17-β estradiol (17-β-E₂) has been implicated to be neuroprotective, yet the mechanisms underlying 17-β-E₂-mediated protection against stroke remain unclear. The purpose of the current study was to elucidate the role of 17-β-E₂ in NADPH oxidase (NOX2) activation during ischemic reperfusion induction of superoxide (O₂⁻) in the hippocampus CA1 region following global cerebral ischemia (GCI) and to investigate the post-translational deacetylation of downstream pro-apoptotic factors by 17-β-E₂. Using a 4-vessel occlusion model to induce GCI, we showed that neuronal NOX2 localizes to the membrane and that NADPH oxidase activity and O₂⁻ production were rapidly and markedly attenuated by 17-β-E₂ following reperfusion, in an estrogen receptor-dependent manner. Inhibition of NADPH oxidase activation via icv administration of a NOX2 competitive inhibitor, gp91ds-tat, strongly attenuated O₂⁻ production and was neuroprotective. The increase of neuronal NOX2 and O₂⁻ following cerebral ischemia was shown to require Rac1 activation, as administration of a Rac1 inhibitor (NSC23766) significantly attenuated these factors following stroke. Interestingly, we found that 17-β-E₂ antioxidant ability to diminish neuronal NOX2-induced O₂⁻ generation involves the attenuation of Rac1 activation. We also provide evidence for 17-β-E₂ post-translational deacetylation of downstream pro-apoptotic p53 and a reduction of p53 transcriptional target, Puma. Our results revealed that p53 acetylation (activation) is markedly increased in ischemic animals 24h after reperfusion and that 17-β-E₂ strongly attenuated that elevation, as well as total p53 protein levels. In support of this suggestion, we also found 17-β-E₂ to strongly attenuate ischemia-mediated Puma upregulation, thus interfering with its transcription-dependent function. We further propose that 17-β-E₂-induced attenuation of p53 levels may involve an upregulation in p53-Mdm2 interactions and p53 mediated degradation via the ubiquitination pathway. Lastly, we provide
evidence showing that treatment with Gp91ds-tat, but not the scrambled tat peptide control, attenuated acetylation of downstream p53 and reduced levels of Puma, thus supporting $O_2^−$p53 crosstalk signaling after stroke. Altogether, our studies reveal a novel, membrane-mediated antioxidant mechanism of 17-β-E2-induced neuroprotection via reduction of neuronal NOX2 activation and $O_2^−$ production, while providing evidence for 17-β-E2–mediated deacetylation and inactivation of p53, thereby protecting the hippocampus CA1 against cerebral ischemia.

INDEX WORDS: Estrogen, neuroprotection, global cerebral ischemia, hippocampus
MECHANISMS OF ESTROGEN NEUROPROTECTION IN STROKE

By

Limor Raz

Submitted to the Faculty of the School of Graduate Studies
of the Medical College of Georgia in Partial Fulfillment
of the Requirements for the Degree of
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MECHANISMS OF ESTROGEN NEUROPROTECTION IN STROKE

This dissertation is submitted by Limor Raz and has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

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.

____________________  ___________________________
Date                               Major Advisor

______________________________
Department Chairperson

______________________________
Dean, School of Graduate Studies
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AF-1: Activation Function 1
AS-ODN: Antisense Oligodeoxynucleotides
BAX: Bcl-2–Associated X Protein
BBB: Blood Brain Barrier
Bcl-2: B-Cell Lymphoma 2
17β-E2: 17β-Estradiol (Estrogen)
BH3: Bcl-2 Homology Domain 3
°C: Degrees Celsius
CBP: P300/CREB-Binding Protein
CNS: Central Nervous System
D: Day
DAB: Diaminobenzidine
DNA: Deoxyribonucleic Acid
DPI: Diphenyleneiodonium
ER: Estrogen Receptor
ER-α: Estrogen Receptor Alpha
ER-β: Estrogen Receptor Beta
ERT: Estrogen Replacement Therapy
GCI: Global Cerebral Ischemia
GDI: GDP-Dissociation Inhibitor
GDP: Guanosine Diphosphate
GEF: Guanine Nucleotide Exchange Factor
GFAP: Glial Fibrillary Acidic Protein
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<tr>
<td>Gp91&lt;sub&gt;phox&lt;/sub&gt;-/-</td>
<td>NOX2 Knock-Out</td>
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<td>GPR30</td>
<td>G-Protein-Coupled Receptor 30</td>
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<td>GTP</td>
<td>Guanosine Triphosphate</td>
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<tr>
<td>H</td>
<td>Hours</td>
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<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
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<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
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<tr>
<td>HEt</td>
<td>Hydroethidine</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-Hydroxynonenal</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone Replacement Therapy</td>
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<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
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<tr>
<td>I.P.</td>
<td>Intraperitoneal</td>
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<tr>
<td>I/R</td>
<td>Ischemia/Reperfusion</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-Out</td>
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<tr>
<td>MCAO</td>
<td>Middle Cerebral Artery Occlusion</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADPH Oxidase</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate-Oxidase</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Superoxide</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>OGD</td>
<td>Oxygen Glucose Deprivation</td>
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<tr>
<td>8-OHdG</td>
<td>8-Hydroxy-2'-Deoxyguanosine</td>
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<tr>
<td>OVX</td>
<td>Ovariectomy</td>
</tr>
<tr>
<td>PCAF</td>
<td>P300/CBP-Associated Factor</td>
</tr>
<tr>
<td>PFTα</td>
<td>Pifithrin α</td>
</tr>
<tr>
<td>p-H2A.X</td>
<td>Phospho-Histone 2AX</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>PUMA</td>
<td>P53-Upregulated Modulator of Apoptosis</td>
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<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RAC1</td>
<td>Ras-Related C3 Botulinum Toxin Substrate 1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>Sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SiRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>SIRT-1</td>
<td>Sirtuin1</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratricopeptide Repeat</td>
</tr>
<tr>
<td>TUNNEL</td>
<td>Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling</td>
</tr>
<tr>
<td>VS.</td>
<td>Versus</td>
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<tr>
<td>W</td>
<td>Week</td>
</tr>
<tr>
<td>WHI</td>
<td>Women Health Initiative</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
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REVIEW OF THE RELEVANT LITERATURE

