), but NOX2 is also known to be expressed in microglia at later time-points following cerebral ischemia (Dubal et al.,
The importance of NADPH oxidase activation to neuronal damage following cerebral ischemia was suggested by the fact that inhibition or knockout of the NOX2 NADPH oxidase enzyme significantly reduced infarct damage (Tang et al., 2008, Zhang et al., 2009a, Brait et al., 2010, Genovese et al., 2011, Yoshioka et al., 2011).

Intriguingly, further work by our laboratory showed that low dose E2 replacement profoundly attenuated NADPH oxidase activation and $O_2^-$ production in the hippocampal CA1 region following reperfusion, an effect blocked by the ER antagonist ICI180,782 (Zhang et al., 2009a),” (pp. 90-91) (Scott et al., 2012).

Scott et al. (2012) also state, “Further work implicated a critical role for extranuclear ERα in E2’s antioxidant effects, as evidenced by the fact that EDC was fully capable of attenuating NADPH oxidase activation and $O_2^-$ production after GCI and that antisense oligonucleotide knockdown of ERα, but not ERβ, blocked the antioxidant effects of E2 (Zhang et al., 2009a). Additional studies showed that E2 inhibited activation of the GTPase Rac1 in an Akt-dependent manner following cerebral ischemia, which is critical for NOX2 NADPH oxidase activation (Zhang et al., 2009a). The finding that E2’s antioxidant effects involve mediation by extranuclear ERα and rapid kinase activation is in agreement with studies in other systems such as bone, where E2 suppression of oxidative stress did not require ERα binding to DNA response elements but instead, resulted from the activation of kinases (Almeida et al., 2010). While the above studies suggest that extranuclear ERα plays a key role in mediating the antioxidant effects of E2, it should be mentioned that there is also evidence that estrogens, in an ER-independent manner, can act as direct hydroxyl radical scavengers, converting captured nonphenolic quinols back into their respective parent estrogens without the production of reactive
oxygen species as a byproduct (Prokai et al., 2003). Thus, E2’s antioxidant effects may
be a sum of both ER-dependent and ER-independent effects,” (pp. 91-92) (Scott et al.,
2012).

In summary, the neuroprotective effect of E2 against cerebral ischemia involves
both nuclear and extranuclear E2 signaling and is mediated primarily by ERα.
Furthermore, as Figure 3 illustrates, E2 neuroprotection is ultimately achieved through
“attenuation of oxidative stress, preservation of mitochondrial bioenergetics, and
regulation of the balance of pro-survival and pro-death factors,” (p.92) (Scott et al.,
2012). In the next sections of the literature review, we will discuss the types of
menopause, examine the neurological consequences that may occur following prolonged
E2 deprivation, and review the literature concerning postmenopausal hormone therapy
(HT) and the “critical period” hypothesis.

2.3 Types of Menopause

According to Scott et al. (2012), menopause is “defined by the complete absence
of menstrual cycles for one year, and women experience menopause at a median age of
51 years in developed countries (Kato et al., 1998, Rocca et al., 2011),” (p. 92). However,
5% of women enter menopause early (< 45), and at least 1% of women enter menopause
prematurely (< 40) due to surgical menopause, premature ovarian insufficiency, or
iatrogenic damage from chemotherapeutics, radiation, or surgery [for review, see
(Shuster et al., 2010, Jin et al., 2012)]. Natural “menopause is physiologically
characterized by gradual ovarian follicle depletion, cessation of menstruation, and a
dramatic decrease in serum levels of ovarian-derived estradiol,” (p.92) (Scott et al.,
2012). On the other hand, “surgical menopause is an ambiguous umbrella term that pertains to surgical removal of either the uterus alone (hysterectomy), removal of one or both ovaries (unilateral or bilateral oophorectomy, respectively), or some combination of the two events (Rocca et al., 2011). While the natural menopausal transition typically occurs over a period of years, surgical menopause is extremely abrupt. Rather than a gradual cessation of menstrual cycles, removal of the uterus and/or the ovaries leads to instantaneous amenorrhea. Therefore, since menopause is defined by the absence of menstrual cycles, these women immediately enter into menopause following surgery, regardless of whether the ovaries are left intact. However, it is important to note that in the subset of women who undergo hysterectomy but retain one or both of their ovaries, the remaining ovary(ies) will continue to produce E2 until follicular depletion occurs at the age of natural menopause, even though these women will not have menses (Rocca et al., 2011),” (p.92) (Scott et al., 2012). “Conversely, in the case of bilateral oophorectomy, instead of the steady decline in serum E2 values seen in naturally menopausal and hysterectomized women, serum E2 values instantly and dramatically plummet once the hormone-producing ovaries are removed,” (p. 92) (Scott et al., 2012).

Regardless of the type of menopause, many undesirable side effects are often experienced due to the decline in serum E2 levels. Scott et al. (2012) state, “common symptoms of E2 deficiency during the menopausal transition include vasomotor “hot flushes,” sweating, vaginal dryness, vaginal atrophy, dyspareunia, urinary incontinence, irritability, depression, and insomnia, all of which range in severity among women (Kato et al., 1998, Woods and Mitchell, 2005),” (p. 92). These bothersome symptoms tend to subside once the menopausal transition period has passed. Prolonged E2 deficiency can
lead to more serious neurological sequelae, but these sequelae take considerably longer to manifest and are often asymptomatic “until significant disease progression has occurred,” (p. 92) (Scott et al., 2012). “Importantly, women who experience surgical menopause through bilateral oophorectomy are, on average, much younger than women who experience natural menopause (Henderson and Sherwin, 2007) and will experience a much longer period of E2 deprivation than traditionally menopausal women, along with the detrimental side effects,” (p.92) (Scott et al., 2012). As such, the following section will address the neurological consequences of menopause that occurs well before the median age of 51.

2.4 Evidence that Premature Menopause Enhances the Risk of Neurological Disease

“Work by several groups has provided evidence that early menopause may negatively impact neurological health [See Table I for Summary]. For instance, the Mayo Clinic Cohort Study of Oophorectomy and Aging revealed that surgical induction of early menopause enhanced the risk of ischemic stroke, doubled the lifetime risk of dementia, and increased the risk of mortality from neurological disorders five-fold (Rocca et al., 2006, Rocca et al., 2007, Shuster et al., 2008, Rivera et al., 2009, Rocca et al., 2009, Rocca et al., 2010, Shuster et al., 2010, Rocca et al., 2011, Rocca et al., 2012). Intriguingly, these detrimental effects increased as the age of menopausal onset decreased. Likewise, the Nurses Health Study found that premenopausal oophorectomy was associated with significantly higher risks for cognitive impairment, dementia, depression, anxiety, stroke, and mortality (Parker et al., 2009, Parker, 2010, 2013). In addition, a recent publication, which combined data from two longitudinal studies
(Religious Orders Study and Rush Memory and Aging Project), also found that earlier age at surgical menopause was associated with faster decline in global cognition (episodic and semantic memory) and an increased presence of Alzheimer’s disease neuropathology (β-amyloid plaques) (Bove et al., 2013). Finally, a Danish cohort study of over 2 million women echoed the findings of American cohort studies and extended the results to include hysterectomy, suggesting that premenopausal hysterectomy and premenopausal hysterectomy with either unilateral or bilateral oophorectomy were all associated with a enhanced risk of developing early-onset dementia (Phung et al., 2010).

On the other hand, some observational studies failed to find any deleterious effects of premature menopause (Table I). For instance, the California Teachers Study (CTS) found that bilateral oophorectomy did not increase the risk of mortality from cancer, cardiovascular disease, or all-causes (Duan et al., 2012). In addition, the Women’s Health Initiative Observational Study (WHIOS) failed to find negative consequences of premenopausal oophorectomy on cardiovascular disease, stroke, hip fracture or total mortality (Jacoby et al., 2011). However, both studies had several caveats that may explain the lack of any negative effects. First, women with bilateral oophorectomy in the WHIOS study were an average age of 49 at the time of surgery and, therefore, cannot be characterized as either early or prematurely menopausal. In addition, these women were compared to hysterectomized women instead of women who had all of their reproductive organs intact. Since hysterectomy is thought to interfere with ovarian function (Siddle et al., 1987, Nahas et al., 2003, Xiangying et al., 2006), and since the aforementioned Danish cohort study found that hysterectomy alone was associated with a 38% increased risk of dementia (Phung et al., 2010, Rocca and Ulrich,
2012), using hysterectomized women as referents could potentially confound the results by making significant differences between groups smaller and harder to detect. Finally, both the CTS and the WHIOS suffered from a potentially insufficient length of follow-up (mean of 11.3 years and 7.6 years, respectively), which is significantly shorter than the 20- to 30-year follow-up periods utilized in the other observational studies described above (Rocca and Ulrich, 2012). It is thus possible that longer follow-up periods may be necessary to observe negative neurological consequences of premenopausal oophorectomy in the CTS and WHIOS studies. Since prolonged deprivation of ovarian-derived E2 is thought to play a key role in the enhanced risk of dementia and neurological disease in prematurely menopausal women, we will next discuss postmenopausal replacement of estrogen and its effects on neurological health,” (Scott et al., 2014).
### Table I. Surgical Menopause Cohort Studies and Neurological Outcomes.

<table>
<thead>
<tr>
<th>Study</th>
<th>Cohort Size</th>
<th>Type of Surgical Menopause</th>
<th>Average Age at Surgery (or Study Initiation)</th>
<th>Length of Follow-Up (Years)</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mayo Clinic Cohort of Oophorectomy and Aging</td>
<td>2,365</td>
<td>Bilateral Oophorectomy</td>
<td>44</td>
<td>25</td>
<td>↑ Risk of Dementia, Stroke, Parkinsonism, and Mortality from Neurological Disorders</td>
</tr>
<tr>
<td>Nurses Health Study</td>
<td>29,380</td>
<td>Bilateral Oophorectomy</td>
<td>43-47</td>
<td>24</td>
<td>↑ Risk of Dementia, Stroke, Depression, Anxiety, and Mortality</td>
</tr>
<tr>
<td>Religious Orders Study and Rush Memory and Aging Project</td>
<td>1,884</td>
<td>Not Available</td>
<td>42.7</td>
<td>18</td>
<td>↑ Cognitive Decline and Neural β-Amyloid Plaques</td>
</tr>
<tr>
<td>Danish Nationwide Cohort Study</td>
<td>2,313,838</td>
<td>Unilateral or Bilateral Oophorectomy ± Hysterectomy</td>
<td>40</td>
<td>Until Death or Diagnosis of Dementia</td>
<td>↑ Risk of Early-Onset Dementia in ALL Types of Surgical Menopause Occurring Before 50 Years of Age</td>
</tr>
<tr>
<td>California Teachers Study (CTS)</td>
<td>42,004</td>
<td>Bilateral Oophorectomy</td>
<td>&lt;45 and ≥45</td>
<td>11.3</td>
<td>No ↑ Risk of Cancer Mortality, Cardiovascular Mortality, or All-Cause Mortality for Either Group</td>
</tr>
<tr>
<td>Women’s Health Initiative Observational Study (WHIOS)</td>
<td>25,448</td>
<td>Bilateral Oophorectomy</td>
<td>49</td>
<td>7.6</td>
<td>No ↑ Risk of Cardiovascular Disease, Stroke, Hip Fracture, or All-Cause Mortality</td>
</tr>
</tbody>
</table>

Adapted from (Scott et al., 2014).
2.5 Postmenopausal Hormone Therapy

_Observational Studies with HT and Prevention of Dementia._ According to Scott et al. (2012), “serum E2 levels have been shown to be directly correlated with cognitive functioning in women, with high serum levels of E2 significantly enhancing verbal working memory (Rosenberg and Park, 2002, Sherwin, 2003). Furthermore, human hippocampal volumes have been demonstrated to be larger in postmenopausal women undergoing current treatment with hormone therapy (HT, any variety of estrogens ± progestogens) when compared to women who had used HT in the past, women who had never used HT, and men (Lord et al., 2008). Cognitive impairment is more prevalent in postmenopausal women who have low serum levels of E2, and several studies demonstrated that postmenopausal women who received some form of estrogens through HT performed better on neuropsychological tests of cognition (Sherwin, 2003). In fact, many promising observational studies showed a decreased risk or delayed onset of cognitive impairment and neurodegenerative diseases in women who used estrogens after menopause (Tang et al., 1996, Brann et al., 2007). A meta-analysis of 12 case-control and prospective cohort studies performed in the 1990s demonstrated a 29-44% reduction in the risk of Alzheimer’s disease development in postmenopausal women who received hormone therapy versus those who never took hormones (Paganini-Hill, 1996, Yaffe et al., 1998, Hogervorst et al., 2000). Specifically, ten of these 12 observational studies found a reduced risk of AD in users of estrogens versus nonusers (Brann et al., 2007). However, in most studies that initiated hormone therapy after symptoms of AD appeared, estrogens were an ineffective treatment for ameliorating cognitive decline, except in a few of the earliest cases (Henderson et al., 2000, Mulnard et al., 2000, Brann et al.,
Considering the positive cognitive effects of estrogens documented by these observational studies, the detrimental effects of LTED, and the possible preventative role of estrogens in postmenopausal dementia, it is no surprise that the next step was to conduct large, prospective clinical trials with HT.

*The WHI Study.* Since laboratory research in animals and observational studies in humans both suggested that estradiol was a neuroprotective hormone that could attenuate the increased risk of stroke and dementia associated with menopause, a large clinical trial of hormone therapy was conducted in postmenopausal women. This study, the Women’s Health Initiative (WHI), was a multi-center, randomized, placebo-controlled trial that enrolled over 16,000 postmenopausal women and looked at the effect of oral HT on the incidence of stroke. However, the WHI was stopped prematurely because instead of seeing the expected decrease in the number of stroke cases in the HT-treated women, conjugated equine estrogens (CEEs) plus medroxyprogesterone acetate (MPA) seemed to increase the risk for ischemic stroke in postmenopausal women (1.8% strokes and a hazard ratio (HR) of 1.44 in CEE plus MPA users versus 1.3% in placebo) (Wassertheil-Smoller et al., 2003). Furthermore, the Women’s Health Initiative Memory Study (WHIMS), which enrolled a subset of the same women from the WHI, saw an increased risk of dementia for women over the age of 65 who were treated with oral CEEs plus MPA (66% dementia, HR 2.05) versus placebo (34% dementia) (Shumaker et al., 2003). Finally, the WHIMS study also examined the effect of oral CEEs alone on cognition in the same women, and they found that the relative risk for having a significant decrease in cognition, measured by Modified Mini-Mental Status Examination score, was 1.47 in women treated with CEEs versus those treated with placebo (Espeland et al., 2004).
Taken together, these findings warned against correcting the E2 deficiency in postmenopausal women through HT, citing possible severe neurological repercussions.

However, several concerns were raised about the WHI studies with regard to statistical analysis, study design, and interpretations. A very basic criticism was the choice of statistics used to interpret the findings of the WHI, the hazard ratio or relative risk. First of all, the 99% confidence interval for the WHI’s hazard ratio for stroke included 1 (0.86-2.31), which is not statistically significant (Clark, 2006). Additionally, upon re-calculating the WHI data, the absolute risk for ischemic stroke was only 0.08%, which is highly unlikely to be statistically significant (Clark, 2006). Other experts also highlighted the fact that oral hormone use is common via oral contraceptives, and oral estrogens/progestogens are already known to incur an increased risk for blood clots and subsequent ischemic strokes, especially in women who smoke or are over the age of 35 (Harman et al., 2004, Klaiber et al., 2005). Therefore, a different route of hormone administration, such as transdermal application, could bypass the first-pass effect and make the formation of more reactive and harmful metabolites less likely in these older women (Harman et al., 2004, Klaiber et al., 2005, Menon and Vongpatanasin, 2006). Another suggestion was that 17β-estradiol (E2) should have been used instead of Premarin (conjugated equine estrogens or CEEs) since all previous positive laboratory results demonstrating the neuroprotective effects of estrogens used E2, not CEEs (Harman et al., 2004, Klaiber et al., 2005). They also posited that the progestogen used, Provera (medroxyprogesterone acetate, MPA) should have been administered in a cyclic fashion (regularly increasing and decreasing doses) to mimic the normal menstrual cycle, even if this might have reinstated menses as a side effect (Harman et al., 2004, Clark,
The final, and quite possibly the most important, criticism of the WHI was the average age of the menopausal women enrolled, 63.3 years. Since the median age of onset of natural menopause is 51, these women were more than a decade past the onset of menopause and had already been E2-deficient for an average of 12 years before the WHI began. Therefore, the negative findings of the WHI may not be applicable to women who are currently experiencing or have recently completed the menopausal transition (Harman et al., 2004, Santen et al., 2010),” (pp. 92-93) (Scott et al., 2012).

2.6 The Critical Period Hypothesis for Estrogen Benefit

The Critical Period Hypothesis and Healthy Cell Bias. Scott et al. (2012) also state, “Soon after the WHI results were published, Sherwin, Maki and others proposed the “critical period hypothesis,” which states that a precise window of opportunity exists for beneficial hormone therapy following menopause (Maki, 2006, Genazzani et al., 2007, Henderson, 2007, Sherwin, 2007, 2009),” (p.93). This hypothesis has also been referred to as the “timing hypothesis” or “critical window hypothesis,” and it proposes “that if hormone therapy is initiated soon after natural or surgical menopause, it may prevent cognitive decline (Maki, 2006, Genazzani et al., 2007, Henderson, 2007, Sherwin, 2007),” (p.93) (Scott et al., 2012). However, according to Scott et al. (2012), it also proposes that “if hormone therapy is initiated after a significant period of time has elapsed following menopause, outside the window of opportunity, then the beneficial effects of estradiol could be significantly attenuated (Sherwin, 2007). Brinton expanded upon this concept with the ‘healthy cell bias of E2 benefit,’ which suggested that E2 only yields neurological benefit if it is applied to healthy neurons (Brinton, 2005, 2008b, a).
Since the health of neurons tends to deteriorate with aging, the benefits of E2 exposure in postmenopausal women wanes as the time since onset of menopause increases, until E2 eventually becomes detrimental and able to exacerbate neurological injury (Brinton, 2005, 2008a, b). The ‘critical period hypothesis’ and the ‘healthy cell bias’ both could plausibly explain the surprisingly negative results of the WHI, which initiated hormone therapy in late postmenopausal women. In the sections below, we will review the basic science and clinical evidence for the critical period hypothesis.

Supporting Animal Studies. Abundant animal studies have been conducted that provide support for the critical period hypothesis, as well as mechanistic evidence for the neurological consequences of long-term E2 deprivation (LTED) (See Figure 4 for summary.) (Daniel and Bohacek, 2010, Gibbs, 2010, Moura and Petersen, 2010, Boulware et al., 2011).
Figure 4. Neurological Consequences of Long-Term Estradiol Deprivation. This figure summarizes the mechanisms currently thought to underlie the three major neurological consequences of long-term E2 deprivation (LTED): neurodegeneration, cognitive impairment, and loss of E2 neuroprotection. There may be some cross-talk between these major events that occur during LTED, as evidenced by connecting arrows. See text for more details. E2, 17β-estradiol; ERα, Estrogen Receptor Alpha; ATP, Adenosine Triphosphate. Adapted from (Scott et al., 2012).
In fact, long-term ovariectomy, a model of surgical menopause, in macaques led to a significant decline in hippocampal response to estrogens (Hamilton et al., 2011). Middle-aged female rats also demonstrated improvement in working memory, a hippocampal-dependent task, when treated with E2 immediately but not 5 months after bilateral ovariectomy (Daniel et al., 2006). Chronic E2 treatment was also found to enhance attention performance in middle-aged ovariectomized rats, and LTED before E2 treatment was found to significantly attenuate this effect (Bohacek and Daniel, 2010). Likewise, E2 replacement beginning up to 15 months post ovariectomy in young adult rats was found to increase long-term potentiation and dendritic spine density at hippocampal CA3-CA1 synapses, two processes which are critical for learning and memory (Smith et al., 2010). However, if E2 treatment was delayed 19 months after ovariectomy, E2’s beneficial effect on hippocampal synaptic physiology was lost, suggesting that a critical period exists for E2 replacement (Smith et al., 2010).

Furthermore, researchers saw that 5 months of E2 deprivation abolished E2’s ability to acutely regulate intrinsic membrane excitability in CA1 hippocampal neurons of rats (Wu et al., 2011b). In fact, the number of action potentials evoked and firing duration per each acute injection of E2 decreased in LTED neurons compared to control (Wu et al., 2011b).

Additionally, E2 was able to increase choline acetyltransferase activity, which is responsible for synthesizing the neurotransmitter acetylcholine, in both the hippocampus and pre-frontal cortex, two neural areas that are critical for memory (Bohacek et al., 2008). However, the same study showed that 5 months after ovariectomy E2 could no longer enhance choline acetyltransferase activity in the hippocampus (Bohacek et al., 2008). Intriguingly, donepezil, an acetyl cholinesterase inhibitor commonly used to treat
Alzheimer’s disease, was demonstrated to restore E2’s enhancement of cognition after LTED via bilateral ovariectomy at 3 months of age in middle-aged (12-17 months), but not aged (22-27 months), female rats (Gibbs et al., 2009). These last two studies are especially fascinating because in addition to providing support for the critical period hypothesis, they further suggest that maintaining a proper balance of acetylcholine in the basal forebrain may restore E2’s ability to enhance task-specific cognitive performance.

Furthermore, Brinton and colleagues provided evidence that LTED promotes a switch to ketogenic profile in the brain (Yao et al., 2010). Specifically, they observed that wild-type mice and transgenic mice that model Alzheimer’s disease both had significant decreases in activity of mitochondrial complexes responsible for oxidative phosphorylation and significant increases in enzymes required for fatty acid formation and ketone body metabolism (Yao et al., 2010, Hamilton et al., 2011). Since these phenomena are thought to contribute to Alzheimer’s disease in humans, this finding could mechanistically explain the increased risk of dementia seen in postmenopausal women (Yao et al., 2010). They also observed that LTED induced formation of mitochondrial β-amyloid and expression of mitochondrial β-amyloid-binding-alcohol-dehydrogenase, further connecting LTED with AD development (Yao et al., 2012). Importantly, this phenotype was prevented by replacement of E2 in ovariectomized mice, which suggests that HT may alleviate the increased risk of AD development in postmenopausal women if treatment is initiated close to the onset of menopause (Yao et al., 2012).

In further support of the critical period hypothesis, E2 was also found to exert profound neuroprotection against ischemic damage if it was replaced immediately but not
10 weeks after ovariectomy (Suzuki et al., 2007, Zhang et al., 2009a),” (pp. 93-94) (Scott et al., 2012). For example our lab observed that a period of LTED (10-week ovariectomy) in the rat was sufficient to cause loss of E2 signaling and neuroprotection from GCI in the hippocampal CA1. However, E2’s trophic effect on the uterus remained unchanged, suggesting that the observed E2 signaling deficit was brain-specific (Zhang et al., 2009a). Scott et al. (2012) state, “Additional work by our group showed that the loss of sensitivity to E2 neuroprotection following LTED was correlated with a significant decrease in ERα, but not ERβ, in the hippocampal CA1 region following LTED (Zhang et al., 2009a), which could explain the decreased hippocampal sensitivity to E2’s neuroprotective actions following LTED. The decrease of ERα in the hippocampal CA1 region after LTED was tissue-specific, as there was no significant decrease observed in the uterus. In follow-up studies, we sought to determine the mechanism underlying the decrease in ERα and to determine whether aging leads to a similar loss of hippocampal ERα and E2 sensitivity. The results of the study revealed that ERα in the rat hippocampal CA1 region, but not the uterus, undergoes enhanced interaction with the E3 ubiquitin ligase, CHIP (carboxyl terminus of Hsc70-interacting protein), which leads to ubiquitination and proteasomal degradation of ERα following LTED (Zhang et al., 2011). E2 treatment initiated before but not after LTED, prevented the enhanced ERα-CHIP interaction and ERα ubiquitination/degradation, and was fully neuroprotective against global cerebral ischemia (GCI). In addition, administration of a proteasomal inhibitor or CHIP antisense oligonucleotides reversed the LTED-induced down-regulation of ERα.
Further work showed that these observations extended to natural aging, since aged (24-month-old) female rats had decreased ERα in the hippocampal CA1 region compared to young (3-month-old) female rats, enhanced CHIP interaction with ERα, and ubiquitination and degradation of hippocampal ERα (Zhang et al., 2011). ERβ also demonstrated enhanced interaction with CHIP, ubiquitination and degradation in the hippocampal CA1 region of 24-month-old female rats. Importantly, these events in aged rats were correlated with loss of E2 neuroprotection against GCI. Interestingly, E2-treated aged (24 month old) rats actually had a 16% increase in mortality (e.g. worse outcome), as compared to placebo controls (Zhang et al., 2011). This is consistent with the WHI results in aged humans, where neurological outcome was worse after E2 treatment. However, of significant importance, E2 administration to young (3-month-old) and middle-aged (10-month-old) rats in our study yielded strong neuroprotection of the hippocampus against cerebral ischemia (Zhang et al., 2011),” (p.95) (Scott et al., 2012). As such, this study provides critical mechanistic support for a “critical period” for E2 neuroprotection in the hippocampus.

Finally, Scott et al. (2012) state, “an additional novel observation derived from our studies was dramatically enhanced hypersensitivity of the hippocampal CA3/CA4 region to ischemic injury and neuronal cell death following long-term E2 deprivation (Zhang et al., 2009a, Zhang et al., 2013a). Compared to the CA1 region, the CA3/CA4 region is normally resistant to ischemic insult and not significantly damaged during GCI. However, our work showed that after LTED, the CA3/CA4 region becomes extensively damaged by the same ischemic insult that caused little to no damage in animals that have not experienced LTED. This is intriguing because long-term ovariectomy (surgical
menopause) in humans has been correlated with an increased risk of cognitive decline, dementia and mortality from neurological disorders (Rocca et al., 2007, Rocca et al., 2009, Rocca et al., 2010, 2011), although the mechanisms underlying these effects have remained unclear. Our study may provide a mechanistic explanation for this increased risk by demonstrating a hypersensitivity of the hippocampal CA3/CA4 to injury after prolonged hypoestrogenicity. Sham animals showed no loss of CA3/CA4 neurons after LTED, suggesting that the ability of the CA3/CA4 to withstand stress following prolonged hypoestrogenicity is severely compromised, and that E2 deprivation itself, per se, does not cause loss of CA3/CA4 neurons. Of significant interest, E2 treatment begun after prolonged hypoestrogenicity did not prevent the induction of the CA3/CA4 hypersensitivity, which may be due to a loss of E2 sensitivity of this region, as was shown in the CA1 region. These findings suggest that LTED can increase sensitivity of the hippocampus to ischemic stress, leading to enhanced damage of hippocampal neurons,” (p. 96) (Scott et al., 2012). A similar hypersensitivity to stroke damage following reproductive senescence has been observed in the cerebral cortex as well (Selvamani and Sohrabji, 2010).

Intriguingly, in our most recent study of the CA3 region, surgically menopausal rats also demonstrated a profound hippocampal induction of Alzheimer’s disease (AD)-related proteins, increased amyloidogenesis, and worse cognitive outcome after ischemic stress (Zhang et al., 2013a). Further work showed that the hippocampal hypersensitivity extended to an AD-relevant insult, as hippocampal CA3 neurons were profoundly hypersensitive to the neurotoxic effects of amyloid-beta 1-42 (Aβ1-42), the most amyloidogenic form of the Aβ peptide. As such, enhanced sensitivity of the brain to
various stressors after premature and prolonged loss of ovarian-derived E2 is a plausible reason for the increased risk of neurological diseases and mortality from these disorders.

With respect to the molecular mechanisms underlying these phenomena, further work revealed that the hippocampal hypersensitivity, AD-related protein induction, and enhanced amyloidogenesis might depend on activation of the pro-apoptotic NADPH oxidase/superoxide/C-Jun N-terminal kinase pathway through both transcriptional and post-translational mechanisms (Zhang et al., 2013a). Brinton and colleagues have also provided compelling evidence that E2 has an additional critical role in sustaining the brain’s bioenergetic capacity by preserving glucose metabolism and mitochondrial function [for review, see (Rettberg et al., 2013)]. Their most recent research showed that ovariectomy in a pre-clinical model of AD was paralleled by a shift to neuronal utilization of ketone bodies as an energy source rather than the more common, and much more effective, energy source, glucose (Yao et al., 2010, Hamilton et al., 2011, Yao et al., 2012). This shift led to a concomitant decline in brain glucose transport and metabolism, and interestingly, occurred simultaneously with compromised oxidative phosphorylation, signs of mitochondrial oxidative stress and an accumulation of Aβ oligomers in the mitochondria (Yao and Brinton, 2012). Importantly, all of these effects were reversed either in part or in full by ET given immediately following ovariectomy (Yao et al., 2012, Ding et al., 2013). Conversely, they posit, if E2 is not replaced and the ketogenic utilization is allowed to continue, eventually fatty acids in myelinated axons may be oxidized and degraded to provide the brain with more alternative fuel, further compromising brain function through the destruction of white matter (Yao and Brinton, 2012). Collectively, the aforementioned basic science studies provide intriguing
mechanistic evidence for the critical period hypothesis, potentially explaining the unexpected surge in cases of stroke and dementia in late-postmenopausal women observed in the treatment arm of the WHI study. In addition, they also lend potential insight to the molecular mechanisms underlying the negative neurological sequelae seen in surgically menopausal women. The following section will review clinical studies that attempt to address one or more criticisms of the WHI, as well as clinical studies that attempt to test the validity of the critical period hypothesis.

Clinical Trials Addressing the Critical Window Hypothesis. According to Scott et al. (2012), “following the results of the WHI, a number of clinical trials were and are still being conducted in an attempt to test the critical period hypothesis and address the aforementioned concerns regarding the WHI study (See Table II for summary). The REMEMBER pilot study provided support for the critical period hypothesis by assessing cognitive function in 428 women aged 60 or older who initiated systemic hormone therapy within five years of natural or surgical menopause (hysterectomy plus bilateral oophorectomy) (MacLennan et al., 2006). Although, they recommended using a larger sample size in the future, the REMEMBER researchers found that women who initiated HT early performed significantly better on cognitive tests than women who initiated HT
Table II. Clinical HT Trials and Their Cardiovascular and Neurological Outcomes.

<table>
<thead>
<tr>
<th>Study Type / # of Subjects</th>
<th>Women Studied</th>
<th>Type of HT</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WHI</strong></td>
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<tr>
<td>RCT: 16,608</td>
<td>Late Menopausal</td>
<td>Oral CEEs +/- MPA</td>
<td>↑ Stroke</td>
</tr>
<tr>
<td><strong>WHIMS</strong></td>
<td></td>
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</tr>
<tr>
<td>RCT – Subsets of WHI: 7,340 total</td>
<td>Late Menopausal</td>
<td>Oral CEEs +/- MPA</td>
<td>CEEs + MPA ↑ dementia. CEEs alone ↓ cognition.</td>
</tr>
<tr>
<td><strong>WHIMS-MRI</strong></td>
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<tr>
<td>RCT - Subset of WHIMS: 1,403</td>
<td>Late Menopausal</td>
<td>Oral CEEs +/- MPA</td>
<td>NO ↑ in volume of ischemic brain lesions, but ↓ average volume of hippocampus and frontal lobe.</td>
</tr>
<tr>
<td><strong>WHISCA</strong></td>
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<tr>
<td>RCT - Subset of WHI/WHIMS: 2,302</td>
<td>Late Menopausal</td>
<td>Oral CEEs +/- MPA</td>
<td>CEEs + MPA ↓ verbal memory. CEEs alone ↓ spatial rotational ability.</td>
</tr>
<tr>
<td><strong>HERS and HERS II</strong></td>
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<tr>
<td>RCT: 2,763</td>
<td>Late Menopausal</td>
<td>Oral CEEs + MPA</td>
<td>HT ↑ VTE risk and does NOT ↓ CHD or ↑ cognition in women with existing CAD.</td>
</tr>
<tr>
<td><strong>WHI 10-year Follow-Up</strong></td>
<td></td>
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<tr>
<td>RCT: 10,739</td>
<td>Early and Late Menopausal</td>
<td>Oral CEEs Alone (No Progestogen)</td>
<td>NO ↑ risk of CHD, DVT, stroke or mortality. Early HT ↓ risk of CHD and MI.</td>
</tr>
<tr>
<td><strong>REMEMBER Pilot</strong></td>
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<td></td>
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<tr>
<td>Cohort: 428</td>
<td>&gt;60 Years of Age</td>
<td>Multiple</td>
<td>Early HT ↑ cognition. Late HT ↓ cognition.</td>
</tr>
<tr>
<td><strong>KPNC</strong></td>
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<tr>
<td>Cohort: 5,504</td>
<td>Early and Late Menopausal</td>
<td>Multiple</td>
<td>Early HT ↓ dementia. Late HT ↑ dementia.</td>
</tr>
<tr>
<td><strong>UK GPRD</strong></td>
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<tr>
<td>Nested Case-Control: 75,668</td>
<td>50-79 Years of Age</td>
<td>Multiple</td>
<td>Low-dose transdermal HT does NOT ↑ stroke, but High-dose and oral HT ↑ stroke.</td>
</tr>
<tr>
<td><strong>ESTHER</strong></td>
<td></td>
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<tr>
<td>Case Control: 881</td>
<td>Early and Late Menopausal</td>
<td>Oral CEEs and Transdermal E2</td>
<td>Oral HT and norpregnanes ↑ risk of VTE.</td>
</tr>
<tr>
<td><strong>E3N</strong></td>
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<tr>
<td>Cohort: 80,308</td>
<td>Early and Late Menopausal</td>
<td>Oral CEEs and Transdermal E2</td>
<td>Transdermal HT does NOT ↑ risk of VTE.</td>
</tr>
<tr>
<td>Study</td>
<td>Cohort</td>
<td>Menopause Type</td>
<td>Estrogen Type</td>
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<tr>
<td>WISE</td>
<td>654</td>
<td>Early and Late Naturally and Surgically Menopausal</td>
<td>Multiple</td>
</tr>
<tr>
<td>KEEPS</td>
<td>RCT</td>
<td>Early Menopausal</td>
<td>Oral CEEs and Transdermal E2</td>
</tr>
<tr>
<td>ELITE</td>
<td>RCT</td>
<td>Early and Late Menopausal</td>
<td>Oral and Transdermal E2</td>
</tr>
</tbody>
</table>

CAD, Coronary Artery Disease; CHD, Coronary Heart Disease; CEEs, Conjugated Equine Estrogens; DVT, Deep Vein Thrombosis; E2, 17β-Estradiol; MPA, Medroxyprogesterone Acetate; RCT, Randomized Placebo-Controlled Trial; VTE, Venous Thromboembolism. Adapted from (Scott et al., 2012).
late or never used HT at all, suggesting that timing of E2 replacement, with regard to menopause, is critical for neurological benefit (MacLennan et al., 2006). Along these lines, a larger population-based study was conducted to further assess the effect of HT timing on prevalence of dementia in postmenopausal women. Whitmer et al. found that, at least in middle-aged women using the Kaiser Permanente Medical Care Program of Northern California (KPNC) between 1964 and 1973, early menopausal HT was associated with a 26% decreased risk of developing dementia (Whitmer et al., 2011). However, in the same set of women, late menopausal HT was associated with a 48% increased risk of dementia (Whitmer et al., 2011), suggesting that a critical window exists for beneficial E2 replacement after menopause, with respect to prevention of dementia.

Dumas et al. conducted a small, randomized, placebo-controlled trial of oral E2 (17β-estradiol) to test the effect of E2 on cognition in younger (50-62) versus older (70-81) postmenopausal women (Dumas et al., 2008). Intriguingly, they showed that pre-treatment with oral E2 attenuated anti-cholinergic drug-induced deficits of episodic memory in the younger postmenopausal women but, conversely, further hindered the older postmenopausal women (Dumas et al., 2008). This critical observation provides supports that E2 may yield neurological benefit if administered within 10 years of menopause but may also exacerbate cognitive deficits if administered more than 20 years after the onset of menopause. Additionally, the Women’s Ischemia Syndrome Evaluation study (WISE) examined the timing of hormone therapy with respect to cardiovascular disease in postmenopausal women having coronary angiography performed during evaluation for ischemia. They found that naturally menopausal women who initiated HT
before the age of 55 had less severe coronary artery disease than never users, and this effect was not observed in naturally menopausal women who initiated HT at or after the age of 55 (Shufelt et al., 2011), another result consistent with the critical period hypothesis. Furthermore, WISE researchers suggested that these conclusions were only valid in the event that the HT user was healthy with little or no pre-existing coronary artery atherosclerosis, which is also consistent with the healthy cell bias of E2 replacement (Shufelt et al., 2011).

Along these lines, Rocca’s recent meta-analyses of clinical studies concerning HT and cognitive aging provided clinical evidence supporting the critical period hypothesis, since the majority of included clinical studies demonstrated neuroprotection in women who underwent treatment with estrogens perimenopausally (50-60 years of age) (Rocca et al., 2010, 2011). Conversely, these meta-analyses also revealed that studies on women who initiated hormone therapy in late menopause (65-79 years of age) demonstrated an increased risk of dementia and cognitive decline (Rocca et al., 2010, 2011). Considering this data, Rocca suggested that the neuroprotective effects of estrogens depend on age at initiation of HT, type of menopause (natural versus surgical) and stage of menopause, and he further recommended that women who experience premature menopause, either naturally or surgically via bilateral oophorectomy, should be treated as ideal candidates for HT until around the age of 51 (Rocca et al., 2011).

It is also important to note that recently, a 10-year follow-up of the WHI’s ‘estrogen-alone trial,’ which treated hysterectomized postmenopausal women with unopposed oral CEEs for a median of 5.9 years, was published. Intriguingly, the study showed no increase in the risk of cardiovascular disease, stroke, hip fracture, colon
cancer, or death and a decreased risk for breast cancer in these women either during or after treatment with oral CEEs (LaCroix et al., 2011). Furthermore, upon examination of results by age group, oral CEEs appeared to provide significant benefit to women aged 50-59. These hormone-treated perimenopausal women experienced a significantly decreased risk of coronary heart disease (approximately 41% decrease) (HR 0.59, 95% CI 0.38-0.90), myocardial infarction (approximately 46% decrease) (HR 0.54, 95% CI 0.34-0.86), and total mortality (HR 0.73, 95% CI 0.53-1.00) versus those treated with placebo (LaCroix et al., 2011). These benefits were not observed in older women aged 60-69 or 70-79. Perimenopausal hysterectomized women treated with unopposed oral CEEs also had relatively neutral risks for deep vein thrombosis, pulmonary embolism, stroke, invasive breast cancer, hip fracture, and colon cancer compared to placebo-treated women (LaCroix et al., 2011). Most importantly, the WHI ten-year follow-up study supports the critical period hypothesis, because the global index of chronic diseases was decreased in women aged 50-59 (HR 0.85, 95% CI 0.70-1.03), neutral in women aged 60-69 (HR 1.00, 95% CI 0.89-1.13) and elevated in women aged 70-79 (HR 1.15, 95% CI 1.01-1.32) (LaCroix et al., 2011). Therefore, the WHI’s estrogen alone trial suggests that HT may provide benefit in perimenopausal women aged 50-59, but this benefit may wane significantly as the time since the onset of menopause increases, until the risks of HT significantly outweigh the benefits.

Finally, there are several ongoing studies concerning the critical period hypothesis, the results of which the scientific community is eagerly awaiting. First is the KRONOS Early Estrogen Prevention Study (KEEPS), which is an ongoing multi-center, placebo-controlled, randomized clinical trial for HT that is restricted to women who are
within 3 years of menopause onset (Miller et al., 2009). In addition to testing the critical period hypothesis, KEEPS will also vary HT regimens to include either low-dose CEEs or transdermal 17β-estradiol (E2) opposed by oral, cyclic, micronized progesterone and measure cardiovascular disease progression via carotid artery intimal-medial thickness and coronary artery calcium build-up in postmenopausal women (Miller et al., 2009). Second is the Early Versus Late Intervention with Estradiol trial (ELITE), which is an ongoing clinical trial that aims to measure the progression of atherosclerosis in postmenopausal women. These researchers plan to focus on the timing of postmenopausal HT initiation, using oral E2 instead of CEEs, in women who are either less than 6 years (early) or greater than 10 years (late) from the onset of menopause (Menon and Vongpatanasin, 2006, Henderson and Brinton, 2010). It is hoped that the results of these two trials will provide more clarity with regard to the timing of HT initiation after menopause and its benefits.

While all of the aforementioned clinical trials focused on the timing of HT in postmenopausal women, other clinical trials attempted to address the remaining concerns of the WHI, such as the type of estrogen/progestogen included and the route of HT administration. The ESTHER study was a multi-center case-control study that measured occurrence of venous thromboembolism (VTE), a pro-thrombotic disease that could contribute to ischemic strokes, in women using either oral or transdermal HT. They found that users of oral estrogens were 4-times more likely to have a VTE than HT nonusers (OR 4.2; 95% CI 1.5-11.6), whereas transdermal E2 users did not demonstrate any increased risk for VTE, compared to nonusers (OR 0.9; 95% CI 0.4-2.1) (Canonico et al., 2007). These data were confirmed in the E3N study, which followed a huge prospective
cohort of menopausal French women for an average of 10 years, documenting HT use and the incidence of first idiopathic venous thromboembolism. The E3N study found that past HT users did not incur a significantly increased risk of VTE compared to never-users (Canonico et al., 2010). They also demonstrated that oral CEEs were associated with an increased risk of VTE, but this increased risk was not observed when transdermal E2 was used instead of oral CEEs (Canonico et al., 2010). Finally, both the ESTHER study and the E3N determined that oral norpregnanes, but not progesterone, pregnanes, or nortestosterones were associated with an increased risk of VTE (Canonico et al., 2007, Canonico et al., 2010). Together, these results could explain why the WHI, which used oral CEEs in late postmenopausal women, saw an increased risk of ischemic stroke and dementia,” (pp. 96-98) (Scott et al., 2012).

2.7 The Canonical Wnt/β-Catenin Signaling Pathway

The first aim of this dissertation focuses on the effect of premature surgical menopause on CA1 hippocampal expression of the Wnt antagonist Dkk1 and E2 regulation status of this neurodegenerative protein following GCI. As such, we will briefly review canonical Wnt/β-Catenin signaling here. In the following section, we will also discuss Dkk1’s association with neurodegenerative disease. Scott and Brann (2013) state, “Wnt is a secreted glycoprotein whose gene was separately discovered in mouse mammary tumors (int-1) and in Drosophila melanogaster (wingless), but due to sequence homology, both int-1 and wingless were determined to encode the same proto-oncogene, which was dubbed Wnt (McMahon and Moon, 1989b, a, Thomas and Capecchi, 1990). The canonical Wnt signaling pathway has since been deemed critical for several
embryonic events, including cell proliferation, cell polarity, and determination of cell fate (Logan and Nusse, 2004, MacDonald et al., 2009). Canonical Wnt/β-Catenin signaling has also been implicated in development of the limbs (Mukhopadhyay et al., 2001, Grotewold and Ruther, 2002), neural tube (Roelink and Nusse, 1991, De Marco et al., 2012), forebrain (Mukhopadhyay et al., 2001), midbrain and cerebellum (McMahon and Bradley, 1990, Thomas and Capecchi, 1990) and in the maintenance of neurotransmission and synaptic plasticity (Speese and Budnik, 2007, Ataman et al., 2008, Avila et al., 2010, Budnik and Salinas, 2011, Jensen et al., 2012). In light of this knowledge, it is not surprising that, in addition to tumorigenesis, mutations that enhance Wnt signaling in humans have been linked to neurological disorders, such as autism, schizophrenia, and bipolar disorder (De Ferrari and Moon, 2006).