1.1 Introduction: Estrogen, Gender and Neuroprotection

Over the past two decades, evidence has emerged in support of a neuroprotective role for the ovarian sex steroid hormone, 17β-Estradiol (17β-E2). Substantial gender differences exist in men and women regarding stroke incidence and outcome. Men are 1.25 times more likely to suffer a stroke before the age of 50, a ratio which reverses once women enter menopause, a period associated with increased incidence of stroke. These findings may be attributed to the dramatic drop in the levels of 17β-E2 to 1% of premenopausal levels. Observational studies of postmenopausal women taking estrogen replacement therapy (ERT) have shown 17β-E2 beneficial effects on cognition [1,2], reduction of osteoporosis [3,4], improvement of cardiovascular health [5] and prevention of neurodegeneration such as in the case of Alzheimer’s disease [6,7], Parkinson’s disease [8,9] and stroke [10]. ERT usage as a potential preventative stroke treatment in postmenopausal women, however, remains a controversial subject due to the Women Health Initiative (WHI) multicenter, clinical studies which found an increased risk of ischemic events upon 17β-E2 treatment in postmenopausal women [11,12]. The average age of subjects in the WHI study was 63 years old, which is far past menopause, and has led many to suggest there may be a “critical period” for 17β-E2 beneficial effect, that is, 17β-E2 must be administered before menopause to produce a favorable outcome. With respect to its neuroprotective effects, there are numerous studies demonstrating the neuroprotective actions of 17β-E2 in cerebral ischemia and other neurodegenerative disorders [13,14,15], thus sparking clinical interest in the steroid hormone’s potential therapeutic function in the prevention of neurodegenerative and cardiovascular diseases in humans.

The primary focus of this dissertation is to elucidate the neuroprotective actions of 17β-E2 in the brain, its underlying molecular signaling cascades and its implications in stroke. To
accomplish this goal, we used the well-characterized global cerebral ischemia (GCI) rat model of stroke as our primary experimental model, as it has high reproducibility with low mortality, which allows detailed studies on mechanisms of delayed neuronal cell death. The following dissertation research has the potential to significantly advance our understanding of how $17\beta$-E$_2$ exerts its signaling and neuroprotective effects in the female brain and may provide the knowledge which could potentially lead to future therapeutic drug development. In the sections below, we will discuss the evidence for $17\beta$-E$_2$ neuroprotection in stroke, the controversies thereof, and provide an overview of the relevant literature pertaining to the dissertation research.

1.2 Evidence for Estrogen Neuroprotective Role in Stroke

1.2.1 In vivo Studies of $17\beta$-E$_2$ Neuroprotection in Cerebral Ischemia-induced Damage

Countless animal studies addressing $17\beta$-E$_2$ neuroprotective effects on stroke outcome, at low, physiological doses, are present throughout the literature. Studies in female gerbils [15,16], rats [17,18] and mice [19] have shown profound neuroprotection against cerebral ischemia-induced neuronal damage upon exogenous $17\beta$-E$_2$ administration, characterized by a reduction in infarct size and an increase in survival following Middle Cerebral Artery Occlusion (MCAO). Evidence for $17\beta$-E$_2$ neuroprotection comes from ovary removal studies, in which ovariectomized (OVX) female rats and mice showed diminished protection following cerebral ischemia, as marked by an increase in infarct size [20]. Support for $17\beta$-E$_2$-mediated neuroprotection was also documented by numerous studies of exogenous $17\beta$-E$_2$ administration, where a significant reduction of infarct volumes in several animal species of both young and old was established in different stroke models [21,22]. Additional elegant aromatase knockout studies in female mice, in which the biosynthetic gene aromatase, which converts androgens to $17\beta$-E$_2$, has been knocked out, have demonstrated the important role of endogenous $17\beta$-E$_2$ production in the reduction of MCAO-induced infarct size [23], as well as aromatase upregulation in the peri-infarct area.
following ischemic insult [24]. Interestingly, Kawato and colleagues have conducted studies in the male rat showing that endogenous 17β-E₂ production by the upregulation of aromatase is protective in neurons [25]. Additional studies using exogenous administration of acute and chronic 17β-E₂ treatment in males demonstrated a significant reduction in cortex infarct size, further validating the neuroprotective role of 17β-E₂ in male animals [26,27]. Moreover, it is important to note that 17β-E₂ has no significant effect on cerebral blood flow [21,22] and that neuronal-specific, estrogen receptor α (ER-α) knockout mice experience larger infarct sizes as compared to their WT controls [28], thus indicating that the observed neuroprotective effect is mediated primarily by a direct effect of 17β-E₂ on neurons via neuronal ERα mediated-signaling.

In recent years, a growing body of literature has emerged, demonstrating 17β-E₂ critical role in the protection of the hippocampus brain region during ischemic injury. Many laboratories, including our own, have found 17β-E₂ to greatly reduce delayed apoptotic neuronal cell death in an experimental model of global cerebral ischemia, while preserving cognitive function [13,29,30]. Importantly, the underlying molecular mechanisms involved in 17β-E₂ neuroprotective effects have been implicated to be mediated by the ERs, more particularly, via rapid, extranuclear ERα rapid signaling involving the PI3K/Akt pathway [31,32,33]. Other studies support the role of ERβ and the putative membrane ER, GPR30, as key players in the 17β-E₂-mediated neuroprotective response, [34,35] as will be discussed in the next section. Taken altogether, these studies support a neuroprotective role of 17β-E₂ in cerebral ischemia.

1.2.2 Estrogen Receptor Physiological Structure and Function

The ovarian steroid hormone, 17β-E₂, produced at high concentrations by the female ovary, exerts its actions on many target tissues such as the breast, ovary, uterus, brain and bone [10]. 17β-E₂ is thought to exert the majority of its biological actions in the body via interaction with two primary estrogen receptors: estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β). The two receptors exhibit significant homology in their structures, but display differential
function, localization and pattern of expression in the brain, characteristics which will be addressed in a later section. Nevertheless, both receptors are localized in the hippocampus, a primary target tissue of 17β-E2 that will be a focus area of our proposed studies. Although controversial [13,36], there is evidence suggesting both ERs may be involved in neuroprotection, as will be discussed in detail in the subsequent sections.

The adult brain contains the two aforementioned ER subtypes: ER-α and ER-β. Structural and physiological differences exist between these two receptors. Among the shared similarities, both receptors exhibit great homology to one another being composed of seven domains, they both bind 17β-E2 with high affinity and they both dimerize and utilize the classical estrogen response elements in a similar if not identical fashion. On the contrary, ERs differ in their tissue distribution (ER-α being localized in breast, ovary, uterus, brain tissue, while ER-β is found in bone, heart, lungs, kidney, endothelial cells and brain), the phenotype of the corresponding knock-out mice and their transcriptional activities [37]. In the brain, localization studies have demonstrated that ER-α is localized most densely in the hypothalamus, hippocampus (especially the CA1 region), and preoptic area, with moderate to light density in the cerebral cortex. Conversely, ER-β localization in the brain has been documented predominantly in the cortex, throughout the hippocampus, in the olfactory bulb, septum, preoptic area, nucleus of striata terminalis, amygdale, paraventricular hypothalamus, thalamus, ventral tegmental area, substantia nigra and cerebellum [38]. Structurally, there are several differences between ER-α and ER-β: 1) They contain different Ligand Binding Domains (LBDs) (Leu 338 as ER-α LBD and Met 384 for ER-β), 2) Each receptor is encoded by a different gene (ESR1 versus ESR2) 3) The receptors signal differently at the Activation Function-1 (AF-1) site in the presence of 17β-E2, where 17β-E2 activates transcription at ER-α and inhibits transcription at ER-β, respectively [39]. Both intracellular receptors serve as ligand-activated transcription factors and have been extensively studied via estrogen receptor knockout mice (ERKO) in order to decipher the specific role and
function for each isoform. A novel yet putative third nongenomic ER, GPR30, has generated much controversy in recent years in regards to its role in 17β-E2-mediated neuroprotection [13]. GPR30 is a seven transmembrane domain G-protein-coupled receptor known to be primarily localized in the plasma membrane [40] and endoplasmic reticulum [41] of neurons in the brain and is highly expressed in several brain regions, including the hippocampus [42]. Evidence supporting the role of GPR30 in neuroprotection is obtained from selective agonist studies (G-1), which have implicated its actions promoting short latency 17β-E2-mediated facilitation of synaptic transmission in the hippocampus [34]. Particularly as relevant to stroke, studies of G-1 pretreatment significantly attenuated neuronal cell death at 1h reperfusion following GCI injury in hippocampal cell cultures, thus demonstrating the neuroprotective effects of 17β-E2 by GPR30 signaling on glutamate-induced neurotoxicity [43]. Nevertheless, the contributions of GPR30 in the brain remain to be elucidated. Work using the G-1 agonist has found increased intracellular calcium concentrations in cultured hypothalamic neurons [42], but unfortunately no functionally relevant endpoints were assessed in the study to determine the physiological significance of the observation. Studies using GPR30 knockout mice may help address this issue and clarify the role of GPR30 in 17β-E2 actions and signaling in the brain, as well as in other estrogen-target tissues in the body. Finally, one must consider the possibility that classical ERα or ERβ may couple to G-protein signaling via an unknown mechanism to mediate rapid estrogen signaling. Navarro and colleagues [44] have demonstrated by coimmunoprecipitation studies that ERα interacts with Galphai3 in immortalized GnRH neurons, and that estrogen-induced inhibition of cAMP can be blocked by pertussis toxin, a G-protein signaling inhibitor. Thus, considering the current controversies surrounding the role of GPR30 in estrogen signaling in the brain and other parts of the body, this area should be an important focal point for future research [33].
1.2.3 Controversies Surrounding Estrogen Receptor Involvement in Ischemic Injury

Support for estrogen receptor (ER) involvement in neuroprotection comes from studies using ICI182,780, a potent and selective ER antagonist that blocks both ER-α and ER-β. Blocking both ERs with ICI182,780 exacerbated infract volume following MCAO and global cerebral ischemia (GCI) in female rats, suggesting a neuroprotective and critical function for the receptors in 17β-E2 regulation of ischemic injury [45]. Evidence for ER-α neuroprotection comes from ER-α mRNA upregulation studies examining the ischemic penumbra in rats following MCAO [46]. Similarly, Wise et al. have demonstrated that 17β-E2 neuroprotection is lost in ER-α, but not ER-β KO mice following MCAO, thus validating ER-α instrumental role in neuroprotection [47]. There is also evidence that ER-β can exert neuroprotective effects in some situations. For instance, ERβKO mice demonstrated a significant reduction in brain size between two month and two year old mice in comparison to wild type controls, with substantial neuronal loss in the somatosensory and pietial cortex, indicating a crucial role for ER-β in the maintenance of neuronal survival and neurogenesis in the adult brain [48]. Nevertheless, Etgen’s group, using PPT (ER-α selective agonist) and DPN (ER-β selective agonist), provided evidence that 17β-E2 may act via both ER-α and ER-β to protect the hippocampus from GCI-induced cell damage [35].

1.3 The Women’s Health Initiative and Controversies Surrounding Hormone Replacement Therapy

While the above studies demonstrate a clear neuroprotective role by 17β-E2 in animal models of stroke, controversy still exist regarding 17β-E2 protective effects in the post-menopausal female. Observational studies in humans predominantly support a cardiovascular protective role for 17β-E2, suggesting that ERT decreases the risk for coronary heart disease [3]. However, the Women’s Health Initiative (WHI) clinical trials, which incorporated a double-blinded, randomized, multi-center study design, failed to find a beneficial cardiovascular effect of
ERT. The long-term WHI studies were initiated to address a fundamental question surrounding the 17β-E₂ controversy and determine the risks and benefits of hormone therapy to cardiovascular and neurological health in postmenopausal women. Unfortunately, the studies were discontinued in 2002 due to an increased risk of stroke, dementia and breast cancer in the postmenopausal women [12]. The negative results of the stroke study showed an increase in the relative risk for ischemic strokes in postmenopausal women between the ages of 60-80 years old (from an incidence of 21 in 10,000 women (control) to 29 in 10,000 women for Premarin and Provera combined therapy) [11,49]. Similarly, this increased risk of ischemic strokes was also noted in the younger, perimenopausal women age group (50-59) [11]. The surprising results of the WHI generated much confusion and additional controversy regarding the prescription of ERT. It is also important to note that the WHI studied stroke “risk” and not “outcome.” Nevertheless, results should be examined carefully, as many caveats and potential limitations have been noted by experts in the field [50,51,52]. A careful reanalysis of the WHI results argues that the study design was flawed. The authors of the study state that there was a 29% increase in coronary heart disease in the women given 17β-E₂ and progesterone treatment as compared to the placebo-treated control group. Since this reported value is expressed as relative risk derived from the Hazard Ratio (1.29) it can be misleading. Data should have been reported as absolute risk (0.07%) and is therefore insignificant [53]. Additionally, the authors of the WHI could not have found cardioprotection in their subjects a decade or more following menopause due to the study design being 10-fold underpowered to show cardioprotection in the perimenopausal women age-group [52,54,55]. As a result, future clinical studies of women during the menopausal transition are needed before definitive conclusions can be drawn. The average age of the women subjects enrolled in the study was also heavily criticized, as the women were 63 years of age, which is well past the age of menopause. This fact becomes important since several groups, including our own, have proposed the necessity of ERT initiation at the inception of menopause to provide
neural and cardiovascular beneficial effects. Brinton proposed a “healthy cell bias of E₂ benefit” hypothesis [56] claiming that 17β-E₂ neuroprotection and survival is achieved in healthy neurons (i.e. perimenopausal women), as was later validated by the MIRAGE clinical trials reporting that a 6 month period of HRT given to postmenopausal women with AD disease is protective of only in the youngest (50-63 years old) age group [6]. Work in our laboratory has found that 17β-E₂ neuroprotection in a 4-vessel occlusion model of GCI is lost after a long period of 17β-E₂ deprivation, and that this loss was correlated with a loss of ER-α in the hippocampus CA1, thus providing important support for the “critical period” of estrogen replacement hypothesis [29].