Wnt initiates an intracellular signaling cascade when it binds to its cognate membrane receptor, frizzled (Fz), and its co-receptor, low density lipoprotein-related protein 5/6 (LRP5/6) (Logan and Nusse, 2004, MacDonald et al., 2009) (See Figure 5 for Summary). The canonical Wnt signaling cascade, which ultimately determines intracellular levels of the transcriptional activator β-Catenin, begins with recruitment of
Figure 5. Summary of the Canonical Wnt Signaling Pathway. This figure briefly summarizes canonical Wnt signaling. **A)** In the absence of Wnt ligand, cytosolic β-Catenin is phosphorylated, ubiquitinated, and degraded by the proteasome to prevent the expression of Wnt target genes. **B)** In the presence of Wnt ligand, cytosolic β-Catenin is stabilized and translocates to the nucleus, where it acts as a co-activator and facilitates the transcription of Wnt target genes. Dkk1 antagonizes canonical Wnt signaling by binding to the Wnt co-receptor LRP5/6 and preventing formation of the Wnt-Frizzled-LRP5/6 complex. APC, adenomatous polyposis coli; β-Cat, β-Catenin; β-TrCP, Beta-Transducing repeat-Containing Protein; Dvl, Disheveled; CKI, Casein Kinase I; Dkk1, Dickkopf-1; GSK3β, Glycogen Synthase Kinase 3β; LRP5/6, Low Density Lipoprotein-Related Protein 5/6; TCF/LEF, T Cell Factor/Lymphoid Enhancing Factor; Ub, Ubiquitin; Wnt, Wingless. (Scott and Brann, 2013)
disheveled (Dvl) to Fz and dual phosphorylation of LRP5/6 by glycogen synthase kinase 3β (GSK3β) and casein kinase I (CKI) (MacDonald et al., 2009). It is important to note that Wnt is also capable of signaling through non-canonical signaling pathways, which are independent of β-Catenin, but these are beyond the scope of this review. As such, the reader is referred to these excellent reviews on the subject (Seifert and Mlodzik, 2007, Wang and Nathans, 2007, Komiya and Habas, 2008, Clark et al., 2012). Intriguingly, GSK3β and CKI are both components of the Axin-adenomatous polyposis coli (APC) complex, which is devoted to the phosphorylation of the transcriptional activator β-Catenin (Logan and Nusse, 2004, MacDonald et al., 2009). As such, in the absence of Wnt ligand, the Axin-APC complex continually phosphorylates cytosolic β-Catenin, priming it for ubiquitination by the E3 ubiquitin ligase Beta-Transducing repeat-Containing Protein (β-TrCP) and subsequent proteasomal degradation in order to prevent expression of Wnt target genes.

However, once Wnt signaling is initiated, GSK3β and CKI are recruited away from the Axin-APC complex to phosphorylate the co-receptor LRP5/6, and once doubly phosphorylated, LRP5/6 becomes a docking site for Axin (MacDonald et al., 2009). These events lead to the temporary disassembly of the Axin-APC complex and the subsequent stabilization of cytosolic β-Catenin, which remains untagged by either phosphate or ubiquitin (Logan and Nusse, 2004, MacDonald et al., 2009). Thus, the canonical Wnt signaling cascade ultimately ends in the elevation, stabilization, and nuclear translocation of cytosolic β-Catenin. Once inside the nucleus, β-Catenin, as a transcriptional activator, is able to convert the T-cell Factor/Lymphoid Enhancing Factor (TCF/LEF) transcriptional repression machinery into an active, mRNA transcription
complex, which then promotes the expression of Wnt target genes, characterized by the presence of a consensus sequence called a Wnt response element (WRE) in their promoter regions (Logan and Nusse, 2004, MacDonald et al., 2009), “(p. 64) (Scott and Brann, 2013).

2.8 Dkk1 and Neurodegenerative Disease

According to Scott and Brann (2013), “Several endogenous ligands, such as Shisa, Wnt inhibitory factor-1 (WIF-1), the secreted Frizzled related proteins (sFRPs), the WISE/SOST family, and the Dickkopf (Dkk) family, can serve as antagonists of canonical Wnt signaling (MacDonald et al., 2009). Arguably, the most important Wnt signaling antagonist is the prototypical Dkk family member Dkk1, which antagonizes Wnt signaling by binding to the LRP5/6 co-receptor and preventing Wnt from forming a signaling complex with Fz and LRP5/6 (Fedi et al., 1999, Wu et al., 2000, Bafico et al., 2001, Mao et al., 2001, Semenov et al., 2001). Similar to Wnt, Dkk1 expression is critical for neurodevelopment during the embryonic period. In fact, Dkk1 is known as a “head-inducer,” as its presence and antagonism of Wnt signaling is required for structures anterior of the midbrain to form (Glinka et al., 1998, Kazanskaya et al., 2000, Mukhopadhyay et al., 2001, Semenov et al., 2001). Importantly, Dkk1 is also responsible for orchestrating the apoptosis necessary for proper limb development (Mukhopadhyay et al., 2001, Grotewold and Ruther, 2002). Along these lines, Dkk1 null mice are not viable, with embryos lacking structures anterior of the midbrain and demonstrating limb polysyndactyly (Mukhopadhyay et al., 2001). Furthermore, doubleridge mice, which have hypomorphic expression of Dkk1, are viable, but they display hemivertebrae fusions
and polysyndactyly of the forelimbs, a phenotype that can be ameliorated by reducing the expression of LRP5/6 (MacDonald et al., 2004).

While transgenic mouse models have overwhelmingly demonstrated that Dkk1 expression is crucial during neurodevelopment, elevation of Dkk1 later in life can be detrimental. In fact, many studies have linked elevated Dkk1 expression in the adult brain to neurodegenerative diseases, such as stroke, Alzheimer’s disease, Parkinson’s disease, and temporal lobe epilepsy (See Table III for Summary). Excitotoxicity has relevance to neurodegenerative disease because stroke leads to neuronal cell death, in part, due to excess release of the excitatory neurotransmitter glutamate, which subsequently leads to NMDA receptor activation and intracellular calcium overload (Choi, 1994a, b, Zipfel et al., 2000). In vitro studies demonstrated that Dkk1 is induced in cultured cortical neurons following an excitotoxic pulse of NMDA and is also capable of potentiating NMDA neurotoxicity in a dose-dependent manner (Cappuccio et al., 2005). Further work confirmed that Dkk1 is, in fact, able to inhibit canonical Wnt signaling and initiate cell death in cultured cortical neurons, which was associated with loss of Bcl-2, induction of Bax, and hyperphosphorylation of the microtubule associated protein tau (Scali et al., 2006). Importantly, the same studies confirmed that these observations are relevant to ischemic insults in vivo, as hippocampal Dkk1 was induced following global cerebral ischemia in both gerbils and rats, and stereotaxic injection of recombinant Dkk1 into either the hippocampal CA1 region or nucleus basalis magnocellularis was sufficient to cause neuronal cell death (Cappuccio et al., 2005, Scali et al., 2006). Intriguingly, intracerebroventricular injection of Dkk1 anti-sense oligonucleotides attenuated the ischemia-induced cell death observed in gerbils, and intraperitoneal administration of
lithium chloride, which rescues canonical Wnt signaling by inhibiting GSK3β, also attenuated the ischemia-induced cell death observed in rats (Cappuccio et al., 2005).
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Aβ, β-Amyloid; Dkk1, Dickkopf-1; GCI, Global Cerebral Ischemia; ET-1, Endothelin-1; MCAO, Middle Cerebral Artery Occlusion; MPP+, 1-methyl-4-phenylpyridinium; NMDA, N-Methyl-D-Aspartate; 6-OHDA, 6-Hydroxydopamine. Adapted from (Scott and Brann, 2013).
A later study demonstrated that neural Dkk1 is also induced in animal models of focal cerebral ischemia (local endothelin-1 infusion and permanent middle cerebral artery occlusion [MCAO]) and reiterated that administration of lithium ions was neuroprotective in rodents (Mastroiacovo et al., 2009). The same authors also performed MCAO in doubleridge mice, which have reduced expression of Dkk1, and noted a significant reduction in cortical infarct volume (Mastroiacovo et al., 2009). As such, these studies demonstrate the importance of the Wnt antagonist Dkk1 in the pathophysiology of cerebral ischemia and suggest that Dkk1 antagonists and/or Wnt agonists may be effective treatments for stroke. Finally, a recent study associated elevated circulating Dkk1 levels with acute ischemic stroke (<24 hours) in humans. While there was no relationship between Dkk1 and stroke severity or outcome, the authors found that plasma levels of Dkk1 were significantly higher in patients presenting with acute ischemic stroke versus healthy controls or patients with clinically stable cerebrovascular disease (Seifert-Held et al., 2011). Intriguingly, this study is in agreement with findings by Kim et al., which suggested that Dkk1 was elevated in the plasma of patients with coronary atherosclerotic plaques, even if they demonstrated low Agatston calcium scores (Kim et al., 2011). Thus, in addition to being a plausible therapeutic target for stroke, the Wnt antagonist Dkk1 may also be a promising biomarker for cardiovascular and/or cerebrovascular disease.

Several studies have also implicated dysregulation of Dkk1 and Wnt/β-Catenin signaling in Alzheimer’s disease (AD), both in familial/early-onset AD and in sporadic/late-onset AD [For review, see (De Ferrari and Moon, 2006) and (Boonen et al., 2009)]. In regard to Dkk1, Caricasole and colleagues noted that the beta-amyloid peptide
induced expression of Dkk1, hyperphosphorylation of tau, and cell death in cultured
cortical neurons (Caricasole et al., 2004). Furthermore, they showed that anti-sense
knockdown of Dkk1 in vitro attenuated beta amyloid neurotoxicity and prevented the
hyperphosphorylation of tau, which forms neurofibrillary tangles, one of the
neuropathological hallmarks of AD (Caricasole et al., 2004). Along these lines, the same
authors also observed enhanced Dkk1 expression in neurons from post-mortem human
AD specimens, which consistently co-localized with neurofibrillary tangles of
hyperphosphorylated tau (Caricasole et al., 2004), suggesting that Dkk1 may play an
important role in human AD neuropathology. A subsequent study revealed that Dkk1 was
also upregulated in transgenic mouse models of AD and fronto-temporal dementia (Rosi
et al., 2010). In particular, Rosi et al. noted that Dkk1 was significantly increased in brain
regions affected by the respective neurodegenerative disease and co-localized with
neurons containing neurofibrillary tangles, similar to what is seen in humans (Rosi et al.,
2010). Additionally, in the TgCRND8 mouse model of AD, Dkk1 was expressed in
choline acetyltransferase-positive neurons of the basal forebrain, neurons thought to be
primarily affected by AD, and in neurons adjacent to beta-amyloid deposits (Rosi et al.,
2010). Recent work also demonstrated that acute treatment with oligomeric beta-amyloid
enhanced Dkk1 expression and led to a loss of synapses, which occurs early in the
pathophysiology of AD and may facilitate cognitive impairment (Purro et al., 2012). The
authors further showed that brief exposure to Dkk1, through the inhibition of Wnt
signaling, decreased the size of both presynaptic and postsynaptic terminals in mature
neurons without affecting cell viability and disassembled synapses within hours by
inducing the release of synaptic vesicles (Purro et al., 2012). Intriguingly, they also
showed that antibodies capable of neutralizing Dkk1 suppressed the aforementioned synapse loss in mouse hippocampal slices (Purro et al., 2012). As such, these results support the idea that Dkk1 could be responsible for synaptic loss in the early stages of AD and further suggest that Dkk1 may serve a feasible target for the treatment of AD. It is important to mention that while no conclusive evidence has been provided, Dkk1 was identified in two screens for late-onset AD susceptibility genes (Morgan et al., 2007, Morgan et al., 2008). Furthermore, reduced Wnt/β-Catenin signaling has already been associated with genetic susceptibility to late-onset AD. Along these lines, a common variant of the LRP6 co-receptor (Val-1062), which has reduced β-Catenin signaling \textit{in vitro}, was shown to interact with apolipoprotein E-epsilon4 (APOE-ε4) carrier status to form a risk haplotype for AD (De Ferrari et al., 2007). These results are in agreement with several studies implicating reduced Wnt signaling and activation of GSK3β, a kinase downstream of Dkk1 that is known to phosphorylate the microtubule associated protein tau, with various neurodegenerative diseases [See (Lei et al., 2011) for Review] and AD, in particular (Rockenstein et al., 2007, Forlenza et al., 2011).

Intriguingly, Dkk1 has also been implicated in two different animal models of Parkinson’s disease. L’Episcopo et al. demonstrated that reactive astrocytes may afford neuroprotection against 1-methyl-4-phenylpyridinium (MPP⁺) neurotoxicity by upregulating Wnt1 in the ventral midbrain and striatum \textit{in vivo} (L'Episcopo et al., 2011). Importantly, the same authors also noted that blocking canonical Wnt signaling with Dkk1 prevented astrocyte-induced neuroprotection \textit{in vitro} (L'Episcopo et al., 2011). Furthermore, Dkk1 was found to be upregulated in rats after stereotaxic administration of the selective dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA), and
administration of Dkk1 was found to potentiate the neurotoxicity of 6-OHDA in the substantia nigra in vivo (Dun et al., 2012). As such, these recent studies suggest that Dkk1 may play a role in the pathobiology of Parkinson’s disease and warrant further study in the human condition. Finally, a single study linked Dkk1 expression and subsequent Wnt inhibition to neurodegeneration caused by temporal lobe epilepsy. Busceti et al. demonstrated that Dkk1 was elevated in rat olfactory and hippocampal neurons following systemic administration of kainate, a compound known to induce seizure activity (Busceti et al., 2007). Intriguingly, Dkk1 was only induced in rats that were characterized as “high responders” to kainate, displayed reduced levels of nuclear β-Catenin, and experienced neuronal cell death following seizures. Furthermore, the researchers showed that either knockdown of Dkk1 or pre-treatment with lithium ions was sufficient to reduce kainate-induced cell death (Busceti et al., 2007). Importantly, Dkk1 was also strongly expressed in brain biopsies from patients with mesial temporal lobe epilepsy and hippocampal sclerosis, further implicating Dkk1 in the neurodegenerative processes associated with this disorder (Busceti et al., 2007). Thus, as a whole, these studies suggest that elevations in the Wnt antagonist Dkk1 in adulthood and subsequent reductions in canonical Wnt signaling are associated with neurodegenerative disease,” (pp. 64-67) (Scott and Brann, 2013). The next section will briefly review E2’s neuroprotective regulation of Dkk1 and Wnt/β-Catenin signaling.

2.9 Estrogen Regulation of Dkk1 and Wnt/β-Catenin Signaling

Intriguingly, E2 is not only capable of attenuating neuronal damage associated with neurodegenerative diseases, but it can also promote a healthy balance of neural
Dkk1 and canonical Wnt signaling. In particular, our laboratory discovered that one mechanism through which E2 prevents GCI-induced apoptosis is through suppressing the post-ischemic elevation of Dkk1 and facilitating pro-survival Wnt/β-Catenin signaling in the CA1 region of the hippocampus (Zhang et al., 2008). Specifically, we demonstrated that Dkk1 was significantly upregulated 24 and 48 hours following GCI and that one week of pretreatment with subcutaneous, low dose E2 prior to ischemia prevented this elevation at both reperfusion time points (Zhang et al., 2008). Additionally, we showed that Dkk1 immunostaining was co-localized with the neuronal nuclear marker NeuN, which suggested that Dkk1 expression was primarily neuronal 24 hours following GCI (Zhang et al., 2008). In the same study, we also measured canonical Wnt signaling in the hippocampal CA1 in the event of GCI and low dose E2 pretreatment through examining protein levels of Wnt3, nuclear β-Catenin, phospho-β-Catenin, and the Wnt pathway product Survivin. Upon treatment with E2, in addition to enhanced hippocampal expression of Wnt3 and nuclear β-Catenin, we saw that E2 not only prevented GCI-induced loss of Survivin but also enhanced neuronal expression of Survivin, a Wnt product that prevents cleavage of pro-apoptotic caspases to promote cell survival, 24 and 48 hours following GCI (Zhang et al., 2008). Since E2 suppressed hippocampal expression of the Wnt antagonist Dkk1 at these same reperfusion time points, these data suggest that E2 maintains a healthy balance of hippocampal Dkk1 and Wnt/β-Catenin signaling in the event of ischemic stress. Finally, it is important to mention that the same study demonstrated that E2’s suppression of post-ischemic Dkk1 expression is required for its ability to exert neuroprotection, as stereotaxic injection of Dkk1 into E2-treated animals was sufficient to prevent E2 neuroprotection (Zhang et al., 2008).
According to Scott and Brann (2013), “While our lab demonstrated that pre-treatment with low-dose E2 increased expression of Wnt3 in CA1 hippocampal neurons 24 and 48 hours following GCI and led to increased expression of Survivin, a Wnt target gene, E2 regulation of β-Catenin-dependent transcription may also occur independently of canonical Wnt signaling. However, regardless of the mechanism involved, the evidence overwhelmingly suggests that E2 stabilizes β-Catenin via inhibition of GSK3β [See (Varea et al., 2010) for review]. In fact, E2 effectively increases the amount of inactive GSK3β in neuronal cells in vitro (Cardona-Gomez et al., 2004, Varea et al., 2009), and E2 treatment protects hippocampal slice cultures from kainate-induced neurotoxicity via rapid, ER-mediated phosphorylation and inactivation of GSK3β at Serine 9 (Goodenough et al., 2005). Cardona-Gomez et al. further demonstrated that acute E2 treatment increased the amount of inactivated GSK3β in the rat hippocampus, which subsequently prevented the hyperphosphorylation of tau (Cardona-Gomez et al., 2004). This is consistent with our previous study, which showed that E2 increased the amount of inactive GSK3β at 24 and 48 hours following GCI and led to the stabilization of β-Catenin and the prevention of tau hyperphosphorylation at these same time points (Zhang et al., 2008). Varea et al. further demonstrated that estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) both directly interact with GSK3β and β-Catenin in vitro and that E2, PPT (an ERα agonist), and DPN (an ERβ agonist) were all capable of inducing β-Catenin-mediated transcription through the TCF/LEF-1 family of transcription factors in primary cortical neurons and neuroblastoma cells (Varea et al., 2009). Importantly, they observed that the ER antagonist ICI 182780 essentially blocked E2’s induction of TCF/LEF-1-mediated transcription, further suggesting that E2’s
stabilization of β-Catenin requires the estrogen receptor, and they noted that PPT was more effective than DPN, suggesting that while both ERα and ERβ can mediate E2’s effect on β-Catenin-mediated transcription in the brain, E2 may be acting primarily through ERα to upregulate TCF/LEF-1-mediated transcription in vitro (Varea et al., 2009). Finally, two independent studies suggested that E2-induced β-Catenin-mediated transcription requires LEF-1 and activates a set of genes that is similar, but not identical to, that activated by canonical Wnt signaling (Varea et al., 2009, Wandosell et al., 2012). These observations further promote the idea that, in addition to regulating the canonical Wnt signaling pathway, E2 can act independently of the canonical Wnt signaling pathway to enhance β-Catenin-mediated transcription in neural cells.

Recently, it has been determined that hypothalamic β-Catenin expression naturally fluctuates across the estrous cycle in rats, with elevations of β-Catenin observed during proestrus and estrus, two days characterized by high, circulating levels of E2 (Barrera-Ocampo et al., 2012). Intriguingly, the peaks in hypothalamic β-Catenin expression were concomitant with increases of activated Akt and inactivated GSK3β, suggesting that these two events are critical for E2-induced stabilization of β-Catenin in the brain (Barrera-Ocampo et al., 2012). E2 is well known to activate the neuroprotective phosphatidylinositol 3 kinase (PI3K)-Akt signaling pathway, either by itself or in concert with IGF-1 signaling, leading to the activation of the serine/threonine kinase Akt through phosphorylation at Serine 473 (Burgering and Coffer, 1995, Singh, 2001, Mendez et al., 2003, Brann et al., 2007, Varea et al., 2010). Importantly, activated Akt has also been shown to inhibit GSK3β via phosphorylation at Serine 9 (Cross et al., 1995), and Mendez et al. demonstrated that blockade of the PI3K-Akt pathway with wortmannin, a specific
PI3K inhibitor, decreased the amount of cytosolic β-Catenin in neuronal cultures (Mendez and Garcia-Segura, 2006). In light of this knowledge, Varea et al. propose a novel, additional mechanism for E2’s regulation of β-Catenin-mediated transcription, where E2 binds to membrane-bound ERα and rapidly activates the neuroprotective PI3K-Akt pathway, possibly through interaction with IGF-1 signaling components. A multi-molecular complex is then formed consisting of ERα, GSK3β, β-Catenin, and others (Varea et al., 2009, Varea et al., 2010). Once activated via phosphorylation at Serine 473, Akt then inactivates GSK3β, which leads to the subsequent stabilization and nuclear retention of cytosolic β-Catenin. Inside the nucleus, β-Catenin then interacts with the TCF/LEF-1 transcription machinery to promote the expression of target genes that are independent of Wnt (Varea et al., 2009, Varea et al., 2010, Wandosell et al., 2012). Thus, it is plausible that E2 may maintain the delicate balance of Dkk1 and Wnt/β-Catenin signaling in the adult brain in three different ways: 1) by suppressing neuronal expression of the neurodegenerative Wnt antagonist Dkk1 2) by enhancing Wnt3 expression and subsequently facilitating canonical Wnt/β-Catenin signaling in neurons, and 3) by promoting Wnt-independent, β-Catenin-mediated transcription through a membrane ER-initiated intracellular signaling cascade involving PI3K/Akt/GSK3β (Figure 6),” (pp. 69-70) (Scott and Brann, 2013).
Figure 6. Summary of Estrogen Regulation of Dkk1 and Wnt/β-Catenin Signaling.