Consequently, to address some of the controversies and concerns of the WHI study design, the KEEPS study was initiated. The rational behind the KEEPS study is to overcome the WHI caveats and clarify controversies concerning HRT by altering three key experimental parameters: 1) Age of subjects reduced to a peri-menopausal state 2) Transdermal route of hormone replacement and 3) Cyclical progesterone administration. The authors hypothesized that early initiation of HRT given to women at the inception of menopause will delay the onset of cardiovascular disease. The KEEPS is a multicenter, 5-year clinical trial that will evaluate the effectiveness of conjugated equine estrogen and progesterone treatment in preventing the progression of carotid intimal medial thickness and the accrual of coronary calcium in perimenopausal women [57,58]. Results are expected to be revealed by 2012.

Finally, a recent 10 year reevaluation of the WHI study of hysterectomized women receiving ERT supports the beneficial effects of 17β-E₂ during the critical period [59]. Interestingly, significant beneficial cardiovascular effects of 17β-E₂ protection were found for women who began 17β-E₂ treatment in their 50s, corresponding to a 41% lower coronary heart disease risk, 46% reduced risk of heart attacks, significantly decreased invasive breast cancer risk and lower overall mortality. On the other hand, women in their 60s experienced neutral effects of 17β-E₂ treatment, while those in their 70s demonstrated increasingly negative outcomes
associated with an increased risk of cardiovascular disease, colorectal cancer and mortality. The study provides strong support for the critical period hypothesis of 17β-E2 neuroprotection, suggesting that 17β-E2 hormone therapy replacement in humans is beneficial when administered early during perimenopausal age and is lost later in life, around the age of 70.

In the next section, we propose a mechanistic hypothesis that 17β-E2 neuroprotective effects in the brain may be due, at least in large part, to an anti-oxidative mechanism involving regulation of the superoxide-generating enzyme, NADPH oxidase.

1.4 The NADPH Oxidase (NOX) Family: NOX2 Complex Formation and Superoxide Generation

The complex underlying cellular and molecular mechanisms responsible for neuronal injury and cell death following ischemia remain unknown and therapeutic interventions are currently lacking. Initial studies conducted by the Brann laboratory in 2007 showed 17β-E2 ability to attenuate superoxide (O$_2^-$) production in the ovariectomized (OVX) female rat cortex 1h following pMCAO [10]. Recent work in the field has described O$_2^-$ as a key player in neuronal cell death following cerebral ischemia and as one of the most potent and damaging free radicals produced during ischemia/reperfusion. The O$_2^-$ anion radical is the product of one electron reduction of oxygen and is the precursor of most reactive oxygen species (ROS) (i.e. H$_2$O$_2$, OH ion, peroxynitrite). Evidence from both tMCAO and pMCAO models shows a significant elevation in ROS following the onset of ischemia. Along the same lines, experiments using hydroethidine (HET), a selectively diffusible in situ measure of O$_2^-$ in central nervous system (CNS) parenchyma, revealed an increase of O$_2^-$ in male mice within two hours of pMCAO [60]. Our own work in the GCI model demonstrates a dramatic elevation of ROS in the hippocampus and cortex after reperfusion in the female rat [29]. An in vitro study has suggested that there may be three distinct mechanisms for generating ROS in hippocampal and cortical neurons during
hypoxia/reoxygenation. The studies provided evidence that the mitochondria generates the initial ROS burst during hypoxia, followed by xanthine oxidase (XO) during the delayed phase, and ending with Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase ROS production in reperfusion. Interestingly, work by our laboratory and others has shown that administration of an inhibitor to NADPH oxidase reduces infarct volume following focal and global cerebral ischemia [29,61], suggesting that NADPH oxidase may be an important contributor to ROS generation and oxidative damage after stroke. In the next section, we will review the biology of NADPH oxidases and their mechanisms of action.

1.4.1 NOX Physiological Structure and Function

The NADPH oxidase enzyme is composed of key subunits from the NOX family, whose primary job is to transport electrons across biological membranes to reduce molecular oxygen to $O_2^-$. The NOX family is composed of five isoforms (NOX1-NOX5). Despite their similar structure and enzymatic function, NOX family isoforms differ in their mechanism of activation. NOX1 activity requires p22phox, NOXO1, NOXA1 and is Rac1 dependent, whereas NOX 3 requires similar subunits for its activation, but is Rac1-independent. The activation of NOX2, the most studied and best characterized NOX isoform and a major focus of our studies, involves interaction with p22phox, p67phox, p40phox and p47phox subunits, the latter being the key complex activator subunit by phosphorylation. In addition, the GTPase, Rac1 has been shown to be critical for NOX2 activation (See Figure 1-1). Finally, NOX4 and NOX5 isoforms do not appear to require many subunits for their activation, for they are constitutively active and Rac1-independent [62]. Localization of the NOX family isoforms has been studied extensively in many tissues throughout the body. In 2001, Lambeth and his group cloned and documented strong NOX2 mRNA expression and faint RT-PCR bands of NOX4 and NOX5 in the brain [63]. Moreover, further studies of the hippocampus by our group and others revealed NOX2 [64] and NOX4 [65] expression and demonstrated additional NOX2 localization in the cortex [29,66]. The
intricate interplay of NADPH oxidase subunit assembly and subsequent $O_2^-$ production has been well-established in phagocytes. As illustrated in Figure 1-1A, NOX2 and p22phox are found primarily on the membrane, in resting cells, existing in close association and stabilizing one another. Upon activation, there is an exchange of GDP for GTP on Rac1, a Rho GTPase, leading to its activation and translocation to the membrane. Simultaneously, phosphorylation of cytosolic p47phox allows for its binding with other membrane subunits (p67phox and p40 phox), leading to conformational changes to allow interaction with p22phox on the membrane (Figure 1-1B). This activates the NOX2 enzyme complex, which transports electrons from cytoplasmic NADPH to oxygen and generates $O_2^-$, leading to enhanced neuronal damage under ischemic conditions as documented in the sections below [62].

1.4.2 Free Radical Elevation Contributes to Cerebral Ischemia Tissue Damage

Reactive oxygen species (ROS) such as superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), the hydroxyl ion ($OH^-$) and Reactive Nitrogen Species (RNS) such as peroxynitrite (ONOO') and nitric oxide (NO'), have been implicated to initiate ischemic damage upon reperfusion onset of acute stroke. These potent anions are highly reactive due to their unpaired electron molecular orbitals. The $O_2^-$ anion is considered a “primary” ROS which can further interact with other molecules to generate a “secondary” ROS either directly or through enzyme or metal-catalyzed processes. During stressful conditions, $O_2^-$ acts as an oxidant, facilitating $OH^-$ production from $H_2O_2$ by making $Fe^{2+}$ available for the Fenton reaction. The hydroxyl radical is also highly reactive, making it a dangerous radical with a very short $in \, vivo$ half life. Additional reactive radicals derived from oxygen that can be formed in living systems are peroxy radicals (ROO'). The RNS, NO', is also an abundant reactive radical that acts as an important oxidative biological signaling molecule. Interestingly, NO' and the $O_2^-$ anion may react together to produce significant amounts of a much more oxidatively active molecule, ONOO', which is a potent oxidising agent that can cause DNA fragmentation and lipid oxidation [62].
The overproduction of ROS and RNS is the common underlying mechanism of many neuropathologies, as they have been shown to damage various cellular components, including proteins, lipids and DNA [67]. For instance, OH$^-$ is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone, measured by the formation of the oxidative DNA damage marker, 8-hydroxy-2'-deoxyguanosine (8-OHdG). Another biological marker of cellular damage is the potent aldehyde produced as consequence of lipid peroxidation, 4-Hydroxynonenal (4-HNE) [29,68]. Additional evidence for ONOO$^-$ contributions to vascular smooth muscle cell damage comes from studies by Cipolla and colleagues of the male rat using Nitrotyrosine to show ONOO$^-$ decreases myogenic tone and induces actin depolymerization, consequently enhancing neurovascular damage following ischemic injury [69,70]. Collectively, the formation of oxidative damage in tissues accompanying an ischemic event results in permanent modifications of genetic material, contributing to the aging process in humans [71,72].

1.4.3 The Contributions of NOX2 Activation to Brain Ischemia

NOX has been implicated as a pro-oxidant enzyme, with its level known to be regulated by ischemia. Results from in vivo and in vitro studies suggest that NOX plays an instrumental role in ischemic brain damage through the production of oxygen radicals, thereby contributing to excitotoxicity and the exacerbation of post-ischemic inflammatory processes. The role of NOX in focal ischemic brain damage was first demonstrated by comparing the reperfusion injuries between wild-type (WT) and gp91$^{phox/-}$ (NOX2 null) mice. The infarct volume was significantly reduced in gp91$^{phox/-}$ null mice as compared to their WT litter mates [73]. The role of NOX was also examined using genetic disruption of p47phox with the pharmacological inhibitor apocynin, a plant derived compound which works by interfering with subunit membrane localization and enzyme activation. Inactivating NOX with apocynin blocked neuronal death in vivo after focal
ischemia [74] and significantly reduced neurological deficit score in mice, thus achieving an improved behavioral cognitive outcome [68,74,75]. Furthermore, NOX2 null and apocynin-treated mice subjected to focal cerebral ischemia experience 50% less brain infarction volumes as compared to controls [68]. In another elegant set of studies, Yenari and colleagues conducted an Apocynin dose response curve to unveil its effectiveness against MCAO-derived damage. Findings revealed that the compound is most effective at reducing infarct size and mortality at a lower dose, while hydroethidine (HEt) fluorescence technique demonstrated that $\mathrm{O}_2^-$ is mainly present in neurons and microglia of the brain, but not in vascular endothelial cells [76]. Additional studies using the GCI stroke model in the gerbil support previous experimental findings by showing that NOX inhibition also reduces brain damage in the hippocampus; in particular, apocynin suppressed ROS by inhibition of NADPH oxidase, protected against ischemia-induced ROS generation and decreased delayed neuronal cell death by attenuating 4-hydroxy-2-nonenal (4-HNE), a marker for lipid peroxidation, at 3h post-reperfusion [77].

1.5 Downstream Pro-Apoptotic Factors: p53 and Puma and their Relevance to Stroke

The pro-apoptotic factor p53 has been implicated as an important downstream mediator of oxidative stress cell death signals following ischemia [78]. P53 is both a tumor suppressor and a transcription factor, known to be regulated within the cell and kept at low, physiological levels by ubiquitination, a cellular mechanism which targets the protein for degradation, thus preventing its accumulation within neurons [79,80]. When the cell is confronted with stress, p53 ubiquitination is suppressed and p53 is stabilized, accumulating in the nucleus, translocating to the mitochondria, inhibiting the anti-apoptotic factor B-cell lymphoma 2 (Bcl-2) and initiating the apoptotic cascade [81]. While much is known regarding p53 role in cancer and other pathologies, its function in neuronal cell death after ischemia remains poorly understood.
1.5.1  P53 Contributions to Neuronal Cell Death after Ischemic Injury

P53 acts biphasically, exerting transcriptional independent and dependent apoptotic responses to stress [82]. The rapid, transcriptional independent effects of p53 are observed in the mitochondria, within minutes, characterized by the formation of p53-Bcl-xL and p53-Bcl-2 inhibitory complexes with anti-apoptotic factors, thereby activating the apoptotic cascade in response to a cell death stimulus [83]. The transcriptional dependent response of p53, on the other hand, is much slower and more involved, lasting for hours and requiring the encoding of transcription factors such as Noxa and Puma [84]. See Figure 1-2 for a detailed mechanism.

Many groups have presented evidence supporting the pro-apoptotic role of p53 in stroke. Studies by Chan and colleagues have shown that mitochondria-localized p53 is elevated at 8 and 24 hours after reperfusion in a model of transient GCI [85]. Other studies using p53 KO mice have shown decreased GCI-induced neuronal cell death in the hippocampus, providing strong evidence for p53 enhancement of neuronal injury [86,87]. Lastly, data obtained from pharmacological inhibition studies of p53 using pifithrin α (PFTα) measured histological and behavioral outcomes. Results revealed a significant improvement in PFTα-treated animals as compared to controls in models of focal and global ischemia [88,89]. There is new emerging evidence that the activational state of p53 can be regulated by epigenetic modifications, such as acetylation. This novel topic and relevant literature are reviewed below.