This figure depicts three currently proposed mechanisms for 17β-estradiol’s regulation of Dkk1 and Wnt/β-Catenin signaling in the brain. 1) E2 can suppress expression of the neurodegenerative Wnt antagonist Dkk1 in the hippocampus, particularly following an ischemic insult. 2) E2 can enhance canonical Wnt signaling in the hippocampus, particularly following an ischemic insult, through induction of Wnt3, which leads to
expression of pro-survival Wnt target genes, such as Survivin, through β-Catenin and TCF. 3) E2, either acting alone or in concert with IGF-1, can initiate a membrane receptor-mediated PI3K/Akt/GSK3β signaling cascade, which leads to the stabilization of cytosolic β-Catenin and Wnt-independent gene expression through β-Catenin and LEF-1. Akt, A Serine/Threonine Kinase (also known as Protein Kinase B); β-Cat, Beta-Catenin; CKI, Casein Kinase 1; Dkk1, Dickkopf-1; Dvl, Disheveled; E2, 17β-Estradiol; ER, Estrogen Receptor; GSK3β, Glycogen Synthase Kinase 3 Beta; IGF-I, Insulin-like Growth Factor-1; IGF-IR, Insulin-like Growth Factor-1 Receptor; IRS-1, Insulin Receptor Substrate 1; LEF-1, Lymphoid Enhancing Factor 1; LRP 5/6, Low-Density Lipoprotein 5/6; PI3K, Phosphatidylinositol 3 Kinase; TCF, T Cell Factor; Wnt, Wingless. (Scott and Brann, 2013)
2.10 Alzheimer’s disease, APP Processing, and Estrogen Regulation

The second aim of this dissertation focuses on the effect of premature surgical menopause on hippocampal amyloidogenesis and E2 regulation of this event following GCI. As such, we will briefly review Alzheimer’s disease, amyloidogenesis, and estrogen regulation here. Alzheimer’s disease was first described by Alois Alzheimer in 1906 (Ramirez-Bermudez, 2012). It is the most common form of dementia and is characterized by the neural accumulation of two neuropathological hallmarks: senile plaques of β-Amyloid (Aβ) and neurofibrillary tangles (NFTs) of hyperphosphorylated tau (Ramirez-Bermudez, 2012). As such, excess deposits of Aβ and NFTs are neurotoxic, causing extensive synapse loss and neurodegeneration, as well as an irreversible cascade of progressive memory loss, psychological disturbances, motor dysfunction, and eventually, death (Defina et al., 2013). There are two types of Alzheimer’s disease (AD): early-onset or familial AD and late-onset or sporadic AD. Early-onset AD, which accounts for approximately 5% of AD cases and usually appears before the age of 65, has a strong genetic component and is most often caused by mutations in genes encoding proteins involved in the synthesis of Aβ, such as amyloid precursor protein (APP), presenilin 1, or presenilin 2 (Defina et al., 2013). On the other hand, late-onset AD typically begins after 65 and, aside from loose associations with genetic variants, such as the epsilon 4 version of the ApoE allele, is largely unrelated to one’s genetic make-up (Defina et al., 2013). Unfortunately, over 5.3 million people currently suffer from this debilitating disease in the U.S., and to date, no effective treatment exists (Defina et al., 2013).

The amount of Aβ present in the brain is largely dependent on the processing of APP, a Type I transmembrane protein that, similarly to Notch, is sequentially cleaved by
enzymes to create intracellular and extracellular fragments (Willnow et al., 2008, De Strooper et al., 2010, Zheng and Koo, 2011). APP has two main processing pathways: non-amyloidogenic and amyloidogenic (See Figure 7 for summary). During non-amyloidogenic processing, APP is sequentially cleaved by an α-secretase, such as ADAM 10 or ADAM 17, followed by gamma secretase, an enzyme complex composed of presenilins 1 and 2, nicastrin, anterior pharynx-defective (APH-1), and presenilin enhancer protein (PEN-2) (De Strooper et al., 2010, Zheng and Koo, 2011). As such, non-amyloidogenic APP processing precludes formation of Aβ and creates three non-toxic fragments: soluble APPα, which is thought to be neuroprotective, p3, a benign, truncated form of Aβ, and APP intracellular domain (AICD), which is purported to travel to the nucleus and modulate gene expression (Willnow et al., 2008, Zheng and Koo, 2011). Conversely, during amyloidogenic processing, APP is cleaved by the β-secretase β-amyloid cleaving enzyme 1 (BACE1) before being cleaved by the gamma secretase machinery. This results in the formation of soluble APPβ, AICD, and the insoluble, neurotoxic 40-42 amino acid Aβ protein. If not successfully cleared from the brain, Aβ monomers form oligomers that then aggregate into extracellular deposits termed senile plaques (Zheng and Koo, 2011).
Figure 7. “Pathways in the Processing and Signaling of Amyloid Precursor Protein.

The inset depicts the alternative processing routes for amyloid precursor protein (APP): sequential cleavage by α- and γ-secretases (non-amyloidogenic pathway; to the right) or by β- and γ-secretases (amyloidogenic pathway; to the left). Functions proposed for APP and its various processing products are shown. APP has a role in cell adhesion, whereas soluble (s) APPα is thought to cooperate with the epidermal growth factor (EGF) receptor pathway to stimulate neuronal proliferation through extracellular signal-regulated kinase (ERK) and AKT. In addition, sAPPα, directly or indirectly, activates high-conduction K+ channels (KCN) and cGMP-dependent protein kinase (PKG) to protect neurons from excitotoxicity. The amyloid-β (Aβ) peptide modulates synaptic transmission, triggers cell death by binding to p75NTR and forms neurotoxic oligomers and plaques. The cytoplasmic tail of full-length APP, as well as the membrane-associated
carboxyl-terminal fragments C99 and C83, interact with multiple adaptors and signaling molecules that are involved in cellular differentiation pathways (for example, SHCA, JIP, X11 and DAB1). Finally, the soluble intracellular domain of APP (AICD) can act as a transcriptional regulator by associating with the adaptor FE65 and the histone acetyltransferase TIP60 in the nucleus,” (p. 905) (Willnow et al., 2008).
Intriguingly, E2 has been credited with a protective role in AD. [For thorough review, please see (Pike et al., 2009).] As mentioned earlier, observational studies revealed that postmenopausal women exposed to exogenous estrogens mid-life had a 29-44% decreased risk of dementia (Paganini-Hill and Henderson, 1996, Hogervorst et al., 2000, Brann et al., 2007). In addition, a recent study suggested that longer cumulative lifetime durations of estrogen exposure, including both endogenous and exogenous sources of E2, were associated with a lowered risk of AD, with each additional month of E2 exposure translating to a 0.5% decrease in AD risk (Fox et al., 2013). With respect to basic science studies, E2 has also been repeatedly shown to protect against the neuropathological hallmarks of AD both \textit{in vitro} and \textit{in vivo} (Simpkins et al., 2005, Pike et al., 2009). For instance, E2 was found to prevent phosphorylation of the microtubule-associate protein tau following cerebral ischemia in rodents, which attenuates formation of NFTs (Simpkins et al., 2005, Zhang et al., 2008). Furthermore, exogenous E2 is well known to protect against Aβ neurotoxicity (Shah et al., 2003, Simpkins et al., 2005, Marin et al., 2008), and brain-specific E2 depletion was found to accelerate Aβ deposition and hinder Aβ clearance in a transgenic mouse model of AD, (Yue et al., 2005). Collectively, these studies suggest that E2 tends to reduce the neural load of Aβ, and they corroborate post-mortem studies, which found significantly reduced levels of E2 in the brains of female AD patients, compared with age-and gender-matched controls (Yue et al., 2005).

In regard to the mechanism(s) through which E2 modulates neural Aβ, scientific evidence supports E2 influence of both Aβ deposition and Aβ clearance. Along these lines, E2 is purported to regulate expression of at least 2 major proteins responsible for
removal of neurotoxic Aβ: insulin degrading enzyme and neprilysin (Huang et al., 2004, Xiao et al., 2009, Liang et al., 2010, Jayaraman et al., 2012, Li et al., 2013). E2 is further thought to enhance microglial phagocytosis of Aβ in cortical neurons (Li et al., 2000). With respect to Aβ deposition, several studies suggest that E2 may regulate APP processing at several steps, thereby promoting the non-amyloidogenic pathway. As evidence, BACE1, the rate-limiting enzyme for Aβ formation, has an estrogen response element (ERE) within its promoter region (Sambamurti et al., 2004), and E2 has been shown to decrease BACE1 expression both in mixed neuronal cultures and in neurons in vivo (Simpkins et al., 2005, Nord et al., 2010, Zhang et al., 2012, Li et al., 2013). Conversely, E2 has also been hypothesized to regulate two putative α-secretases ADAM 10 (Fernandez et al., 2010, Sun et al., 2012, Zhang et al., 2012, Anukulthanakorn et al., 2013, Zhang et al., 2013a) and ADAM 17 (Nord et al., 2010, Nadadur et al., 2012), which is also known as TNF α-converting enzyme (TACE). It should be mentioned here that although some controversy exists in the literature regarding which putative α-secretase, ADAM 10 or ADAM 17, is mainly responsible for APP processing (De Strooper et al., 2010), recent studies provided evidence that ADAM 10 is the primary α-secretase and that ADAM 17 plays a more secondary role in the non-amyloidogenic processing of APP (Schroeder et al., 2009, Epis et al., 2010, Gil-Bea et al., 2012).

While E2’s neuroprotective role in Alzheimer’s disease has been well studied in vitro, E2’s neuroprotection from AD has not been completely characterized in vivo, particularly when considering the development of AD neuropathology following GCI. Furthermore, aside from a single observed decrease of neprilysin expression in the brain 45 days post ovariectomy (Huang et al., 2004), and my colleagues’ recent finding of
enhanced amyloidogenesis in the hippocampal CA3 region following GCI in long-term ovariectomized females (Zhang et al., 2013a), the effect of long-term E2 deprivation (surgical menopause) on critical pathways affecting Aβ load in non-transgenic rodents is largely unknown. Along these lines, the second aim of the current study attempted to determine whether long-term ovariectomy enhanced amyloidogenesis in the hippocampal CA1 following a stressor (GCI). Through this aim, we also aspired to definitively characterize acute E2 regulation of APP processing (ADAM 10, ADAM 17, and BACE1 expression) in the hippocampal CA1 following GCI and to determine whether E2 regulation of APP processing is lost following surgical menopause, as these events could mechanistically explain the enhanced risk of dementia and mortality from neurological disorders seen in prematurely menopausal women (Scott et al., *In Press*).

### 2.11 PELP1 – A Critical Estrogen Receptor Co-regulator?

Proline-, Glutamate-, and Leucine-rich Protein 1 (PELP1) is a 160 kD nuclear hormone receptor co-regulator with 10 LXXLL domains, which allow it to interact with several types of nuclear hormone receptors, including the androgen receptor, the progesterone receptor, and ERα/ERβ, as well as 3 PXXP motifs that allow it to bind SH2/SH3 domains in kinases (Vadlamudi and Kumar, 2007). PELP1 is a widely expressed phospho-protein, and intriguingly, some kinases to which PELP1 binds and/or activates can reciprocally phosphorylate PELP1 in return, thereby facilitating its protein-interacting ability (Vadlamudi and Kumar, 2007, Nair et al., 2010). To date, no enzymatic activity has been reported for PELP1 (Vadlamudi and Kumar, 2007). However, PELP1 has been suggested to modulate chromatin modification, cell cycle progression, activation
of cytosolic kinases Src and PI3K via phosphorylation, and mediation of mitogenic signaling via protein-protein interactions (Brann et al., 2007, Vadlamudi and Kumar, 2007, Cheskis et al., 2008, Mann et al., 2013, Roy et al., 2013). Due to its involvement with cell cycle progression and mitogenic signaling, PELP1 has been heavily studied in the cancer field, where it has been shown to be a proto-oncogene that mediates E2 signaling and tumor cell survival in ER-positive carcinomas (Rajhans et al., 2007, Vadlamudi and Kumar, 2007, Cortez et al., 2010, Nair et al., 2010, Roy et al., 2013). Our collaborators have also provided evidence that PELP1 may promote local E2 production in breast cancer cells via modulating aromatase expression (Vadlamudi et al., 2010). Previous work in our lab and others’ has shown that PELP1 is highly expressed in the brain, co-localizes with ERα+, but not GnRH+, neurons, and forms an extranuclear E2 signalsome with ERα, Src kinase, and the P85 subunit of PI3K (Barletta et al., 2004, Khan et al., 2005, Brann et al., 2008, Chakravarty et al., 2010). However, despite PELP1’s high expression in key E2-responsive brain regions, such as the cortex and hippocampus (Khan et al., 2005), and its potential connection with E2 neuroprotection, very little is known regarding PELP1’s functional role in the brain or in vivo.

We hypothesize that in CA1 hippocampal neurons, PELP1 may act as a scaffold protein to bring liganded, membrane-bound ERα into close proximity with P85, thereby facilitating E2’s rapid phosphorylation and subsequent activation of the neuroprotective PI3K-Akt signaling pathway. Currently, the precise mechanism through which E2 prevents post-ischemic phosphorylation of JNK is unknown. However, Akt has been shown to phosphorylate another scaffold protein called POSH (Plenty of SH3 Domains) in the Rac1 binding site at Ser 304 (Lyons et al., 2007). As such, it is possible that E2-
induced phosphorylation of Akt could, in turn, prevent activation of pro-apoptotic JNK by deactivating its associated regulatory kinase, POSH. This would, ultimately, prevent JNK’s subsequent nuclear activation of the pro-apoptotic transcription factor c-Jun, which is implicated in transcriptional control of the neurodegenerative Wnt antagonist Dkk1. Thus, it is plausible that PELP1 may play a mediatory role in E2’s regulation of pro-apoptotic JNK/c-Jun signaling and resultant Dkk1 expression in hippocampal neurons, thereby allowing E2 to exert neuroprotection in the event of GCI. Along these lines, the third and final aim of the dissertation was to determine the effect of surgical menopause (10-week ovariectomy) on CA1 hippocampal expression of PELP1 and to determine whether PELP1 plays a critical role in E2’s regulation of JNK/c-Jun signaling, Dkk1 expression, stress-induced amyloidogenesis, and E2 neuroprotection.
II. METHODS

1. Animals

All procedures were approved by the Georgia Regents University Institutional Animal Care and Use Committee (Animal Use Protocols: 09-03-174 and 2012-0474) and were conducted in accordance with the National Institutes of Health guidelines for animal research. Young adult (3-month-old) female Sprague Dawley rats were utilized for most studies. However, in a subset of studies in Aim 1, both young (3-month-old) and aged (24-month-old) female Fisher344 rats were used. All animals were group housed on a 10h/14h light-dark cycle and fed *ad libitum* using Harlan’s 8604 Teklad Rodent Diet.

2. Surgical Menopause and Estrogen Therapy

To induce surgical menopause, all female rats were bilaterally ovariectomized under isoflurane anesthesia either one week (STED: short-term E2-deprivation) or 11 weeks (LTED: long-term E2 deprivation) before induction of global cerebral ischemia. At the time of menopausal onset (STED) or 10 weeks later (LTED) the animals had subcutaneous, osmotic mini-pumps (0.5 μL/hr, 14-day release; Alzet, CA) implanted between their scapulae containing either placebo (20% β-Cyclodextrin) or 17β-estradiol (0.0167 mg E2 in 20% β-Cyclodextrin) to mimic physiological E2 levels produced
during Diestrus I (10-15 pg/mL serum) (Zhang et al., 2008). As such, STED rats modeled surgical menopause with immediate E2 therapy and LTED modeled surgical menopause with delayed E2 therapy. For the reproductive senescence study in Aim 1, both Young and Aged Fisher 344 rats followed the STED paradigm of surgical menopause with immediate E2 therapy. Sham animals were bilaterally ovariectomized for either one week (STED) or 11 weeks (LTED) but did not receive pumps.

3. Global Cerebral Ischemia

Global cerebral ischemia (GCI) was performed using 4-vessel occlusion as described previously (Pulsinelli and Brierley, 1979, Pulsinelli and Buchan, 1988, Zhang et al., 2006). Briefly, the day before GCI, animals were anesthetized using intraperitoneal chloral hydrate (350 mg/kg) or intraperitoneal ketamine/xylazine (60mg/kg and 8 mg/kg, respectively), and both vertebral arteries (VA) were permanently occluded at the level of the alar foramina via electrocauterization. Immediately following bilateral VA occlusion, both common carotid arteries (CCA) were carefully isolated and loosely ligated with suture thread without interrupting blood flow. After a 24-hr recovery period, animals were re-anesthetized with intraperitoneal chloral hydrate (300 mg/kg) or isoflurane (1-4%), and the bilateral CCA were exposed and occluded with hemostatic clips to induce 10 minutes of complete forebrain ischemia. Only animals which lost their righting reflex within 30 seconds and whose pupils were dilated and unresponsive to light during cerebral ischemia were selected for the experiments. After 10 minutes, the clips were removed, and reperfusion was confirmed before the wound was sutured. During GCI,
rectal temperature was maintained at 36.5 to 37.5°C with a thermal blanket. Sham animals underwent identical surgical procedures except that the CCA were simply exposed but not occluded.

4. Histochemical Analysis of Neuroprotection

For brain harvesting, animals were deeply anesthetized with chloral hydrate or isoflurane and transcardially perfused with 0.9% saline at 3d, 5d, or 7d post ischemia-reperfusion, followed by fixation with cold 4% paraformaldehyde in 0.1M phosphate buffer (PB). Brains were post-fixed in the same fixative overnight at 4°C and cryoprotected with 30% sucrose in 0.1 M PB, pH 7.4 for 24-36 hr. Coronal sections (25µm) were collected throughout the entire dorsal hippocampus (~2.5-4.5 mm posterior from Bregma, ~100 sections per brain) for each animal.

For analysis of neuroprotection, every fifth section was collected and stained. Briefly, sections were washed for 10 min in PBS followed by 0.1 % PBS-Triton-X100 for additional 10 min. After incubation with blocking solutions containing 10% donkey serum for 1h at room temperature in PBS containing 0.1% Triton X-100, sections were exposed to anti-NeuN mouse monoclonal antibody (1:500, MAB377, Millipore) overnight at 4°C. Sections were washed for 4 x 10 min, followed by incubation with Alexa Fluor 488 donkey anti-mouse antibody (1:500, Invitrogen) for 1 h at room temperature. Staining solution was removed, and sections were washed with PBS-Triton-X100, followed by PBS and water. Then, sections were mounted using a water-based mounting medium containing anti-fading agents (Fluoro-Gel, Electron Microscopy
Sciences) and images were captured on an LSM510 Meta confocal laser microscope (Carl Zeiss, Germany). The number of NeuN-positive CA1 neurons per 1mm length of the medial CA1 pyramidal cell layer was counted bilaterally in five sections per animal.

Alternately, neuroprotection status was ascertained by washing sections 2 x 10 min with 1X PBS and staining with Cresyl Violet for 10 minutes. Afterward, slides were washed briefly with distilled water, dehydrated in increasing concentrations of ethanol, cleared in xylene, and mounted using a xylene-based mounting medium (Cytoseal XYL, Richard-Allan Scientific). Images were captured on an Axiophot-2 visible/fluorescence microscope using an AxioVision4Ac software system (Zeiss, Germany). Regardless of whether NeuN or Cresyl Violet staining was used, cell counts from the right and left hippocampus on each of the five sections were averaged to provide the mean value. A Mean ± SE was calculated from the data in each group, and statistical analysis was performed as described below.

5. DAB Staining

For diaminobenzidine staining, sections were incubated with 10% normal horse serum in PBS containing 0.1% Triton-X100 and 0.3% H2O2 for 1h at room temperature to block nonspecific surfaces. Sections were then incubated with a single primary antibody: anti-Dkk1 (1:50, sc-25516, Santa Cruz Biotechnology, Inc.), anti-survivin (1:100, 2803, Cell Signaling Technology, Inc.), anti-p-β-Catenin (1:100, 9561S, Cell Signaling Technology, Inc.), anti-PHF1 (1:1000, Gift from Peter Davies), anti-β-Amyloid (1:100, MAB8768, Millipore), or anti-PELP1 (1:2000, A300-180A, Bethyl Laboratories,
Inc.), overnight at 4°C in PBS containing 0.1% Triton-X100 and 1% Horse serum.  
Afterward, sections were washed with PBS containing 0.1% Triton-X100, followed by incubation with secondary biotinylated horse anti-rabbit/anti-mouse antibodies (1:500, Vector Laboratories, Inc., CA) in the same buffer for 1 h at room temperature. Sections were then washed and incubated with ABC reagent for 20 minutes at room temperature.

Finally, sections were rinsed, mounted onto a slide, and incubated with DAB reagent for 2-10 min., according to the manufacturer’s instructions (Vector Laboratories, Inc.). Following DAB incubation, slides were washed briefly with distilled water, dehydrated in increasing concentrations of ethanol, cleared in xylene, and mounted using a xylene-based mounting medium (Cytoseal XYL, Richard-Allan Scientific). Images were captured on an Axiophot-2 visible/fluorescence microscope using an AxioVision4Ac software system (Carl Zeiss, Germany). Analysis was performed by either counting the number of immunopositive neurons per 250µm length of the medial CA1 pyramidal cell layer or by analyzing the integrated density of immunostaining using ImageJ analysis software (Version 1.45s; Wayne Rasband, NIH, USA). Regardless of the method of analysis, a Mean ± SE was calculated for each treatment group, which consisted of 4-7 animals each and 3-5 sections per animal. Statistical analysis was then performed as described below, and all results were expressed as Mean ± SE.

6. Immunofluorescence and TUNEL Staining

Coronal sections were incubated with 10% normal donkey serum for 1h at room temperature in PBS containing 0.1% Triton X-100, followed by incubation with primary
antibody: anti-Dkk1 (1:50, sc-25516, Santa Cruz Biotechnology or 1:50, NBP1-95560, Novus Biologicals), anti-BACE1 (1:100, AHB0241, Invitrogen), anti-ADAM10 (1:50, sc-25578, Santa Cruz Biotechnology, Inc.), anti-ADAM17/TACE (1:50, sc-6416, Santa Cruz Biotechnology, Inc.), anti-p-APP (1:400, 3823S, Cell Signaling Technology, Inc.), anti-p-JNK (1:50, sc-12882, Santa Cruz Biotechnology, Inc.), or anti-p-cJun (1:50, sc-7981, Santa Cruz Biotechnology, Inc.), for 1-3 nights at 4°C in the same buffer. After primary antibody incubation, sections were washed for 3 x 10 min at room temperature, followed by incubation with the appropriate combination of secondary antibodies: Alexa-Fluor488/568/647 donkey anti-rabbit/anti-mouse/anti-goat (1:500, Invitrogen). For Terminal deoxynucleotidyl transferase-mediated dUTP-X Nick-End Ligase (TUNEL) staining, an In Situ Cell Death Detection Kit with TMR Red (Roche Diagnostics, Manheim, Germany) was used during addition of secondary antibody, according to manufacturer’s instructions. Sections were then washed with PBS containing 0.1% Triton X-100 for 3 x 10 min, followed by 2 x 5 min with 1X PBS and briefly with water. Then, sections were mounted with water-based mounting medium containing anti-fading agents (Fluoro-Gel, Electron Microscopy Sciences).