1.5.2  Epigenetic Regulation of p53 by Post-Translational Modifications

Post-translational modifications are chemical changes to a protein following its translation. Epigenetic regulation, notably acetylation and deacetylation of p53, has been documented throughout the literature and p53 acetylation, which enhances its deoxyribonucleic acid (DNA)-binding activity and stability at specific Lysine residues, including Lysine$^{373}$ and Lysine$^{382}$, has been confirmed [81,90,91]. Interestingly, 17β-E$_2$ has been found to regulate p53
acetylation (i.e. activation) in breast cancer cells. Das and his group reported that ER-α binds directly to p53, acting as a negative regulator and leading to a reduction in p53 transcriptional activity [92,93]. Along the same lines, 17β-E2-mediated regulation of p53 post-translational acetylation attenuated p53-dependent DNA binding and prevented the initiation of apoptosis in breast cancer cells [94]. However, it is still unknown whether p53 acetylation state changes in the brain following cerebral ischemia and whether 17β-E2 can regulate it. This issue was addressed in \textit{Aim3} of my dissertation studies.

Recently, scientists have begun elucidating a novel level of protein regulation, whereby post-translational modifications may serve a neuroprotective function in neurodegenerative disease. More specifically, the molecular machinery responsible for the regulation of p53 acetylation has been studied extensively. Histone acetyltransferases (HATs), enzymes responsible for the transfer of acetyl groups and the activation of p53 (i.e. CBP, p300, PCAF) and histone deacetylases (HDACs) (i.e. Sirtuin, HDACs 1-10), enzymes which remove acetyl groups resulting in the silencing of gene transcription have been implicated in neuroprotection [95]. Recently, new evidence has emerged documenting the disruption of HAT and HDAC expression and activity in neurological disorders [96]. HDAC inhibitors have been proposed as important therapeutic tools to rescue the brain from neuronal cell death and restore proper levels of acetylation both \textit{in vitro} and \textit{in vivo} models of neurodegenerative diseases [96]. For instance, post-MCAO injections with HDAC inhibitors valproic acid, sodium buterate, trichostatin A [97], or SAHA [98] significantly decreased brain infarct volumes in the rodent cortex. Furthermore, it is important to note that HDAC inhibitors work at the level of the histones to achieve neuroprotective effects, and as such, may operate through a different mechanism of action than proposed in our studies [97]. Another well known mechanism of p53 transcriptional regulation is through the ubiquitin-proteasome degradation pathway [99]. P53 is destabilized and is targeted for degradation upon its binding to Mdm2, its negative regulator. A thorough investigation of the
underlying molecular mechanism responsible for p53 regulation by 17β-E₂ will be performed in Aim3 of the dissertation.

5.3 Puma: A p53-Induced Transcription Factor

Recent studies indicate the dependence of p53-mediated apoptosis on the presence of Puma. Puma is a Bcl-2 homology domain 3 (BH3) p53-upregulated modulator of apoptosis, known to couple the nuclear and cytoplasmic pro-apoptotic functions of p53, allowing for its shuttling between cell compartments and the induction of the apoptotic signaling cascade [100]. During a stressful cellular event accompanied by DNA damage, such as ischemia, nuclear p53 results the transcriptional activation of Puma expression. Puma then binds to the anti-apoptotic protein Bcl-xL, thus freeing p53 to activate pro-apoptotic Bcl-2–associated X protein (Bax) and induce mitochondrial permeabilization followed by neuronal cell death [78]. See Figure 1-2 for a detailed mechanism.

Support for the pro-apoptotic role of Puma in stroke was documented in the literature. Using a model of GCI, Chan and colleagues found Puma protein upregulation in the mitochondria of vulnerable hippocampal CA1 neurons at 4h and 24h post-reperfusion, an effect which was reversed by PFTα, thus suggesting that Puma is partly controlled by the p53 transcriptional pathway [101]. Similarly, another study measuring Puma mRNA expression after MCAO in mice documented Puma upregulation at 8 hours, but surprisingly, failed to show a decrease in infarct size within the cortex of Puma-deficient mice at the 24 hour time point [102].

Noxa, an additional BH3-only pro-apoptotic Bcl-2 family member, has been similarly implicated to contribute to neuronal cell death [103]. Nevertheless, it has been noted that Puma plays the most dominant role in oxidative stress-induced cell death, as studies of cortical neurons revealed that Puma⁻/⁻ neurons but not Noxa⁻/⁻ neurons were remarkably resistant to the induction of apoptosis by multiple oxidative stressors [104].
1.5.4 Superoxide Crosstalk Signaling with Downstream p53

Crosstalk signaling between ROS and downstream pro-apoptotic factors has been implicated under stressful conditions [105]. A careful search of the literature demonstrates a clear gap of knowledge in regards to $\text{O}_2^-$ p53 crosstalk signaling in the brain, especially in neurodegenerative disease. Interesting work by Chan and colleagues in a model of focal ischemia presented evidence for crosstalk signaling between NOX and SOD, finding increased levels of Gp91phox expression in SOD overexpression transgenic mice as compared to their WT counterparts [68]. Other studies of highly stressful circumstances such as starvation found starvation-induced NOX2 mRNA upregulation in endothelial cells, accompanied by a two-fold increase in $\text{O}_2^-$ production and a dramatic increase in p53, resulting apoptosis [106]. Along the same lines, evidence from a mouse skin carcinogenesis model utilized the pharmacological inhibitor, diphenyleneiodonium (DPI) to prevent NADPH oxidase activation and observed a corresponding suppression of p53 activation, altogether supporting the importance of crosstalk signaling in the body [107]. Yet more studies focused on crosstalk signaling between $\text{O}_2^-$ and p53 in the brain are necessary to address this novel area of research, particularly as relevant to stroke.