7. Confocal Microscopy and Image Analysis

All images were captured on an LSM510 Meta confocal laser microscope (Carl Zeiss, Germany) using a 40X oil immersion Neofluor objective (NA, 1.3) with the image size set at 1024 x 1024 pixels. The following excitation lasers/emission filters settings were used for various chromophores: argon/2 laser was used for Alexa Fluor488, with
excitation maximum at 490 nm and emission in the range of 505-530 nm, HeNe1 laser was used for Alexa Fluor 568 and TMR with excitation maximum at 543 nm and emission in the range of 568-615 nm, and HeNe2 laser was used for Alexa Fluor647 with excitation maximum at 633 nm and emission in the range of 650-800 nm. The captured images were viewed and analyzed using LSM510 Meta imaging software. Simultaneous examination of negative controls confirmed the absence of nonspecific immunofluorescent staining, cross-immunostaining, or fluorescence bleed-through. Images were analyzed either by counting the number of immunopositive neurons per 250µm length of the medial CA1 pyramidal cell layer or by measuring the integrated density of fluorescent staining using ImageJ analysis software (Version 1.45s; Wayne Rasband, NIH, USA) for each animal (2-5 sections/animal), and a Mean ± SE was calculated from the data in each group (n = 3-7 animals/group). Statistical analysis was performed as described below.

8. Brain Homogenization and Protein Extraction

For brain tissue preparation, rats were euthanized by rapid decapitation under deep anesthesia 30m, 3h, or 1d post ischemia-reperfusion. Once the whole brains were removed, the hippocampal CA1 regions were microdissected from both sides of the hippocampal fissure, immediately frozen in dry ice or liquid nitrogen, and stored at -80°C until use. Tissues were homogenized with a Teflon-glass homogenizer in ice cold homogenization medium consisting of 50 mM HEPES (pH 7.4), 150 mM NaCl, 12 mM β-glycerophosphate, 3 mM dithiothreitol (DTT), 2 mM sodium orthovanadate (Na3VO4),
1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, and inhibitors of proteases and enzymes (0.5mM PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin A). The homogenates were centrifuged at 15,000g for 30 min at 4°C, and supernatants were collected and stored at -80°C until use. Protein concentrations were determined with a Modified Lowry Protein Assay Kit (Thermo Scientific), using bovine serum albumin as a standard.

9. Co-Immunoprecipitation and Western Blotting

For co-immunoprecipitation, 200μg total CA1 hippocampal lysates were diluted four-fold in homogenization buffer and incubated with 5μg PELP1 antibody (A300-180A, Bethyl Laboratories) for 4 hours at 4°C. After adding Protein A/G sepharose beads (sc-2003, Santa Cruz Biotechnology), samples were incubated for 2 more hours at 4°C. Immunoprecipitates were then centrifuged, washed in homogenization buffer, eluted in Laemmli loading buffer, and boiled for 5 minutes in preparation for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For Western blotting, aliquots of 20-50 μg of total hippocampal protein lysate were separated via 4-20% SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore), blocked for 3 h, and incubated with 1° antibody against Dkk1 (1:200, sc-25516, Santa Cruz Biotechnology or 1:500, NBP1-95560, Novus Biologicals), Survivin (1:1000, 2803, Cell Signaling Technology), p-APP (1:400, 3823S, Cell Signaling Technology), APP (1:1000, 366900, Invitrogen), β-Amyloid Oligomers (1:500, AB9234, Millipore), PHF-1 (1:1000, Gift from Peter Davies), Tau (1:200, sc-1995, Santa Cruz
Biotechnology), PELP1 (1:1000, A300-180A, Bethyl Laboratories), p-JNK (1:200, sc-12882, Santa Cruz Biotechnology), JNK (1:200, sc-572, Santa Cruz Biotechnology), POSH (1:200, sc-8280, Santa Cruz Biotechnology), ERα (1:200, sc-542, Santa Cruz Biotechnology), p85α (1:200, sc-1637, Santa Cruz Biotechnology), JIP1 (1:500, sc-25267, Santa Cruz Biotechnology), Rac1 (1:1000, 610650, BD or 1:200, sc-217, Santa Cruz Biotechnology), pMLK3 (1:1000, 2811, Cell Signaling Technology), or MKK7 (1:50, sc-7103, Santa Cruz Biotechnology), overnight at 4 °C. α-Tubulin (1:500, sc-5286, Santa Cruz Biotechnology) served as a loading control.

The membrane was then washed with Tween20-PBS to remove unbound antibody and incubated with 2° antibody: Alexa Fluor 680/800 goat anti-rabbit/mouse IgG (1:10,000, Invitrogen) or Alexa Fluor 680/800 donkey anti-goat IgG (1:10,000, Invitrogen), for 1 h at room temperature. Bound proteins were visualized using the Odyssey Imaging System (LI-COR Bioscience), and semi-quantitative analysis of the bands was performed using ImageJ analysis software (Version 1.45s; Wayne Rasband, NIH, USA). To quantitate hippocampal protein abundance, band densities of the indicated total proteins were analyzed and expressed as ratios relative to either full-length protein or α-tubulin signals, as appropriate, and a Mean ± SE was calculated from each group for graphical presentation and statistical comparison.

10. Administration of Drugs and Antisense Oligonucleotides

For studies requiring inhibition of JNK, the JNK inhibitor SP600125 was dissolved in PPCES vehicle (30% PEG–400/20% polypropylene glycol/15% cremophor EL/5%
ethanol/30% saline) as described previously (Bennett et al., 2001), and delivered to LTED Placebo females by tail vein injection 15 minutes prior to GCI at a dose of 10 mg/kg. Controls consisted of LTED females that received a tail vein injection of PPCES vehicle alone 15 minutes prior to GCI (Vehicle) and LTED animals that received placebo mini-pumps only, but no tail vein injection, before being subjected to GCI (Placebo).

For PELP1 knockdown studies, prior to GCI, E2-treated STED rats were further subjected to 4 daily stereotaxic intracerebroventricular injections (from Bregma: AP – 0.08 cm, Lateral ± 0.15 cm, Depth – 0.35 cm) of PELP1 antisense oligonucleotides (E2+AS: 10uL, 10nmol, 5’-TCCACCTTTCCACTTCCC-3’) with a Hamilton syringe at a rate of 1uL/min, starting 72h prior to GCI, with the fourth and final injection given 3h prior to ischemia. The syringe was left undisturbed for 5 min before removal. Missense oligonucleotides (E2+MS: 10uL, 10nmol, 5’-CCCCTTTCCCTTCAACT-3’) served as a control in E2-treated STED rats. For some studies, STED animals that received placebo mini-pumps only, but no stereotaxic injections, before being subjected to GCI (Placebo) were also included as an additional control.

11. Statistical Analysis

Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA), as appropriate, followed by a Student-Newman-Keuls post-hoc test. Where only two groups were compared, a Student’s t-test was used. Statistical significance was accepted at the 95% confidence level (P < 0.05). All data are expressed as Mean ± Standard Error (SE).
III. RESULTS

1. Surgical Menopause Leads to Dysregulation of Dkk1 Expression and Wnt/β-Catenin Signaling in the Hippocampal CA1

1.1. Surgical Menopause Leads to Basal Elevation of Dkk1 Expression and Loss of E2 Regulation in the Hippocampal CA1

Previously, our lab demonstrated that pre-treatment with low, Diestrus I levels of E2 afforded robust neuroprotection from GCI, in part, through preventing the post-ischemic hippocampal elevation of the neurodegenerative Wnt antagonist Dkk1 (Zhang et al., 2008). Since we observed a loss of E2 neuroprotection in surgically menopausal (10-week ovariectomized) female rats (Figure 1) (Zhang et al., 2009a, Zhang et al., 2011, Zhang et al., 2013a), we aimed to determine whether chronic loss of ovarian-derived E2 would attenuate E2’s regulation of post-ischemic Dkk1 and/or affect basal levels of Dkk1 expression in hippocampal CA1 neurons as well. Two experimental paradigms were used: 1) Short-term E2 deprivation (STED, one-week ovariectomy with immediate Pla or E2 treatment) and 2) Long-term E2 deprivation (LTED, 10-week ovariectomy with delayed Pla or E2 treatment). One week following initiation of E2 therapy, we subjected all animals to 10 min of GCI to selectively damage the CA1 region of the hippocampus.
For these studies, we chose to examine the 24h reperfusion time point after GCI because our previous study showed a significant increase in Dkk1 in hippocampal CA1 neurons 24h following GCI (Zhang et al., 2008). As shown in Figure 8A and 8C, both western blotting and immunohistochemistry demonstrated that GCI induced a significant elevation of Dkk1 in the hippocampal CA1 24h post GCI and that this change was attenuated by immediate, low-dose E2 treatment in STED animals (Fig. 8A and Fig. 8C: a-c, STED). However, if E2 therapy was delayed following 10 weeks of ovariectomy (LTED), it was no longer effective in preventing the post-ischemic rise of Dkk1 (Fig. 8B and Fig. 8C: d-f). Intriguingly, immunohistochemistry also revealed a modest, but significant, basal upregulation of Dkk1 expression in LTED non-ischemic sham rats (Fig. 8C: d, LTED Sham) (Scott et al., 2013).
(Scott et al., 2013)
Figure 8. “Long-Term Estrogen Deprivation Leads to Loss of Estradiol Suppression of Neurodegenerative Dkk1 in CA1 Hippocampal Neurons. (A) Western blotting shows Dkk1 expression 24 hours post global cerebral ischemia in total CA1 hippocampal lysates of animals treated with placebo (Pla) or low-dose estradiol (E2) immediately following ovariectomy (STED). Quantifications of band densities (Means + SE, n = 3-6 animals per group) are expressed as densitometric ratios of Dkk1 to α-Tubulin. (B) Western blotting shows Dkk1 expression 24 hours post global cerebral ischemia in total CA1 hippocampal lysates of long-term E2-deprived (LTED) female rats treated with placebo (Pla) or low-dose estrogen (E2) 10 weeks following bilateral ovariectomy. Quantifications of band densities (Means + SE, n = 3-4 animals per group) are expressed as densitometric ratios of Dkk1 to α-Tubulin. (C) Representative DAB staining (top) demonstrates Dkk1 expression in the CA1 region 24 hours post global cerebral ischemia. Quantitative summary (Bottom, Means + SE, n = 4-6 animals per group) indicates the raw integrated density of Dkk1 immunostaining in the medial CA1. * = p < 0.05 compared to Sham; # = p < 0.05 compared to Placebo; ^ = p < 0.05 compared to STED E2. Magnification = 20X; Scale bar = 50μm,” (p. 628) (Scott et al., 2013).
1.2. Surgical Menopause Leads to Basal Antagonism of Wnt/β-Catenin Signaling and Loss of E2 Regulation in the Hippocampal CA1

We next examined the phosphorylation status of β-Catenin, as canonical Wnt signaling promotes de-phosphorylation and nuclear translocation of β-Catenin, while GSK3β, on the other hand, promotes phosphorylation and subsequent degradation of β-Catenin. Immunohistochemistry showed that one week of immediate low-dose E2 therapy prevented the ischemia-induced phosphorylation of β-Catenin in STED rats (Fig. 9A:a-c, 9B) (Zhang et al., 2008). In contrast, following LTED, E2 was no longer able to prevent the elevation of phospho-β-Catenin 24h post GCI (Fig. 9A:e-f, 9B). Interestingly, we also observed a significant increase in the basal amount of phospho-β-Catenin in hippocampal CA1 neurons (Fig. 9A:d, B), which corroborates the aforementioned Dkk1 findings and provides evidence that ovarian-derived E2 may regulate basal Wnt/β-Catenin signaling (Scott et al., 2013).
Figure 9. “Long-Term Estrogen Deprivation Promotes Phosphorylation of β-Catenin.

(A) Representative DAB staining demonstrates expression of β-catenin phosphorylated at Ser33, Ser37, and Thr41 24 hours post global cerebral ischemia. (B) Quantification (Means + SE, n = 4-6 animals per group) shows the raw integrated density of phospho-β-catenin immunostaining in the medial CA1. * = p < 0.05 compared to STED Sham; # = p < 0.05 compared to Placebo; ^ = p < 0.05 compared to STED E2. Magnification = 20X; Scale bar = 50μm,” (p. 629) (Scott et al., 2013).
Survivin is a protein product of canonical Wnt/β-Catenin signaling, which prevents the cleavage and activation of pro-apoptotic caspases (Wang et al., 2004). Since we previously showed that one week of E2 pre-treatment enhances Survivin expression in CA1 hippocampal neurons following GCI (Zhang et al., 2008), we decided to examine whether E2’s ability to regulate Survivin is lost after LTED. **Figure 10** confirms our previously published study, showing that Survivin expression in the hippocampal CA1 is dramatically reduced 24h post GCI and that immediate E2 therapy not only prevents loss of Survivin, but even enhances Survivin expression in the hippocampus, relative to sham levels (Zhang et al., 2008). In addition, both immunohistochemistry and western blotting demonstrated a statistically significant *basal* reduction in hippocampal CA1 Survivin expression in LTED sham rats (**Fig. 10A**:d, B-D). Importantly, this finding agrees with the *basal* elevation of Dkk1 expression (**Figure 8**) and the *basal* increase in phosphorylation of β-Catenin (**Figure 9**) observed in LTED sham animals. Furthermore, it suggests that long-term ovariectomy may promote *basal* antagonism/dysregulation of the Wnt/β-Catenin signaling pathway in the hippocampus (Scott et al., 2013).
Figure 10. “Delayed Estrogen Replacement Following Ovariectomy Fails to Induce Survivin Expression in CA1 Hippocampal Neurons. (A) Representative DAB staining demonstrates survivin expression in the hippocampal CA1 region 24 hours post ischemia-reperfusion. (B) Quantitative summary of data (Means + SE, n = 4-6 animals per group) displays the raw integrated density of survivin immunostaining in the medial CA1. (C) Western blotting of survivin present in total CA1 hippocampal lysates corroborates DAB staining and is compared to α-Tubulin. (D) Quantification of Western blotting via densitometric band analysis is expressed as ratio of Survivin to α-Tubulin (Means + SE, n = 4-6 animals per group). * = p < 0.05 compared to STED Sham; # = p < 0.05 compared to Placebo; ^ = p < 0.05 compared to STED E2; Magnification = 10X; Scale bar = 50μm,” (p. 629) (Scott et al., 2013).
1.3. C-Jun N-Terminal Kinase (JNK) is a Critical Mediator of Ischemia-Induced Dkk1 Elevation, Survivin Depression, and Neuronal Apoptosis Following Surgical Menopause

Our laboratory previously demonstrated that C-Jun N-Terminal Kinase (JNK) activation plays a critical role in the post-ischemic hippocampal elevation of Dkk1 via the phosphorylation and activation of the transcription factor c-Jun (Zhang et al., 2008). We thus hypothesized that JNK activation may mediate the ischemia-induced changes observed in Dkk1 and Survivin in LTED females as well. To test this hypothesis, we administered a tail vein injection of the commercialized JNK inhibitor SP600125 to LTED female rats 15 min prior to inducing GCI. As shown in Figure 11, while GCI induced dramatic elevation of Dkk1 (Fig. 11A-B: Vehicle) and reduction of Survivin (Fig. 11C-D: Vehicle), pharmacological inhibition of JNK prevented these two events in long-term ovariectomized females (Fig. 11A-B and 11C-D: SP600125). These findings suggest that JNK is, indeed, a critical mediator of the post-ischemic elevation of the neurodegenerative factor Dkk1 and the post-ischemic reduction of the pro-apoptotic factor Survivin observed in the hippocampal CA1 of LTED females (Scott et al., 2013).
Figure 11. “JNK is required for Ischemia-Induced Elevation of Dkk1 and Loss of Survivin in Long-Term Estrogen-Deprived Female Rats. Representative DAB staining demonstrates Dkk1 (A) or Survivin (C) expression in CA1 hippocampal neurons of LTED female rats 24 hours following ischemia-reperfusion. The JNK inhibitor SP600125 was administered 15 minutes prior to ischemia via tail vein injection (10mg/kg in PPCES Vehicle). Controls consisted of LTED females that received a tail vein injection of PPCES alone 15 minutes prior to GCI (Vehicle). Quantification (Means + SE, n = 4-6 animals per group) displays raw integrated density of Dkk1 (B) or Survivin (D) immunostaining in the medial CA1. * = p < 0.05 compared to Sham; # = p < 0.05 compared to Vehicle (PPCES alone). Magnification = 20x; Scale bar = 50μm,” (p. 630) (Scott et al., 2013).
Since we previously showed that pharmacological inhibition of JNK was neuroprotective against GCI in short-term ovariectomized female rats (Zhang et al., 2008), we utilized the current LTED experimental paradigm to ascertain whether acute inhibition of JNK activation remained sufficient to protect against GCI following long-term ovariectomy. Intriguingly, confocal microscopy demonstrated that the number of healthy, NeuN-positive neurons in the hippocampal CA1 of ischemic LTED females treated with a single tail vein injection of SP600125 was significantly higher than the number of NeuN+ neurons in Placebo- or Vehicle-treated ischemic LTED females and was, in fact, comparable to non-ischemic sham levels (Figure 12). Along with the other JNK inhibition data shown in Figure 11, these data suggest that SP600125 remains an effective neuroprotective agent in LTED females, in whom E2 neuroprotection is lost. Furthermore, these data suggest that JNK remains a critical mediator of GCI-induced apoptosis in the hippocampal CA1, regardless of the length of surgical menopause (Scott et al., 2013).
Figure 12. “The JNK Inhibitor SP600125 is Neuroprotective in the Hippocampal CA1 Region of Long-Term Estrogen-Deprived Female Rats Following Global Cerebral Ischemia.” (A) The JNK inhibitor SP600125 was administered 15 minutes prior to ischemia via tail vein injection (10mg/kg in PPCES Vehicle). Controls consisted of LTED females that received a tail vein injection of PPCES alone (Vehicle) and LTED Placebo females that did not receive a tail vein injection (Placebo). Typical photomicrographs of hippocampal CA1 region depicting NeuN staining 7 days following ischemia-reperfusion in LTED females are shown. (B) Quantitative summary of data (Means + SE, n = 6 animals per group) shows the number of surviving neurons per 250 µm of medial CA1 expressed as % of Sham. NeuN-positive pyramidal cells showing intact, round nuclei were counted as surviving cells. Magnification = 40X; Scale bar = 50 µm. * p < 0.01 vs. sham; # p < 0.05 vs. Vehicle (PPCES alone) and Placebo,” (p. 630) (Scott et al., 2013).
1.4. Natural Aging Leads to Basal Elevation of Dkk1 and Loss of E2 Regulation in the Hippocampal CA1

Since we observed a loss of E2’s ability to regulate both basal and post-ischemic hippocampal expression of the neurodegenerative Wnt antagonist Dkk1 following surgical menopause, we aimed to determine whether the same events occurred in the hippocampus following natural aging and reproductive senescence. Furthermore, since previous studies have revealed that aged rats are more susceptible to GCI-induced neuronal damage (Xu et al., 2007, Xu et al., 2010, Shetty et al., 2011, Zhang et al., 2011) and are no longer protected from GCI by low, physiological levels of E2 (Zhang et al., 2011), we hypothesized that elevation of the neurodegenerative factor Dkk1 may play a key role. To investigate, we utilized Young (3-month-old) and Aged (24-month-old, acyclic) Fisher344 female rats, which were both subjected to one week of bilateral ovariectomy, immediate subcutaneous Placebo or E2 therapy, and 10-minute GCI, and we examined the co-localization of Dkk1 with TUNEL, a marker of DNA damage and apoptosis (Li et al., 2011a, Thal et al., 2011, Suman et al., 2012). Intriguingly, double immunohistochemistry for Dkk1 (green) and TUNEL (red), revealed that both markers were significantly elevated in the hippocampal CA1 region of Young and Aged rats several days following GCI, relative to Young, non-ischemic shams, and tended to co-localize to the same neurons (Figure 13A: b, e and B) (Scott et al., 2013).

While immediate low-dose E2 treatment following ovariectomy largely prevented the robust post-ischemic elevation and co-localization of both markers in Young female rats, (Figure 13A:c and B), immediate low-dose E2 treatment after ovariectomy was
ineffective in preventing the post-ischemic elevation and co-localization of Dkk1 and TUNEL in hippocampal CA1 neurons of Aged female rats (Figure 13A:f and B).

Furthermore, similar to events observed in young, long-term ovariectomized females, we detected a modest, basal elevation of Dkk1, in addition to TUNEL, in Aged, non-ischemic sham females (Figure 13A: d and B), suggesting that some amount of Dkk1 dysregulation and DNA damage may occur following natural aging and reproductive senescence in the absence of ischemia. It is important to note, however, that these two markers did not co-localize until after exposure to ischemic stress (Figure 13A:d and B) (Scott et al., 2013).
Figure 13. “Neurodegenerative Dkk1 Co-Localizes with the Apoptotic Marker TUNEL in CA1 Hippocampal Neurons After Global Cerebral Ischemia in Aged, Reproductively Senescent Female Rats. (A) Representative photomicrographs depicting Dkk1 (green) and TUNEL (red) expression in the hippocampal CA1 7 days (Young) and 3 days (Aged) following global cerebral ischemia. (B) Quantitative summary of data (Means + SE, n = 5-7 animals per group) indicates the number of neurons expressing both Dkk1 and TUNEL per 250μm medial CA1. * = p < 0.05 compared to Young Sham; # = p < 0.05 compared to Placebo; ^ = p < 0.05 compared to Young E2. Magnification = 40X; Scale bar = 50μm,” (p. 630) (Scott et al., 2013).
2. Surgical Menopause Enhances Post-Ischemic Amyloidogenesis in the Hippocampal CA1 Region

2.1 Surgical Menopause Leads to Post-Ischemic Loss of Alpha-Secretase Expression in the Hippocampal CA1

Prematurely menopausal women have a *doubled* lifetime risk of developing dementia and a 5-fold increased risk of mortality from neurological disorders (Rocca et al., 2007, Rivera et al., 2009). In light of these significant observations, we aimed to determine whether premature and chronic loss of ovarian E2 would enhance the development of AD-like neuropathology following an ischemic insult. Importantly, β-amyloid, one of the neuropathological hallmarks of AD, is well known to be neurotoxic and could augment basal damage to neurons, making them more vulnerable to injury and/or death following a stressor. To investigate, we utilized the STED and LTED animal models, with immediate (STED) or delayed (LTED) E2 therapy, and used GCI as the stressor, as described in the previous section. First, we decided to focus on hippocampal deposition of neurotoxic β-amyloid (Aβ) after ischemia, and in the first experiment, we examined hippocampal CA1 expression of two putative α-secretases: ADAM 10 and ADAM 17. These secretases are thought to be the driving forces of non-amyloidogenic processing of amyloid precursor protein (APP). In the studies, we searched for evidence of acute E2 regulation of ADAM 10 and 17 expression following GCI and/or dysregulation of ADAM 10 and ADAM 17 expression following LTED. Intriguingly, confocal microscopy revealed that ADAM 10 expression was significantly
downregulated in the hippocampal CA1 3h post GCI (Figure 14A:b, e and B) and that immediate E2 replacement following ovariectomy was able to prevent this loss in STED females (Figure 14A:c and B). In contrast, delayed E2 therapy was no longer able to mitigate the post-ischemic loss of ADAM 10 in the hippocampal CA1 following LTED (Figure 14A:f and B). Of significant interest, we noted an even more robust lost of hippocampal CA1 ADAM 10 expression 3h post GCI in LTED females than in STED females (Scott et al., In Press).
Figure 14. Hippocampal ADAM 10 Expression is Acutely Regulated by Global Cerebral Ischemia and Estrogen but Dysregulated after Long-Term Ovariectomy. (A) Representative photomicrographs depict ADAM 10 expression in the hippocampal CA1 of short-term E2-deprived (STED) and long-term E2-deprived (LTED) female rats 3 hours following global cerebral ischemia. (B) Quantitative summary of data (Means + SE, n = 4–6 animals per group) indicates the raw integrated density of ADAM 10 immunostaining in the medial CA1. * = p < 0.05 compared to STED Sham; # = p < 0.05
compared to STED Placebo; ^ = p < 0.05 compared to STED E2. Magnification = 40X;

Scale bar = 50μm. Adapted from (Scott et al., In Press).
While recent literature touts ADAM 10 as the main α-secretase responsible for the non-amyloidogenic processing of APP (Schroeder et al., 2009, Epis et al., 2010), other studies maintain that ADAM 17 or TACE plays a major role in the same process (Pietri et al., 2013). Therefore, we also examined expression of ADAM 17 following GCI and long-term ovariectomy. In contrast to ADAM 10, there was no ischemia-induced decrease of ADAM 17 expression in the hippocampus 3h post GCI in STED females (Figure 15A:b and B). Furthermore, there was no E2 regulation of ADAM 17 expression in the hippocampal CA1 at the same time point after ischemia (Figure 15A:c and B). Following 10-week ovariectomy, non-ischemic LTED sham animals displayed a pattern for increased basal ADAM 17 immunostaining, but this trend did not reach statistical significance. However, confocal microscopy did reveal a marked loss of ADAM 17 expression 3h post GCI in long-term ovariectomized (LTED) females, and importantly, delayed E2 therapy was unable to prevent this loss (Figure 15A:e-f and B). As a whole, our data suggest that non-amyloidogenic processing of APP may be significantly impaired in the event of ischemia following long-term ovariectomy, as hippocampal CA1 expression of both α-secretases ADAM 10 and ADAM 17 are significantly decreased upon exposure to ischemic stress in long-term ovariectomized rats. Furthermore, the observed impairment of non-amyloidogenic processing of APP suggests that a switch to amyloidogenic processing of APP may occur in LTED females in the event of ischemic stress (Scott et al., In Press).
Figure 15. Hippocampal ADAM 17 Expression is diminished after Long-Term Ovariectomy in the Event of Global Cerebral Ischemia. (A) Representative photomicrographs depict ADAM 17 expression in the hippocampal CA1 region 3 hours after global cerebral ischemia. Note that reduction in ADAM 17 expression was only observed in ischemic long-term ovariectomized (LTED) females. (B) Quantitative summary of data (Means + SE, n = 4-6 animals per group) indicates the raw integrated density of ADAM 17 immunostaining in the medial CA1. * = p < 0.05 compared to STED
Sham; \# = p < 0.05 compared to STED Placebo; ^ = p < 0.05 compared to STED E2.

Magnification = 40X; Scale bar = 50\mu m (Scott et al., In Press).
2.2 Surgical Menopause Enhances Basal and Post-Ischemic BACE1 Expression in the Hippocampal CA1

Since the β-secretase BACE1 is thought to be the rate-limiting step for Aβ formation via the amyloidogenic processing of APP (Sambamurti et al., 2004), we next aimed to determine how GCI, exogenous E2, and long-term ovariectomy influence BACE1 expression in the hippocampal CA1. Confocal microscopy analysis revealed that neuronal BACE1 expression was acutely upregulated in the hippocampal CA1 of STED females 3h following GCI, and this elevation was prevented by pretreatment with low-dose E2 (Figure 16A:a-c and B). However, we observed a robust loss of E2 regulation of ischemia-induced BACE1 expression in the hippocampal CA1 of LTED female rats (Figure 16A:e-f and B), suggesting that E2 is no longer able to prevent the ischemia-induced increase in hippocampal BACE1 following prolonged loss of ovarian E2. Furthermore, we noted a modest increase in basal expression of BACE1 in the hippocampal CA1 region of non-ischemic LTED sham animals, but this trend did not reach statistical significance (Figure 16A:d and B). These results agree with the aforementioned α-secretase results, suggesting that non-amyloidogenic processing of APP is significantly impaired and that amyloidogenic processing of APP is significantly enhanced following long-term ovariectomy (LTED), particularly in the event of GCI. These results also extend a previous finding by our laboratory, which described post-ischemic BACE1 elevation and a switch to amyloidogenic processing in the hippocampal CA3 region of LTED females subjected to GCI (Zhang et al., 2013a), to the critical CA1 region (Scott et al., In Press).
Figure 16. Long-Term Ovariectomy Enhances BACE1 Expression in the Hippocampal CA1. (A) Representative photomicrographs demonstrate BACE1 expression in the hippocampal CA1 3 hours post global cerebral ischemia. Note the elevated expression of BACE1 in long-term ovariectomized (LTED) females. (B) Quantitative summary of data (Means + SE, n = 4-6 per group) indicates the raw integrated density of BACE1 immunostaining in the medial CA1. * = p < 0.05 compared to STED Sham; # = p < 0.05 compared to STED Placebo; ^ = p < 0.05 compared to STED E2. Magnification = 40X; Scale bar = 50μm (Scott et al., In Press).
2.3 Surgical Menopause Promotes Development of Alzheimer’s disease-Related Neuropathology in the Hippocampal CA1 Following GCI

Collectively, our previous evidence for decreased alpha-secretase expression and increased beta-secretase expression suggested that the hippocampal load of Aβ should be enhanced in LTED females subjected to GCI. Importantly, using DAB staining to visualize neuronal Aβ, we observed a marked increase in the number of neurons immunopositive for intracellular Aβ 24 hours post GCI in the hippocampal CA1 region of LTED females (Figure 17A:e-f and B). Furthermore, western blotting analysis also revealed significantly increased Aβ oligomer formation in the hippocampal CA1 of LTED female rats 24 hours post GCI, relative to α-Tubulin expression (Figure 17B), suggesting that neuronal Aβ load is, in fact, increased in long-term surgically menopausal rats subjected to cerebral ischemia (Scott et al., In Press).
Figure 17. Post-Ischemic Aβ Load is enhanced in the Hippocampus Following Long-Term Ovariectomy. (A) Representative DAB staining (top) demonstrates Aβ expression in the CA1 region 24 hours post global cerebral ischemia. Quantitative summary (Bottom, Means + SE, n = 4-7 animals per group) indicates the number of Aβ-positive neurons per 250μm medial CA1. (B) Western blotting shows Aβ expression 24 hours post global cerebral ischemia in total CA1 hippocampal lysates of female rats treated with placebo (Pla) or low-dose estrogen (E2) either immediately (STED) or 10 weeks (LTED) following bilateral ovariectomy. Quantifications of band densities (Means + SE, n = 4-7 animals per group) are expressed as densitometric ratios of Aβ to α-Tubulin. * = p < 0.05 compared to Sham; # = p < 0.05 compared to STED Placebo; ^ = p < 0.05 compared to STED E2. Magnification = 20X; Scale bar = 50μm (Scott et al., In Press).
Finally, since neurofibrillary tangles are another major neuropathological hallmark of AD, we chose to examine E2’s ability to regulate the hyperphosphorylation of tau following chronic loss of ovarian E2. Cerebral ischemia is a well-known tauopathy (Wen et al., 2004a, Wen et al., 2004b, Wen et al., 2007, Pluta et al., 2013). In fact, we previously demonstrated that GCI induces significant hyperphosphorylation of tau 24 hours post GCI and that E2 pretreatment attenuates this event (Zhang et al., 2008). Along these lines, we hypothesized that E2 regulation of tau hyperphosphorylation would be lost following LTED. To investigate, we examined paired helical filaments (PHF) of microtubule-associated tau phosphorylated at Ser 396 and Ser 404, two residues implicated in human AD neuropathology. Both DAB staining (Figure 18A) and western blotting (Figure 18B) confirmed our previously published findings that GCI led to phosphorylation of tau 24 hours later (Figure 18A:b,e and B). In addition, both methods confirmed that one week of E2 pre-treatment, initiated immediately following ovariectomy in STED rats, was able to prevent this event (Figure 18A:c and B). In contrast, delayed E2 treatment in LTED rats was unable to mitigate the phosphorylation of tau at these two pathological residues (Figure 18A:f and B), suggesting that E2 regulation of tau phosphorylation is, indeed, lost following LTED. As such, along with our data showing enhanced post-ischemic hippocampal amyloidogenesis in LTED females, this finding provides evidence that chronic loss of ovarian function may predispose women to the development of AD-like neuropathology in the event of a stressor, such as cerebral ischemia. Furthermore, since Aβ itself is neurotoxic, and since LTED female rodents sustain more damage from Aβ (Zhang et al., 2013a), an increased
hippocampal load Aβ after ischemic stress could potentially explain, in part, the increased risk of dementia and mortality from neurological disorders seen in prematurely menopausal women (Scott et al., *In Press*).
A) Paired Helical Filaments (PHF)

**STED**
- Sham (a)
- Placebo (b)
- E2 (c)

**LTED**
- Sham (d)
- Placebo (e)
- E2 (f)

**Graph:**
- PHF+ Neurons
  - Bar graphs for Sham, Pla, and E2 conditions.
- STED and LTED conditions indicated with different markers (*, #).

B) Proteins

**STED**
- PHF1 (50-70 kD)
- Tau (50-70 kD)
- α-Tubulin (55 kD)

**LTED**
- PHF1 (50-70 kD)
- Tau (50-70 kD)
- α-Tubulin (55 kD)

**Graph:**
- PHF1/Tau Ratio
  - Bar graphs for Sham, Pla, and E2 conditions.
  - STED and LTED conditions indicated with different markers (*, #).
Figure 18. Estrogen Regulation of Ischemia-Induced Tau Phosphorylation is Lost Following Long-Term Ovariectomy. Representative DAB staining (top) demonstrates expression of microtubule-associated protein tau phosphorylated at Ser 396 and Ser 404 (Paired Helical Filaments: PHF) in the hippocampal CA1 region 24 hours after 10-minute global cerebral ischemia. Quantitative summary (Bottom, Means + SE, n = 4-7 animals per group) indicates the number of PHF-positive neurons per 250μm medial CA1. (B) Western blotting shows PHF and total tau expression 24 hours post global cerebral ischemia in total CA1 hippocampal lysates of female rats treated with placebo (Pla) or low-dose estrogen (E2) either immediately (STED) or 10 weeks (LTED) following bilateral ovariectomy. α-Tubulin is shown as a loading control.

Quantifications of band densities (Means + SE, n = 4-7 animals per group) are expressed as densitometric ratios of PHF to Tau. * = p < 0.05 compared to Sham; # = p < 0.05 compared to STED Placebo; ^ = p < 0.05 compared to STED E2. Magnification = 20X; Scale bar = 50μm (Scott et al., In Press).
3. Surgical Menopause Leads to an E2 Signaling Deficit in the Hippocampal CA1: A Role for the ER Co-regulator PELP1

3.1 Surgical Menopause Leads to a Decreased Expression of PELP1 in the Hippocampal CA1

Previously, our laboratory demonstrated that degradation of unliganded ERα occurs in the hippocampal CA1 of both 10-week ovariectomized and aged, reproductively senescent female rats (Zhang et al., 2011). Since we also used in vivo antisense knockdown studies to show that ERα was the main receptor responsible for E2’s neuroprotective effect against GCI (Zhang et al., 2009a), we believe that selective degradation of hippocampal ERα could, in part, mechanistically explain the observed loss of E2’s neuroprotective effects in surgically menopausal and aged rodents (Figure 1) (Zhang et al., 2009a, Zhang et al., 2011). However, additional factors may play important roles in this loss of E2 neuroprotection as well. In fact, these findings led us to further hypothesize that an E2 signaling deficit could develop in the hippocampus in response to chronic deprivation of ovarian E2. In the next set of studies, we examined hippocampal expression of the ER co-regulator Proline-, Glutamate-, and Leucine-Rich Protein 1 (PELP1), another member of the neural E2 signaling machinery that has been identified as a key mediator of E2 signaling in breast cancer (Mann et al., 2013). Again, STED and LTED female rats were used for these studies. Intriguingly, immunohistochemistry for PELP1 revealed that PELP1 expression was decreased approximately 40% in the
hippocampal CA1 of non-ischemic LTED female rats, compared to non-ischemic STED females (Figure 19).
Figure 19. Expression of the Estrogen Receptor Co-Regulator PELP1 is decreased in the Hippocampal CA1 Following Long-Term Ovariectomy. (A) Representative DAB staining demonstrates PELP1 expression in the hippocampal CA1 of non-ischemic rats either 1 week (STED) or 11 weeks (LTED) following ovariectomy. (B) Quantitative summary of data (Means + SE, n = 5-7 animals per group) shows raw integrated density of PELP1 immunostaining in the medial CA1. * = p < 0.05 vs. STED; Magnification = 20X; Scale bar = 50μm.
3.2 PELP1 is Successfully Knocked Down *In Vivo*

Since we observed a significant decrease of *basal* PELP1 expression in the hippocampal CA1 region following long-term ovariectomy, we wanted to determine the effect of decreased hippocampal PELP1 expression on E2’s neuroprotective signaling. To investigate, we knocked down the ER co-regulator PELP1 *in vivo* with intracerebroventricular (icv) injections of PELP1 anti-sense oligonucleotides in ovariectomized, E2-treated female rats prior to inducing GCI. Missense oligonucleotides served as a control. Daily icv injections began 72 hours prior to GCI, with the fourth and final injection administered 3 hours before ischemia. Importantly, we verified successful knockdown of PELP1 via western blot analysis at 3 reperfusion time points: 30 minutes (*Figure 20A*), 3 hours (*Figure 20B*), and 24 hours (*Figure 20C*), where we noted approximately 50%, 40%, and 30% decreases in PELP1 (E2+AS), respectively, relative to missense controls (E2+MS).
Figure 20. Effectiveness of PELP1 Anti-sense Knockdown in the Hippocampal CA1 30 Minutes, 3 Hours, and 24 Hours Following Global Cerebral Ischemia. (A) Western blotting shows PELP1 and α-Tubulin expression in the hippocampal CA1 30 minutes following GCI. Quantifications of band densities (Means + SE, n = 5-8 animals per group) are expressed as densitometric ratios of PELP1 to α-Tubulin. (B) Western blotting shows PELP1 expression in the hippocampal CA1 3 hours following GCI. Quantifications (Means + SE, n = 5-7 animals per group) are expressed as densitometric ratios of PELP1 to α-Tubulin. (C) Western blotting shows PELP1 and α-Tubulin expression in the hippocampal CA1 24 hours following GCI. Quantifications of band densities (Means + SE, n = 4-5 animals per group) are expressed as densitometric ratios of PELP1 to α-Tubulin. * = p < 0.05 compared to Sham; # = p < 0.05 compared to Placebo; ^ = p < 0.05 compared to E2+MS.
3.3 *In Vivo* Knockdown of PELP1 Leads to Loss of E2 Regulation of JNK/c-Jun/Dkk1 Signaling in the Hippocampal CA1 after GCI

We next examined whether hippocampal knockdown of PELP1 was sufficient to cause loss of E2’s neuroprotective signaling following GCI. Since previous work by our laboratory revealed that E2 could attenuate JNK/c-Jun/Dkk1 signaling following ischemia (Zhang et al., 2008), and since Aim 1 of the current study showed that E2 regulation of the same pathway is lost following LTED, we examined JNK/c-Jun/Dkk1 signaling status in PELP1 knockdown animals. Intriguingly, both western blotting and confocal microscopy analyses demonstrated that knockdown of hippocampal PELP1 significantly attenuated E2’s ability to prevent the post-ischemic phosphorylation of JNK (Figure 21). As seen in Figure 21A, immunohistochemistry shows that di-phosphorylated JNK typically increases in the hippocampal CA1 3 hours post GCI (Figure 21A: Placebo) and that E2 normally mitigates this event (Figure 21A: E2+MS), a finding that agrees with our previous study (Zhang et al., 2008). However, in the event of PELP1 knockdown, we observed that E2 could no longer prevent the di-phosphorylation, and subsequent activation, of pro-apoptotic JNK (Figure 21A: E2+AS). As shown in Figure 21B, western blotting corroborates the finding that E2 is unable to attenuate phosphorylation of JNK 3 hours post GCI in PELP1 knockdown animals.
Figure 21. Hippocampal PELP1 Knockdown Causes Loss of E2 Regulation of JNK Phosphorylation Following Global Cerebral Ischemia. (A) Representative confocal microscopy pictures depict expression of JNK phosphorylated at Thr 183 and Tyr 185 in the hippocampal CA1 3 hours following GCI. Quantitative summary of data (Means ± SE, n = 4-7 per group) is expressed as raw integrated density of di-phosphorylated-JNK immunostaining. (B) Western blotting shows di-phosphorylated JNK and Total JNK expression in total hippocampal CA1 protein lysates 3 hours following GCI. α-Tubulin is shown as a loading control. Quantifications of band densities (Means ± SE, n = 4-7 animals per group) are expressed as densitometric ratios of p-JNK to Total JNK. * = p < 0.05 compared to Sham; # = p < 0.05 compared to Placebo; ^ = p < 0.05 compared to E2+MS. Magnification = 40X; Scale bar = 50μm.
Since we observed a loss of E2 regulation of post-ischemic JNK phosphorylation in PELP1 knockdown animals, we decided to test whether E2 regulation of downstream c-Jun was lost in PELP1 knockdown animals as well. JNK is a serine/threonine kinase, and di-phosphorylation of JNK at Thr 183 and Tyr 185 is purported to activate JNK’s enzymatic activity, leading to downstream phosphorylation of the transcription factor c-Jun at Ser 63 and Ser 73 (Weston and Davis, 2007, Thevenin et al., 2011). Intriguingly, di-phosphorylation of JNK is thought to activate c-Jun as well, which subsequently leads to transcription of several pro-apoptotic genes, including that of the neurodegenerative Wnt antagonist Dkk1 (Grotewold and Ruther, 2002, Cappuccio et al., 2005). Along these lines, we performed double immunohistochemistry for both di-phosphorylated c-Jun and Dkk1 on brains harvested following our PELP1 knockdown paradigm. As seen in Figure 23, p-c-Jun immunostaining, shown in green, was mostly nuclear, which is in line with its role as a transcription factor, and Dkk1 immunostaining, shown in red, was extranuclear, which is in line with its role as a secreted glycoprotein. Interestingly, both p-c-Jun and Dkk1 expression, which co-localized to the same neurons, were elevated in the hippocampal CA1 24 hours after GCI (Figure 22: Placebo) and attenuated at the same reperfusion time point in E2-treated controls (Figure 22: E2+MS), two findings that agree with our previously published study (Zhang et al., 2008) and with Aim 1 of the current study (Figure 8). However, in PELP1 knockdown animals, E2 was no longer able to mitigate the post-ischemic elevation of p-c-Jun or Dkk1 (Figure 22: E2+AS), which suggests that PELP1 plays a key role in E2’s prevention of c-Jun phosphorylation and Dkk1 expression in the hippocampus after ischemia. We further confirmed the loss of
E2’s ability to attenuate post-ischemic Dkk1 elevation in PELP1 knockdown animals through western blot analysis, which showed that when hippocampal PELP1 was knocked down in E2-treated female rats prior to ischemia, E2 could no longer prevent the GCI-induced increase in Dkk1 expression in the hippocampal CA1 (Figure 23: E2+AS).
Figure 22. Hippocampal PELP1 Knockdown Causes Loss of E2 Regulation of c-Jun Phosphorylation and Dkk1 Expression Following Global Cerebral Ischemia. (A) Representative photomicrographs depict hippocampal CA1 expression of c-Jun phosphorylated at Ser 63 and Ser 73 (green) and Dkk1 (red) 24 hours after global cerebral ischemia. Note the nuclear localization of p-c-Jun and the extranuclear localization of Dkk1. (B) Quantitative summary of data (Means ± SE, n = 4-5 per group) is expressed as raw integrated density of p-c-Jun (black) or Dkk1 (gray) immunostaining. * = p < 0.05 compared to Sham; # = p < 0.05 compared to Placebo; ^ = p < 0.05 compared to E2+MS. Magnification = 40X; Scale bar = 50μm.
Figure 23. Hippocampal PELP1 Knockdown Leads to Loss of E2 Regulation of Ischemia-Induced Dkk1 Elevation. (A) Western blotting shows Dkk1 expression in total CA1 hippocampal lysates of E2-treated animals exposed to missense (E2+MS) or PELP1 anti-sense oligonucleotides (E2+AS) 24 hours after 10-minute global cerebral ischemia. α-Tubulin is shown as a loading control. (B) Quantifications of band densities (Means + SE, n = 4-5 animals per group) are expressed as densitometric ratios of Dkk1 to α-Tubulin. ^ = p < 0.05 vs. E2+MS.
These results suggest that E2 prevents the ischemia-induced phosphorylation of JNK and the subsequent activation of the JNK/c-Jun signaling pathway via a PELP1-mediated mechanism. To better understand how PELP1 may mediate E2’s effects, we next examined key kinase signaling pathway factors, including those in the JNK pathway, for potential interactions with PELP1, since PELP1 has several protein-protein interaction motifs. To investigate, total CA1 hippocampal protein lysates were collected at 30 minutes and 3 hours after GCI and subjected to PELP1 co-immunoprecipitation (co-IP). Intriguingly, PELP1 co-IP demonstrated that, in addition to known interactions with ERα and p85, PELP1 was capable of physically binding to several members of the JNK signaling pathway in the brain, including JNK itself (Table IV). Importantly, PELP1’s interaction with JNK and its upstream signaling effector MKK7 in the hippocampus was confirmed in the mouse, using a proteomics and mass spectrometry approach, by our collaborators (Dr. Ratna Vadlamudi’s Laboratory) at the University of Texas Health Science Center in San Antonio. In addition, mutational analysis at the Vadlamudi Lab further determined that PELP1 binds to JNK1 via amino acid residues 400-600 (data not shown). While we remain unsure of the precise mechanism through which E2 regulates the pro-apoptotic JNK signaling pathway, since PELP1 has no enzymatic activity (Vadlamudi and Kumar, 2007), we speculate that PELP1 may serve as a scaffolding protein that promotes formation of a large signaling complex. As such, this “signalsome” may facilitate the rapid post-translational modification of the JNK pathway proteins by alternate kinases, like Akt, or phosphatases, like MAP Kinase Phosphatase 1 (MKP-1),
thereby indirectly permitting E2 to rapidly modulate the activity of JNK after ischemic stress.
Table IV. PELP1 Physically Binds to Several JNK Signaling Pathway Members in the Hippocampal CA1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>PELP1 Interaction – 3h Reperfusion</th>
<th>PELP1 Interaction – 30m Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P85α</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>JNK*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M KK7*</td>
<td>+</td>
<td>+</td>
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<tr>
<td>pMLK3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>POSH#</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>JIP1</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Co-immunoprecipitation of the ER co-regulator PELP1 in total protein lysates at 30 minutes and 3 hours after GCI demonstrated that PELP1 could interact with JNK and several of its upstream signaling effectors in the hippocampal CA1 region of the rat. PELP1 exhibited known interactions with ERα and p85α in the rat hippocampal CA1 as well. A plus sign (+) signifies a protein-protein interaction was observed, and a minus sign (-) signifies that no protein-protein interaction was observed at the designated reperfusion time point. ERα, Estrogen Receptor Alpha; JIP1, JNK-Interacting Protein 1; JNK, c-Jun N-terminal kinase; M KK7, mitogen-activated protein kinase kinase 7; MLK3, mixed-lineage kinase 3; N/A, Not Applicable; POSH, plenty of SH3 domains (POSH). * = Interaction confirmed in mice at 30 minutes post GCI; # = p < 0.05 in E2+AS vs. E2+MS.
3.4 *In Vivo* Knockdown of PELP1 Leads to Loss of E2 Regulation of APP Processing in the Hippocampal CA1 after GCI