1.6 Objectives and Specific Aims

The overall objective of this research is to elucidate the signaling mechanisms underlying 17β-E2–mediated neuroprotection at the onset of reperfusion following ischemic injury. Since stroke remains the third leading cause of death in the United States and the number one cause of long-term adult disability, it is of utmost importance to address this clinical problem of global dimensions by finding effective therapeutic targets. 17β-E2, classically known as the “reproductive hormone,” has many non-reproductive, beneficial effects, as exemplified by its remarkable, neuroprotective properties documented by in vivo and in vitro animal studies [10]. However, it is currently unclear by which mechanism 17β-E2 protects the brain after an ischemic event and the issue of whether women should be given HRT during the menopausal transition
remains highly controversial. Since a hallmark of many neurodegenerative diseases is the induction of ROS which play a key role in the initiation of necrotic and pro-apoptotic cell death cascades following reperfusion in stroke [108], my dissertation studies focused on exploring the membrane contributions of the NOX2 enzyme to free radical production and whether 17β-E2 antioxidant effects involve the attenuation of potent O$_2^-$ and downstream p53. Unveiling the mechanisms responsible for 17β-E2 protective effects could potentially have immense clinical implications, suggesting a common mechanism for estrogen-mediated neuroprotection in stroke and a variety of neurodegenerative diseases characterized by O$_2^-$ elevation. To address the mechanisms of 17β-E2 protection following cerebral ischemia, three specific aims were proposed:

**Specific Aim #1.** To establish the temporal pattern of NOX2 activation, O$_2^-$ production and oxidative stress neuronal damage in global ischemia and determine whether 17β-E2 regulatory effects are ER-dependent.

**Hypothesis.** 17β-E2 protects the brain by a novel antioxidant ER-mediated effect to suppress membrane neuronal NOX2 complex activation and O$_2^-$ production.

**Rationale.** Work by several laboratories has established that physiological doses of 17β-E2 protect the rodent brain against cerebral ischemia and the accompanying oxidative stress. Previously, it was believed that the primary contributions of O$_2^-$ free radicals are generated by the mitochondria, acting as the main culprit of apoptotic neuronal cell death following an ischemic event [68,76]. Only recently, work by Duchen and others has demonstrated the importance of membrane NADPH oxidase in the production of O$_2^-$ free radicals, contributing significantly to ischemic damage [61]. Yet the aforementioned studies were conducted in the male rat and 17β-E2 regulation of NOX after ischemic injury has not been examined. Preliminary work by our laboratory revealed 17β-E2 ability to profoundly attenuate O$_2^-$ production in the cortex at 1d following MCAO-induced injury in the female rat brain [10]. Yet how 17β-E2 was able to achieve
these actions remains to be elucidated. While it is known that the protective effect is not due to changes in cerebral blood flow, the precise cellular signaling mechanisms at the level of the neurons are unknown and could potentially lead to the future development of therapeutic targets. Therefore, to further clarify the mechanisms underlying 17β-E2-induced protection and determine the involvement of the ERs, a well-characterized global ischemia (stroke) animal model will be utilized. The model represents a reproducible in vivo system, which allows for the controlled study of the susceptible hippocampus CA1 brain region, in a reperfusion model for the study of delayed neuronal cell death. In essence, this aim will test whether 17β-E2 can inhibit NOX2 complex activation on the neuronal membrane, thus preventing O2− generation and serving a neuroprotective function in the female rat brain during ischemia. Subsequent aims will test how 17β-E2 exerts its neuroprotective actions via the regulation of Nox2 complex formation and potent O2− anion production.

Specific Aim #2. To elucidate the signaling mechanisms underlying 17β-E2-mediated regulation of NOX2 activation and O2− generation in the brain after cerebral ischemia.

Hypothesis. 17β-E2 protects the brain by a rapid signaling mechanism involving the suppression of Rac1 GTPase activation.

Rationale. Rac1 has been implicated as an important GTPase activator of NADPH oxidase throughout the body [109,110]. Data from our laboratory has previously shown that Rac1 GTPase activation peaks at early reperfusion timepoints after stroke, while Rac1 expression remains unchanged. Rac1 elevation greatly enhanced delayed neuronal cell death within the hippocampus by facilitating JNK pro-apoptotic signaling as confirmed by Rac1 inhibitor studies [111]. Yet the precise functional role of Rac1 in ischemia and its contributions to neuronal demise in the context of O2− generation have not been explored. For these set of studies, we will use the a Rac1 pharmacological inhibitor, NSC23766, to test its effects in the male and female rat brain and shed
some light on Rac1 mechanistic involvement in cerebral ischemia. If successful, our studies could lead to the potential use of NSC23766 post-treatment in stroke. The subsequent aim will examine pro-apoptotic factors located downstream of the NOX2 complex and address how 17β-E₂ may prevent their activation and facilitate neuroprotection.

**Specific Aim #3. To determine whether 17β-E₂ affects downstream pro-apoptotic factors p53 and Puma levels or activation and establish the underlying regulatory mechanism.**

**Hypothesis.** 17β-E₂ will deacetylate p53 and attenuate Puma expression, thus providing a novel neuroprotective mechanism downstream of O₂⁻ free radical production.

**Rationale.** P53 post-translational modifications have been mainly documented in the cancer literature, supporting a role for p53 acetylation and activation in direct association with apoptotic neuronal cell death [81,91]. Interestingly, it has been suggested that 17β-E₂ may suppress this pro-apoptotic p53 function by interfering with p53 transcriptional activity in breast cancer cells [92,112]. Yet, to our knowledge, no-one has examined 17β-E₂ role in p53 deacetylation following ischemia, which could provide a novel level of regulation of the apoptotic cascade and potentially serve as a beneficial therapeutic target in stroke treatment. Consequently, we turned our attention to examine two important mediators of oxidative stress cell death following ischemia, p53 and Puma, found downstream of O₂⁻ production. In this aim, we will utilize specific antibodies that target known acetylation sites on the p53 protein to detect overall activation and regulation by 17β-E₂.
Mechanism of Superoxide Production by NADPH Oxidase

A

Mechanism of Superoxide Production by NADPH Oxidase

B
Figure 1-1: Mechanism of superoxide production by NADPH oxidase.