Activated JNK is thought to promote the amyloidogenic processing of APP in two ways: 1) via phosphorylation of APP at Thr 668 (Colombo et al., 2009) and 2) via c-Jun-mediated elevation of BACE1 (Tamagno et al., 2005, Guglielmotto et al., 2009, Tamagno et al., 2009). In Aim 2 of the current study, we observed enhanced elevation of post-ischemic BACE1 expression ([Figure 16](#)) in the hippocampal CA1 of LTED females, in whom E2’s regulation of JNK signaling is lost. In light of this finding, we next decided to examine E2’s ability to regulate endogenous APP processing after GCI in the event of PELP1 knockdown. Immunohistochemistry revealed that GCI promoted APP phosphorylation 3 hours after reperfusion ([Figure 24: Placebo](#)) and that E2 was able to prevent this event in missense-treated controls ([Figure 24: E2+MS](#)). However, when the ER co-regulator PELP1 was knocked down in the hippocampus, E2 was no longer able to attenuate ischemia-induced APP phosphorylation at this time point ([Figure 24: E2+AS](#)).
Figure 24. Hippocampal PELP1 Knockdown Leads to Loss of E2 Regulation of Ischemia-Induced Amyloid Precursor Protein Phosphorylation. (A) Representative photomicrographs depict hippocampal CA1 neurons immunopositive for Amyloid Precursor Protein phosphorylated at Threonine 668 (p-APP) 3 hours post 10-minute global cerebral ischemia. (B) Quantitative summary of data (Means ± SE, n = 4-7 per group) is expressed as raw integrated density of p-APP immunostaining in the medial CA1. * = p < 0.05 compared to Sham; # = p < 0.05 compared to Placebo; ^ = p < 0.05 compared to E2+MS. Magnification = 40X; Scale bar = 50μm.
Similarly, in the event of hippocampal PELP1 knockdown, immunohistochemistry demonstrated that E2 could no longer attenuate expression of BACE1 in the hippocampal CA1 3 hours post GCI (Figure 25: E2+AS), while it could attenuate post-ischemic BACE1 elevation in E2-treated missense controls (Figure 25: E2+MS). As such, these data suggest that PELP1 plays a critical role in E2’s regulation of endogenous APP processing following GCI.
Figure 25. Hippocampal PELP1 Knockdown Leads to Loss of E2 Regulation of Post-Ishemic BACE1 Elevation. (A) Representative photomicrographs depict hippocampal CA1 neurons immunopositive for Beta-Amyloid Cleaving Enzyme 1 (BACE1) 3 hours post 10-minute global cerebral ischemia. (B) Quantitative summary of data (Means ± SE, n = 4-7 per group) is expressed as raw integrated density of BACE1 immunostaining in the medial CA1. * = p < 0.05 compared to Sham; # = p < 0.05 compared to Placebo; ^ = p < 0.05 compared to E2+MS. Magnification = 40X; Scale bar = 50 μm.
3.5 *In Vivo* Knockdown of PELP1 Reverses E2 Neuroprotection Status in the Hippocampal CA1

Finally, to determine whether the ER co-regulator PELP1 is a critical mediator of E2 neuroprotection, we assessed E2 neuroprotection status in PELP1 knockdown animals 7 days following GCI. Intriguingly, as seen in Figure 27, *in vivo* knockdown of hippocampal PELP1 was sufficient to reverse E2 neuroprotection status, suggesting that PELP1 is, indeed, a critical mediator of E2 neuroprotection from GCI. As mentioned earlier, transient GCI selectively damages the hippocampus, leading to widespread, delayed loss of pyramidal neurons after the ischemic event (Pulsinelli et al., 1982, Petito et al., 1987). Furthermore, as repeatedly shown by our lab and others, pretreatment with subcutaneous low-dose E2 prior to GCI affords robust neuroprotection of the CA1 region in female rats (Brann et al., 2007, Zhang et al., 2008, Zhang et al., 2009a, Zhang et al., 2011, Scott et al., 2013, Zhang et al., 2013a). Importantly, this finding was reproduced by the current study, which used NeuN (Figure 26 A) and Cresyl Violet staining (Figure 26 B) to show that low-dose E2 prevented GCI-induced cell death in the hippocampal CA1 of missense-treated controls (Figure 26: E2+MS). In contrast, PELP1 knockdown led to a loss of E2’s ability to exert neuroprotection in the hippocampal CA1 region after GCI (Figure 26: E2+AS). These findings suggest that the ER co-regulator PELP1 is required for E2 to exert its neuroprotective effects in the hippocampus during an ischemic event. They further suggest that a significant decrease in hippocampal expression of PELP1, such as that observed in non-ischemic LTED sham animals (Figure 19), could partially explain the loss of E2 neuroprotection seen following long-term ovariectomy.
Figure 26. Hippocampal PELP1 Knockdown Reverses E2 Neuroprotection from Global Cerebral Ischemia. (A) Representative photomicrographs depict hippocampal CA1 neurons immunopositive for the neuronal nuclear marker NeuN 7 days post 10-minute global cerebral ischemia. Quantitative summary of data (Means + SE, n = 5-7 per group) is expressed as a percentage of NeuN-positive neurons per 250μm medial CA1 of E2+MS animals. ^ = p < 0.05 compared to E2+MS. Magnification = 40X; Scale bar = 50μm. (B) Representative Cresyl Violet staining in the hippocampal CA1 7 days post 10-minute global cerebral ischemia. Quantitative summary of data (Means + SE, n = 5-7 per group) is expressed as a percentage of Cresyl Violet-positive neurons per 250μm medial CA1 of E2+MS animals. ^ = p < 0.05 compared to E2+MS. Magnification = 40X; Scale bar = 50μm.
IV. DISCUSSION

1. Elevation of Neurodegenerative Factors

The results presented in this dissertation all center around the three molecular mechanisms proposed to underlie the enhanced risk of dementia and mortality from neurological disorders observed in prematurely menopausal women: 1) elevation of neurodegenerative factors, 2) enhanced stress-induced amyloidogenesis, and 3) a neural E2 signaling deficit (Figure 2). In regard to the elevation of neurodegenerative factors, Aim 1 was to test the hypothesis that long-term ovarian E2 deprivation (LTED) leads to dysregulation of neurodegenerative Dkk1 and Wnt/Beta-Catenin signaling in the hippocampal CA1 region and to decreased sensitivity of this pathway to E2 regulation.

According to Scott et al. (2013), “the current study yielded 2 major findings - 1) LTED (either naturally via aging or surgically via bilateral ovariectomy) leads to dysregulation of basal Dkk1-Wnt/β-catenin signaling in the hippocampal CA1 region of female rats, and 2) E2 loses its ability to attenuate ischemia-induced Dkk1 elevation following LTED.

With respect to the first finding, up-regulation of basal Dkk1 and a corresponding down-regulation of basal survivin in the hippocampal CA1 region of LTED rats could suggest that there is increased neuronal cell death occurring after LTED in the non-ischemic state. However, immunostaining for the neuronal marker NeuN in the
hippocampal CA1 region failed to show any evidence of increased neuronal cell death in non-ischemic LTED animals as compared to non-ischemic STED animals (Figure 1). Furthermore, the modest Dkk1 elevation in the CA1 region of non-ischemic aged animals did not show significant co-localization with the DNA damage/apoptotic marker TUNEL. In contrast, the robust induction of Dkk1 observed following GCI in aged rats was highly co-localized with TUNEL. Taken as a whole, these findings suggest that the basal elevation of Dkk1 in LTED and aged non-ischemic rats is not sufficient to induce neuronal cell death.

However, it is possible that basal changes in the hippocampal expression of Dkk1 and survivin in LTED and aged non-ischemic rats may cause these animals to be more sensitive to [ischemia and other types of] insults. In support of this contention, we (Zhang et al., 2009a, Zhang et al., 2011) and others (Xu et al., 2007, Xu et al., 2008, De Butte-Smith et al., 2012, Inagaki et al., 2012) have reported that the LTED and aged rat hippocampus is more susceptible to damage from global cerebral ischemia. Obviously, in regard to the current study, lower survivin levels could make the hippocampal neurons more susceptible to ischemic stress because survivin is known to prevent cell death by inhibiting the cleavage and, thus, activation of pro-apoptotic caspases (Shin et al., 2001, Li et al., 2004, Wang and Zheng, 2004, Wang et al., 2004). Along these lines, the decrease in survivin observed in the current study is likely due to the fact that Dkk1, as a Wnt/β-catenin pathway antagonist, prevents expression of critical β-catenin pro-survival genes, such as survivin. It is interesting to note that there is also a report of basal changes in activation of cAMP response element binding protein (CREB), Akt, and STAT3 in the hippocampal CA1 of LTED female rats (De Butte-Smith et al., 2012), which may also
play a role in the observed increased sensitivity of the LTED hippocampus to damage from GCI.

Intriguingly, Dkk1 is thought to be under transcriptional control of the stress sensor p53 (Wang et al., 2000, Cappuccio et al., 2005), and we have reported that p53 appears to be stabilized via acetylation in non-ischemic LTED animals (Raz et al., 2011). Therefore, it is possible that the modest increase we observed in basal Dkk1 levels following LTED could be due to enhanced p53-mediated transcription of Dkk1 in the absence of ischemic stress. Once ischemia occurs, however, Dkk1 is then dramatically elevated above the threshold required to induce cell death. We propose that this event occurs due to robust activation of the pro-death JNK/c-Jun signaling cascade, which ultimately leads to neuronal apoptosis in the hippocampal CA1 region following GCI. Along these lines, Dkk1 is also thought to be under transcriptional control of c-Jun (Grotewold and Ruther, 2002, Cappuccio et al., 2005), and we hypothesized that JNK/c-Jun activation might be responsible for the dramatic, post-ischemic elevation of Dkk1. In support of these contentions, we demonstrated that inhibition of JNK strongly attenuates GCI-induced Dkk1 elevation, survivin depression, and neuronal apoptosis in the CA1 region of LTED rats. Thus, in the current study, we provide evidence that JNK/c-Jun activation plays a critical role in the regulation of Dkk1 and survivin and the resultant hippocampal neuronal cell death in LTED animals after GCI.

The second major finding of the current study was that the ability of E2 to modulate Dkk1/Wnt-β-catenin signaling is lost in LTED rats. As such, the loss of E2’s ability to regulate Dkk1 and Wnt-β-catenin signaling after prolonged hypoestrogenicity may help explain previous reports that LTED is associated with a loss of E2 neuroprotection in the
hippocampal CA1 region following GCI (Zhang et al., 2009a, Zhang et al., 2011). Since Dkk1 has been found to be elevated in human neurodegenerative disorders, such as stroke and Alzheimer’s disease, the current findings, if applicable to humans, could also help explain the doubled lifetime risk of dementia and 5-fold increase in mortality from neurological disorders in women who enter menopause prematurely due to bilateral oophorectomy (Rivera et al., 2009, Rocca et al., 2010, Shuster et al., 2010, Rocca et al., 2011). Furthermore, E2’s inability to suppress ischemia-induced Dkk1 following LTED supports the critical period hypothesis of E2 replacement (Maki, 2006, Sherwin, 2007), which holds that E2 replacement must be initiated at peri-menopause to be beneficial. Along these lines, this observation could also help shed light on the unexpected negative results of the WHI study, which suggested that oral hormone replacement therapy led to an increased risk of ischemic stroke and dementia in postmenopausal women aged 65 and older (Shumaker et al., 2003, Wassertheil-Smoller et al., 2003), and provide critical support for the implementation of perimenopausal, transdermal E2 replacement,” (p. 629-631) (Scott et al., 2013).

2. Enhanced Stress-Induced Amyloidogenesis

In regard to enhanced stress-induced amyloidogenesis, Aim 2 was to test the hypothesis that LTED leads to enhanced amyloidogenesis after ischemic injury to the hippocampus and to decreased sensitivity of this pathway to E2 regulation. Along these lines, the current study yielded several novel observations: 1) acute regulation of the α-secretase ADAM 10 in the hippocampal CA1 by both GCI and E2, 2) evidence of an ischemia-induced switch to amyloidogenic processing of APP in the hippocampal CA1,
3) a robust increased hippocampal Aβ load in LTED female rats after an ischemic insult, and 4) a loss of E2’s ability to regulate post-ischemic changes in ADAM 10, ADAM17, BACE1, and PHF following LTED. Collectively, these findings suggest that E2’s ability to prevent post-ischemic hippocampal AD-related protein induction is, indeed, lost after chronic loss of ovarian function (10-week ovariectomy) (Scott et al., In Press).

With respect to the first finding (acute regulation of ADAM 10 by GCI or E2), expression of the α-secretase ADAM 10 has been shown to be decreased by oxygen-glucose-deprivation, chronic hypoxia, and chronic anoxia in primary cortical neurons, neuroblastoma cells, and cerebral microvascular smooth muscle cells, respectively, in vitro (Auerbach and Vinters, 2006, Lee et al., 2006, Marshall et al., 2006). However, this is the first study, to our knowledge, demonstrating an acute loss of hippocampal ADAM 10 expression following ischemia in vivo. On the other hand, E2 signaling has been recently linked with modulation of ADAM 10 in the brain. In fact, two green tea derivatives, (-)-epigallocatechin-3 gallate (EGCG) and octyl gallate, were recently reported to reduce Aβ plaque load in transgenic AD mouse models via an ERα/PI3K/Akt signaling mechanism that led to maturation and increased α-secretase activity of ADAM 10 (Fernandez et al., 2010, Zhang et al., 2013b). An additional study revealed that administration of 100mg/kg E2 to an ovariectomized, D-galactose-injected rat model of AD led to elevation of ADAM 10, reduction of BACE1, and alleviation of spatial memory deficits (Zhang et al., 2012). The current study corroborates these findings by showing that low, Diestrus I levels of E2 are capable not only of preventing GCI-induced loss of hippocampal ADAM 10 in vivo. Furthermore, our results expand upon these findings by demonstrating E2 regulation of ADAM 10 expression in wild type, non-
transgenic rodents, suggesting that E2 may play a key role in *endogenous* non-amyloidogenic processing of APP in the hippocampus. It should be mentioned here that a single study noted that 4-month ovariectomy led to an *increase* in ADAM 10 mRNA in the absence of ischemia (Anukulthanakorn et al., 2013). While the current study did not find an elevation of ADAM 10 expression in non-ischemic LTED sham animals, it used a much shorter ovariectomy period (10 weeks). Thus, these findings are not necessarily in disagreement with our results (Scott et al., *In Press*).

The second novel finding of Aim 2 was evidence of a post-ischemic switch to amyloidogenic processing of APP in the hippocampal CA1 region following LTED. Along these lines, we observed an ischemia-induced loss of hippocampal protein expression of both α-secretases ADAM 10 and ADAM 17, as well as a concurrent, ischemia-induced increase of hippocampal protein expression of the β-secretase BACE1 in LTED females. While neuronal expression of the α-secretase ADAM 10 has not been previously studied in the context of ischemia *in vivo,* neuronal expression of ADAM 17, or TNF α-converting enzyme (TACE), has been demonstrated to be enhanced following ischemic preconditioning (Cardenas et al., 2002, Pradillo et al., 2005). Since this suggests that upregulation of ADAM 17/TACE is a neuroprotective mechanism responsible for the phenomenon of ischemic tolerance, downregulation of an α-secretase, like ADAM 17, in the hippocampus following ischemic stress could be detrimental to neurons. As such, a marked decrease in expression of *two* neuroprotective α-secretases in LTED females after ischemia could partially explain the hippocampal hypersensitivity to GCI-induced cell loss observed in LTED females (Zhang et al., 2009a, Zhang et al., 2011, Zhang et al., 2013a). Exogenous E2 has been shown to modulate expression of both ADAM 10 and
ADAM 17 *in vitro* and *in vivo* (Nord et al., 2010, Nadadur et al., 2012, Zhang et al., 2012). However, this is the first study to suggest that ovarian-derived E2 may promote non-amyloidogenic processing of APP following ischemic stress via modulation of α-secretase expression in hippocampal neurons *in vivo*. Along with the significant increase in amyloidogenic BACE1 in LTED females subjected to GCI, robust loss of non-amyloidogenic ADAM10 and ADAM 17 suggests that prolonged loss of ovarian E2 may promote a switch to amyloidogenic processing of APP in the event of ischemia. This observation extends a recent report by our laboratory, which described a post-ischemic switch to amyloidogenic processing of APP in the hippocampal CA3 of LTED females, which become hypersensitive to both GCI and Aβ neurotoxicity (Zhang et al., 2013a).

The current study demonstrates that this process also occurs in the critical CA1 region of LTED females. Furthermore, it shows that E2 is capable of regulating of two putative α-secretases (ADAM 10 and ADAM 17) in addition to its known regulation of the β-secretase BACE1. These additional findings are particularly important because they suggest that the post-ischemic switch to amyloidogenic APP processing, which occurs following LTED, is not region-specific. Along these lines, it will be important for future studies to determine whether long-term ovariectomy only enhances stress-induced amyloidogenesis in the hippocampus or if this event occurs in other critical regions of the brain, such as the cerebral cortex, as well (Scott et al., *In Press*).

The third major finding of Aim 2 was an increased Aβ load in the hippocampal CA1 of LTED females subjected to GCI. This observation corroborates the changes seen in both α-secretase and β-secretase expression following ischemia in LTED females, suggesting that the post-ischemic switch to amyloidogenic processing of APP does, in
fact, enhance amyloidogenesis in the LTED female hippocampus. Furthermore, this finding agrees with our previous study, which observed a switch to amyloidogenic APP processing and a resulting increase in Aβ immunoreactivity in the hippocampal CA3 of LTED females following GCI (Zhang et al., 2013a). While not examined in this study, it is possible that a loss of E2 regulation of Aβ clearance mechanisms could occur following LTED as well. Considering that E2 has been shown to upregulate both nepriysin and insulin-degrading enzyme (Huang et al., 2004, Xiao et al., 2009, Liang et al., 2010, Jayaraman et al., 2012, Li et al., 2013), as well as microglial phagocytosis of Aβ (Li et al., 2000), it would be interesting to determine whether long-term loss of ovarian E2 increases post-ischemic hippocampal Aβ load only through enhancing amyloidogenesis or whether it prevents the clearance of insoluble Aβ as well. One concern is that premature surgical menopause, which is associated with a doubled lifetime risk of dementia (Rocca et al., 2007), alone may enhance neurotoxic Aβ deposition in the brain. However, neither the current study nor our previously published work (Zhang et al., 2013a) found an increase of Aβ in the hippocampus of LTED sham animals. Furthermore, an unrelated study found that the total hippocampal BACE1/ADAM10 mRNA ratio, which reflects the status of amyloidogenic processing of APP, was unchanged in 4-month ovariectomized females (Anukulthanakorn et al., 2013). Together, these studies suggest that LTED alone does not promote a switch to amyloidogenic processing of APP and that an acute stressor is required for hippocampal amyloidogenesis to occur (Scott et al., In Press).

The fourth and final finding of Aim 2 was a loss of E2 regulation of post-ischemic changes in hippocampal ADAM 10, ADAM 17, BACE 1, and PHF following LTED.
This finding agrees with the previous study of our colleagues, which found a loss of E2 regulation of BACE1 and PHF in the hippocampal CA3 region of LTED females after ischemia (Zhang et al., 2013a). Furthermore, it extends the aforementioned study to the critical CA1 region of the hippocampus and shows, for the first time, that E2’s ability to regulate α-secretase expression is lost following LTED as well. Importantly, this finding is also in agreement with a growing body of literature that suggests E2 loses its ability to regulate neural factors following long-term ovariectomy (Bohacek and Daniel, 2010, Hamilton et al., 2011, Ding et al., 2013) ((Suzuki et al., 2007, Zhang et al., 2009a, Raz et al., 2011, Zhang et al., 2011, Scott et al., 2013, Zhang et al., 2013a). It should be mentioned here that one important concern is whether enhanced post-ischemic development of AD-like neuropathology in a region critical for learning and memory would worsen neurocognitive outcome following an ischemic insult. Indeed, our colleagues found that ischemic LTED female rats, which displayed enhanced amyloidogenesis and Aβ load in the CA3 region of the hippocampus, performed worse on the Morris water maze than their ischemic STED counterparts (Zhang et al., 2013a). As such, this suggests that the enhanced post-ischemic AD-like neuropathology seen in LTED female rats further impairs neurocognitive functioning. Thus, collectively, the novel observations of Aim 2 provide evidence that prolonged loss of ovarian-derived E2 could predispose the hippocampus to the development of AD-like neuropathology (increased hippocampal Aβ and PHF) in the event of ischemic stress. This could occur due to the loss of E2’s ability to regulate post-ischemic changes in AD-related proteins, such as the α- and β-secretases and the microtubule-associated protein tau (Scott et al., In Press).
3. Neural E2 Signaling Deficit

Aim 3 was to test the hypothesis that LTED leads to an E2 signaling defect in the hippocampus, which involves dysregulation of the ER coregulator protein, PELP1. Along these lines, we made three novel observations in support of this hypothesis – 1) hippocampal PELP1 expression is downregulated after 10-week ovariectomy, 2) in vivo knockdown of PELP1 interrupts neuroprotective E2 signaling, and 3) PELP1 physically binds to members of the JNK signaling pathway. Our lab previously reported that unliganded ERα was selectively degraded in the hippocampus of 10-week ovariectomized and aged, reproductively senescent (24-month-old) female rats, in whom E2’s neuroprotective ability is lost (Zhang et al., 2011). This finding was proposed to mechanistically explain the “critical period hypothesis,” since it suggested that once circulating levels of ovarian E2 have decreased, the hippocampus may become less sensitive to E2 due to a decrease in neuronal expression of its cognate receptor. However, the study did not address whether other members of the E2 signaling machinery were altered following long-term ovariectomy or whether these potential changes could signify development of a neural E2 signaling deficit following LTED. The findings of the current study provide evidence that this, indeed, may be the case, as our first observation was a 40% decrease in protein level of the ER co-regulator PELP1 in the hippocampal CA1 of LTED Sham animals (Figure 19). We next used an in vivo PELP1 antisense knockdown approach to determine exactly how downregulation of hippocampal PELP1 expression would affect neural E2 signaling in short-term ovariectomized rats, in which E2’s neuroprotective ability remains intact. Along these lines, our second, related finding was that PELP1 knockdown interrupts E2’s neuroprotective signaling following GCI.
Specifically, the current study detailed a loss of E2’s ability to prevent post-ischemic activation of the pro-apoptotic JNK/c-Jun/Dkk1 signaling pathway, a loss of E2’s ability to regulate post-ischemic AD-related protein induction, and a reversal of E2 neuroprotection status in the hippocampus of PELP1 knockdown females.

While several studies have previously identified a decrease in neural expression of ERα following LTED (ovariectomy or natural aging) and the consequences of this event on E2’s neuroprotective signaling (Zhang et al., 2011, Foster, 2012, Navarro et al., 2013), we have shown, for the first time, that LTED leads to a decrease in neural expression of the ER co-regulator PELP1. Furthermore, we have provided direct evidence that decreased PELP1 expression reverses E2’s neuroprotective signaling in vivo. However, it is important to note that the current study only examined PELP1 expression and E2 signaling status in hippocampal CA1 neurons. Along these lines, it would be interesting to determine whether the LTED-induced decrease in PELP1 expression and the resulting dysregulation of E2’s neuroprotective signaling are region- and/or cell-specific. In addition, while these two findings extend the aforementioned study of LTED’s effects on hippocampal ERα, they do not entirely resolve the questions of whether other members of the E2 signaling machinery, besides ERα and PELP1, are affected by prolonged loss of ovarian E2 and how these potential changes could impact neural E2 signaling. Furthermore, they raise additional questions as to whether selective degradation of unliganded ERα and downregulation of PELP1 are related events and whether these two events act independently or synergistically in neurons to impair E2’s neuroprotective signaling following LTED.
An intriguing aspect of the PELP1 knockdown-induced neural E2 signaling deficit is the similarity to the neural E2 signaling deficit observed in LTED females. In Aim 1, we demonstrated that both long-term ovariectomy and natural aging interfered with E2’s ability to prevent a post-ischemic increase of the neurodegenerative Wnt antagonist Dkk1 in the hippocampal CA1 (Figures 8 and 13). Furthermore, in Aim 2, we showed that E2 could no longer prevent the ischemia-induced increase in BACE1 expression in LTED females (Figure 16), and our colleagues showed that the same event occurs in the hippocampal CA3 after LTED (Zhang et al., 2013a). Similarly, in Aim 3, we observed a loss of E2’s ability to prevent post-ischemic elevations of p-JNK (Figure 21), Dkk1 (Figures 22 and 23), and BACE1 (Figure 25) in PELP1 knockdown females.

In addition, both the current study and previous reports have provided evidence that LTED is associated with a loss of E2 neuroprotection from GCI (Figures 1 and 13) (Suzuki et al., 2007, Zhang et al., 2009a, Zhang et al., 2011, Zhang et al., 2013a), and in Aim 3 of the current study, we also demonstrated that in vivo knockdown of the ER co-regulator PELP1 was sufficient to cause loss of E2 neuroprotection from GCI (Figure 26). Since both LTED and in vivo PELP1 knockdown resulted in a 40-50% decrease in PELP1 hippocampal CA1 protein levels (Figure 19), the current study suggests that PELP1 is a critical mediator of E2 neuroprotection and that a decrease in hippocampal expression of PELP1, regardless of whether it occurs as a result of LTED or antisense knockdown, severely disrupts E2’s neuroprotective signaling in the hippocampus, leading to a neural E2 signaling deficit.

The third and final novel observation of this aim was a physical interaction between the ER co-regulator PELP1 and JNK signaling pathway members JNK, MKK7,
MLK3, and POSH in the brain (Table IV). While previous studies have shown that E2 prevents activation (di-phosphorylation) of JNK following GCI (Zhang et al., 2008) and that this ability is lost following LTED (Zhang et al., 2013a), exactly how E2 signaling influences this event remains unclear. Our lab previously reported that GCI-induced JNK signaling in the hippocampal CA1 region critically depends on the upstream activation of Rac1, which leads to Rac1-POSH-MLK3 complex formation, enzymatic activation of the MLK3-MKK4/7-JNK signaling cascade, and neuronal apoptosis after GCI (Zhang et al., 2009b). Importantly, our lab has also shown that both E2 and EDC can prevent post-ischemic Rac1 activation, thereby mediating neuroprotection from GCI (Zhang et al., 2009a). While the previous study focused on E2 and EDC’s ability to attenuate Rac1-induced NADPH oxidase activation and superoxide production after GCI, a logical conclusion that follows is that E2/EDC’s prevention of Rac1 activation can also attenuate Rac1-induced pro-apoptotic JNK signaling in the hippocampal CA1 in the event of GCI. However, this hypothesis has not been tested directly. Aim 3 of the current study found that the ER co-regulator PELP1 can physically bind to POSH, MLK3, MKK7, and JNK, all of which are downstream of Rac1, in the hippocampal CA1 region following ischemia. Furthermore, we also observed a POSH-Rac1 interaction in the hippocampal CA1 3h post GCI, a finding that agrees with our previous study (Zhang et al., 2009b), and PELP1 was found to interact with both POSH and ERα at the same reperfusion time point (Table IV). Collectively, these findings suggest that E2 may regulate the pro-apoptotic JNK signaling pathway at multiple levels via ERα and the ER co-regulator PELP1. As mentioned earlier, an interesting theory is that non-enzymatic PELP1 functions as an scaffold that brings ERα in close proximity to JNK signaling pathway members to form a
Thus facilitating E2-induced post-translational modulation of the JNK pathway via alternate kinases and phosphatases, such as Akt and MKP-1, respectively. Involvement of the aforementioned alternative proteins is plausible, as E2 is well known to activate the PI3K-Akt signaling pathway to afford neuroprotection (Scott et al., 2012), and E2’s inhibition of Rac1 activation, as well as its neuroprotective effects, can be abrogated with an Akt inhibitor (Zhang et al., 2009a). Furthermore, preliminary data from our laboratory suggest that E2 can regulate expression and activation of MKP-1, a neuroprotective phosphatase shown to de-phosphorylate, and thus directly inhibit the activation of, pro-apoptotic JNK (Koga et al., 2012), following GCI (Unpublished Observation). However, more work is needed to determine the precise mechanism(s) through which E2 signaling regulates the JNK signaling pathway.

Estrogen receptor knockdown and knockout studies have demonstrated that ERα is the principal mediator of E2 neuroprotection from cerebral ischemia (Dubal et al., 2001, Merchenthaler et al., 2003, Zhang et al., 2009a). Additional studies have also provided evidence that extranuclear ERs are critical mediators of E2’s neuroprotective signaling (Zhang et al., 2009a, Yang et al., 2010). The current study agrees with these findings, as ERα was found to form a signaling complex with PELP1, POSH, MLK3, MKK7 and JNK 3h post GCI, a reperfusion time point at which anti-sense knockdown of PELP1 was shown to reverse E2’s ability to prevent ischemia-induced phosphorylation of pro-apoptotic JNK. Furthermore, the current study showed that E2’s ability to exert neuroprotection via regulating post-ischemic Dkk1/Wnt-β-Catenin signaling and AD-related protein induction was lost following LTED (10-week ovariectomy or natural aging), when ERα is significantly downregulated in the brain (Zhang et al., 2011,
Navarro et al., 2013). Conversely, the rapidity of ischemia-induced changes observed in ERα-Pelp1 signalsome formation (30 minutes – 3 hours) and in phosphorylation states of JNK and APP (3 hours) also suggest the involvement of rapid extranuclear E2 signaling, even though EDC and/or E2-BSA were not employed in the current study. Along these lines, it is intriguing to consider the possibility that both nuclear and extranuclear ERα may play key roles in neuroprotective E2 signaling. However, other extranuclear E2 receptors have been proposed to exist, such as GPR30, ER-X, and Gq-mER, and these receptors may significantly contribute to the observed neuroprotective effects of E2 (Roepke et al., 2011). As such, future studies employing extranuclear ER-specific ligands (EDC, E2-BSA, G1, STX) and extranuclear ER knockdown/knockout approaches will significantly advance the field. Furthermore, while the ER co-regulator Pelp1 has been extensively studied as a mediator of E2 signaling in breast cancer cells (Vadlamudi and Kumar, 2007, Vadlamudi et al., 2010, Mann et al., 2013) and has been shown to co-localize with ERα-positive neurons in the brain (Khan et al., 2005), for the first time, the current study has directly implicated Pelp1 in neural E2 signaling. Along these lines, future studies should continue to characterize the physiological role of Pelp1 in the brain, both dependent and independent of E2, and study pathological changes in Pelp1 expression, subcellular localization, and post-translational modification.

4. Translational Relevance

While observational studies have shown that women who enter menopause prematurely via bilateral oophorectomy have a doubled lifetime risk of dementia and a 5-fold increased risk of mortality from neurological disorders (Rocca et al., 2007, Rivera et
al., 2009), due to ethical constraints, it is impossible to prospectively study the
detrimental neurological effects of premature surgical menopause in women. Thus, the
current study employed a rat model of premature surgical menopause, in which young-
adult female rats (3-months old) were bilaterally ovariectomized and allowed to age for
10 weeks (5 ½ months old) in order to identify molecular mechanisms that could
potentially underlie these enhanced neurological risks. We chose this model because: 1)
women who have bilateral oophorectomy are, on average, much younger than naturally
menopausal women (Shuster et al., 2008, Rocca et al., 2011), 2) removal of both ovaries
is a common form of premature surgical menopause in humans (Howe, 1984, Henderson
and Sherwin, 2007), and 3) 10-week ovariectomy was previously demonstrated to be
sufficient to induce loss of E2 neuroprotection in rats (Suzuki et al., 2007, Zhang et al.,
2009a, Zhang et al., 2011). We also chose GCI as our stressor because 1) GCI selectively
damages the hippocampus, a critical learning and memory structure targeted by
Alzheimer’s disease, 2) GCI models cardiac arrest and resuscitation, an event that is more
likely to occur with age in humans, 3) GCI-induced neuronal damage is highly
reproducible, which makes comparison between animals easier, and 4) E2 is highly
neuroprotective in this model. However, there are several caveats, which make our
models less than ideal.

The most important caveat is that GCI is a fairly severe stressor. Along these
lines, most of the prematurely menopausal women who eventually developed dementia in
the aforementioned observational studies probably did not do so as a result of cardiac
arrest. More likely culprits for neuronal stress in these women would be more chronic
and include sub-clinical transient ischemic attacks (TIAs or “mini-strokes”) and/or
asymptomatic exposure to Aβ. Along these lines, it would be interesting to see if less severe stressors following GCI (hypoxia or Aβ) would be sufficient to induce the same changes observed in the current study. Intriguingly, another study in our lab stereotaxically injected Aβ into the hippocampal CA3 region of short-term and long-term ovariectomized female rats and found that these neurons were more susceptible to Aβ-induced cell death after LTED (Zhang et al., 2013a). As such, this study supports the current hypothesis, as it suggests that after prolonged loss of ovarian E2, hippocampal neurons become hypersensitive to less severe, non-ischemic stressors. Thus, we believe that the findings of the current study do yield significant translational relevance to prematurely menopausal women.

5. Therapeutic Considerations and Future Directions

The results of the current study echo the findings of observational studies in prematurely menopausal women, suggesting that bilateral oophorectomy carries significant negative neurological risks if performed well before the age of natural menopausal onset (Phung et al., 2010, Rocca et al., 2010, Bove et al., 2013). Furthermore, our data agree with the assessment that premenopausal oophorectomy should be reserved as a last resort of treatment for ovarian disease or ovarian cancer prevention, with ET initiated at the time of menopausal onset and continued until the median age of natural menopausal onset to offset the potential for negative neurological sequelae (Rocca et al., 2011, Rocca and Ulrich, 2012, de Villiers et al., 2013), as delayed ET was ineffective in affording neuroprotection in the current study. However, an important question remains: how do the assessments of the current study relate to natural
menopause? While studies have not yet found a significant detrimental effect of *natural* menopause on neurological health, with our aging population, negative neurological consequences of natural menopause may become apparent 30 or 40 years following the menopausal transition.

In this regard, menopausal hormone therapy (MHT) for traditionally menopausal women remains quite controversial. As mentioned earlier, the surprisingly negative results of the WHI study led to development of the “critical period” or “critical window” hypothesis for E2 benefit (Maki, 2006, Sherwin, 2007). The current study supports this hypothesis, as delayed E2 was unable to prevent the ischemia-induced elevation of the neurodegenerative factor Dkk1, hyperphosphorylation of tau, and enhanced amyloidogenesis in the hippocampal CA1 in LTED females. While there is no provision for utilizing E2 for the protection of neurological health in *naturally* menopausal women, the current global consensus statement asserts that use of MHT within 10 years of menopausal onset, or before the age of 60, is a highly effective treatment for vasomotor symptoms of E2 deficiency; may decrease the risk of osteoporosis, coronary heart disease, and all-cause mortality; only yields a low absolute risk of VTE or stroke, particularly if transdermal preparations are used; and should be a highly-individualized decision (de Villiers et al., 2013). Along these lines, women and physicians should be vigilant and mindful of menopausal status, so as to ensure *timely* initiation of MHT, if desired.

Furthermore, as discussed earlier, the caveats of the WHI led to re-examination of the “ideal” MHT regimen. The current study utilized low-dose, subcutaneous 17β-estradiol in bilaterally oophorectomized rats, and this preparation is highly relevant to
MHT in humans. In fact, clinical trials have shown that low-dose, transdermal E2 is far superior to oral E2, with respect to risk of VTE (Canonico et al., 2007, Canonico et al., 2010). However, the preferred type of estrogen, or estrogen plus and progestogen for women with an intact uterus, is still subject to debate. An important consideration of hormone-based therapy is the development of hormone-sensitive cancers, such as breast cancer. According to the global consensus statement, “the risk of breast cancer in women over 50 years associated with MHT is a complex issue. The increased risk of breast cancer is primarily associated with the addition of a progestogen to estrogen therapy and related to the duration of use. The risk of breast cancer attributable to MHT is small and the risk decreases after treatment is stopped,” (de Villiers et al., 2013). Along these lines, creation of an estrogen that selectively targets the brain and/or vasculature without exerting trophic effects on the breast would be ideal. Importantly, selective estrogen receptor modifiers (SERMs) and brain-selective SERMs (neuroSERMs) are under development, and these compounds could possibly provide all the benefits of traditional E2 without exerting carcinogenic or feminizing side-effects (Brinton, 2004, Simpkins et al., 2005, Simpkins et al., 2009). As such, potential success of these compounds could lead to a revision of the global consensus statement on MHT, making it a preventative neuroprotectant agent as well.

Future directions for the field also include firmly defining the “critical window” for E2 benefit. Key clinical trials of perimenopausal E2 therapy, such as KEEPS and ELITE, are currently being analyzed and will, hopefully, shed more light on this important timing issue (Harman et al., 2005a, Menon and Vongpatanasin, 2006, Miller et al., 2009, Henderson and Brinton, 2010). Another goal of future study is to identify
additional molecular mechanisms underlying the enhanced risk of neurological disease in prematurely menopausal women, with the hope that doing so would lead to identification of non-hormonal therapeutic targets, which could protect the neurological health of postmenopausal women in whom the critical window has passed or in whom MHT is otherwise contraindicated. Furthermore, as mentioned previously, since the current study has, for the first time, implicated the estrogen receptor co-regulator PELP1 in neural E2 signaling and found a change in PELP1 following long-term ovariectomy, it would be beneficial to further characterize the physiological role of PELP1 in the brain. As such, the current study, along with those proposed, should significantly advance the field and hopefully inform clinical practice regarding the preservation of neurological health in menopausal women.
V. SUMMARY

In summary, the current study provides evidence that premature surgical menopause enhances the risk of dementia and mortality from neurological disorders via three mechanisms: 1) permitting elevation of neurodegenerative factors, 2) enhancing stress-induced amyloidogenesis, and 3) establishing an E2 signaling deficit in the hippocampus, thereby enhancing cell death and worsening cognitive outcome following a stressor. In particular, we observed a basal upregulation of the neurodegenerative Wnt antagonist Dkk1, with a concurrent downregulation of pro-survival Wnt/β-Catenin signaling in the hippocampal CA1 region following 10-week ovariectomy (LTED). We also observed a post-ischemic switch to amyloidogenic processing of APP in LTED female rats, with an elevation of BACE1 and a decrease of the two putative α-secretases ADAM 10 and ADAM 17, resulting in an increased hippocampal Aβ load following GCI. Furthermore, following LTED, we observed significant downregulation of the ER co-regulator Proline-, Glutamate- and Leucine-Rich Protein 1 (PELP1) in the hippocampal CA1, and through in vivo knockdown of hippocampal PELP1, we demonstrated loss of E2 regulation of JNK/c-Jun/Dkk1 signaling, loss of E2 regulation of APP processing, and loss of E2 neuroprotection, similar to events observed in LTED female rats. Finally, we demonstrated that PELP1 physically binds to several members of the JNK signaling pathway (JNK, MKK7, MLK3, and POSH) in the hippocampal CA1,
which may lend some mechanistic insight into how E2 may regulate this pro-apoptotic pathway. In conclusion, as shown in Figure 27, E2 normally signals through both classical genomic (left) and rapid, extranuclear signaling (right) to afford neuroprotection in hippocampal CA1 neurons. However, following prolonged deprivation of ovarian E2 (surgical menopause), these neurons undergo critical changes,
Figure 27. Summary of Molecular Mechanisms Underlying Enhanced Risk of Dementia and Mortality from Neurological Disease. See text for further details. AD, Alzheimer’s disease; Dkk1, Dickkopf-1; E2, 17β-estradiol; ER, Estrogen Receptor; ERE, Estrogen Response Element; LXXLL, Nuclear Hormone Receptor Binding Domain; PELP1, Proline-, Glutamate-, and Leucine-Rich Protein 1; PI3K, Phosphatidyl Inositol 3-Kinase; SH2/SH3, Kinase Binding Domain.
VI. REFERENCES OF LITERATURE CITED


Li GY, Fan B, Ma TH (2011a) Visible light may directly induce nuclear DNA damage triggering the death pathway in RGC-5 cells. Molecular vision 17:3279-3289.


Pluta R, Jablonski M, Ulamek-Koziol M, Kocki J, Brzozowska J, Januszewski S,
Sporadic Alzheimer's disease begins as episodes of brain ischemia and
ischemically dysregulated Alzheimer's disease genes. Molecular neurobiology
48:500-515.

Pradillo JM, Romera C, Hurtado O, Cardenas A, Moro MA, Leza JC, Davalos A, Castillo
J, Lorenzo P, Lizasoain I (2005) TNFR1 upregulation mediates tolerance after
brain ischemic preconditioning. Journal of cerebral blood flow and metabolism :
official journal of the International Society of Cerebral Blood Flow and
Metabolism 25:193-203.

Proc Natl Acad Sci U S A 100:11741-11746.

Pulsinelli WA, Brierley JB (1979) A new model of bilateral hemispheric ischemia in the

Pulsinelli WA, Brierley JB, Plum F (1982) Temporal profile of neuronal damage in a

complete occlusion of vertebral arteries and control of collateral circulation.
Stroke 19:913-914.


Scott EL, Zhang QG, Han D, Desai BN, Brann DW (2013) Long-term estrogen deprivation leads to elevation of Dickkopf-1 and dysregulation of Wnt/beta-Catenin signaling in hippocampal CA1 neurons. Steroids 78:624-632.


Thomas KR, Capecchi MR (1990) Targeted disruption of the murine int-1 proto-
oncogene resulting in severe abnormalities in midbrain and cerebellar

Vadlamudi RK, Kumar R (2007) Functional and biological properties of the nuclear
receptor coregulator PELP1/MNAR. Nuclear receptor signaling 5:e004.

Vadlamudi RK, Rajhans R, Chakravarty D, Nair BC, Nair SS, Evans DB, Chen S,
Tekmal RR (2010) Regulation of aromatase induction by nuclear receptor
coregulator PELP1. The Journal of steroid biochemistry and molecular biology
118:211-218.

Vallet P, Charnay Y, Steger K, Ogier-Denis E, Kovari E, Herrmann F, Michel JP, Szanto
I (2005) Neuronal expression of the NADPH oxidase NOX4, and its regulation in

Interaction of estrogen receptors with insulin-like growth factor-I and Wnt
signaling in the nervous system. Steroids 75:565-569.

Estradiol activates beta-catenin dependent transcription in neurons. PLoS One
4:e5153.