(A) Physiologically, inactive state of the NOX2 enzyme where NOX2 and p22 phox are present in the neuronal plasma membrane, stabilizing one another, while the rest of the organizational (p47, p67, p40 phox) and activating (Rac1) subunits are inactive in the cytoplasm. (B) Upon ischemic stress, simultaneous p47 phox phosphorylation and Rac1 activation induce the translocation of all subunits to the membrane, which facilitates NOX2 complex formation, activation and $O_2^-$ generation.
Figure 1-2: Apoptotic signaling cascade of p53 acetylation and Puma induction following ischemic insult. During ischemic stress, nuclear p53 will induce the transcriptional activation of Puma, which will bind to the anti-apoptotic protein Bcl-xL, thus freeing p53 to activate pro-apoptotic Bcl-2–associated X protein (Bax) and stimulate mitochondrial permeabilization resulting in apoptotic neuronal cell death. We postulate that the free radical elevation which accompanies ischemic injury will trigger the acetylation of downstream p53 and induce apoptosis.
2.1 Introduction

Numerous studies to date have demonstrated the neuroprotective effect of 17β-Estradiol (17β-E2) in multiple neurodegenerative disorders [10,18,35,113,114,115], although the mechanism for such broad-based neuroprotection remains unclear. With respect to stroke, 17β-E2 has been shown to be neuroprotective in rodent models of both focal and global cerebral ischemia (GCI) [10,18,26,35,116]. Furthermore, it is well known that women are “protected” against stroke relative to men – at least until menopause [117,118,119], and that after menopause, women reportedly have a worse stroke outcome as compared to males [119,120]. How does 17β-E2 exert a broad-based neuroprotective effect in different neurodegenerative disorders such as stroke? The Brann lab had previously shown that superoxide (O$_2^-$) is elevated rapidly in the cerebral cortex following focal cerebral ischemia and that 17β-E2 attenuates this elevation [10], but the mechanism underlying the O$_2^-$ elevation and 17β-E2 regulation was unknown.

We hypothesized that O$_2^-$ would be similarly rapidly elevated in the hippocampal CA1 region following GCI, and that the enzyme, NADPH oxidase, plays an important role in the elevation of O$_2^-$ and production of downstream ROS. We further hypothesized that 17β-E2 acts to attenuate activation of NADPH oxidase, and that this effect plays a key role in its neuroprotective effects in cerebral ischemia.

As discussed in the Introduction, NOX2 NADPH oxidase is highly localized in the hippocampal CA1 region [64], and its activation is dependent upon forming an active complex with several cytosolic factors (p47phox, p67phox, p40phox) and activated Rac1, which
translocate to the membrane after activation [62,64]. Previous studies in male rats using the focal cerebral ischemia model have shown that NADPH oxidase is elevated in the cerebral cortex after reperfusion, and that NOX2 NADPH oxidase knockout mice have a reduced infarct size as compared to wild type mice [68]. However, comparatively little is known about NADPH oxidase activation in the hippocampus, and it is also unknown whether 17β-E₂ can regulate NADPH oxidase activation and \( O_2^- \) production as a mechanism of its neuroprotection against cerebral ischemia neuronal damage. Furthermore, since the previous studies on NADPH oxidase and cerebral ischemia all used male animals; there is no information on the role of NADPH oxidase in cerebral ischemia in female animals.

Thus, the goal of this aim was to address the issue of whether 17β-E₂ can directly influence neuronal survival in the female rat brain in an experimental model of GCI and assess NOX2 NADPH oxidase enzymatic contributions to oxidative stress in the hippocampal CA1 region.

**Specific Aim #1. To establish the temporal pattern of NOX2 activation, \( O_2^- \) production and oxidative stress neuronal damage in global ischemia and determine whether 17β-E₂ regulatory effects are ER-dependent.** In an attempt to determine the role of reactive oxygen species (ROS), in particular, \( O_2^- \), following global ischemia, the molecular mechanisms by which 17β-E₂ intervenes in GCI-induced apoptotic cell death will be examined, while assessing the contributions of the ERs to neuroprotection.
2.2 Materials and Methods

2.2.1 Global Cerebral Ischemia Model

Animals. Adult (3-month old) Sprague Dawley female rats were bilaterally ovariectomized. Placebo or estrogen (17β-E₂) Alzet minipumps (0.025mg; 14-21 day release) were implanted subcutaneously in the upper mid-back region under the skin at the time of ovariectomy and global cerebral ischemia (GCI) performed one week later. The dose of 17β-E₂ used produces serum 17β-E₂ levels of ~10-15 pg/ml, which represents physiological low Diestrus I levels of 17β-E₂ [116].

Surgical Protocol. For GCI, all animals (except sham control) underwent 4-vessel occlusion (4-VO) GCI performed as described previously [116,121,122,123]. Briefly, 24h after electrocautery of the vertebral arteries, the common carotid arteries (CCAs) were occluded with aneurysm clips to induce 10 min forebrain ischemia. Animals which lost their righting reflex within 30 seconds and whose pupils were dilated and unresponsive to light during GCI were selected for the experiments, as this indicates successful GCI. The clips were then removed, and the blood flow through the arteries was confirmed before the wound was sutured. Rectal temperature was maintained at 36.5 to 37.5°C throughout the experiment with a thermal blanket. The animals of the sham group underwent identical procedures except that the CCAs were simply exposed, but not occluded.

Administration of Drugs. The following compounds were bilaterally infused into the lateral ventricles (from the bregma: anteroposterior, ±0.8 mm; lateral, 1.5 mm; depth, 3.5 mm) 30 min before induction of GCI: the estrogen receptor antagonist ICI182,780 (50 µg; Tocris, Ellisville,
MO, dissolved in 5 µl of 50% dimethylsulfoxide, DMSO), gp91ds-tat and its scrambled peptide control (100 ng each, 5 µl in saline, synthesized by AnaSpec Inc, San Jose, CA). For intracerebroventricular (i.c.v.) injections, anesthetized rats were placed on a stereotaxic instrument. All drug infusions, as listed above, were performed using a Hamilton microsyringe at a flow rate of 1 µl/min. Following injections, the needle was left in situ for 5 min before the complete 2 min retraction.

2.2.2 Histology and Immunohistochemistry

Histology Analysis. Histological examination of the ischemic brain was performed by NeuN and Fluoro Jade B as described previously by our lab [116]. Briefly, after perfusion with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), the brains were post-fixed, cryoprotected with 30% sucrose until they sank and frozen sectioned (20 µm) in the coronal plane of the dorsal hippocampus (~2.5-4.5 mm posterior from bregma). Every fifth section was collected and utilized for staining. Staining for NeuN and Fluoro Jade B was performed using a mouse anti-NeuN monoclonal antibody (1:500, Chemicon, MA, USA) and Fluoro Jade B (AG310, Chemicon) as described in detail previously by our laboratory [116]. Images were captured on an LSM510 Meta confocal laser microscope (Carl Zeiss, Thornwood, NY) as described previously by our laboratory [124]. Cells that positively stained with NeuN and negatively stained with Fluoro Jade B were identified as “surviving neurons”; in contrast, double-stained yellow-colored cells represent CA1 neurons undergoing degeneration.

TUNEL Staining. TUNEL staining was performed on the free-floating coronal sections using the In Situ Cell Death Detection Kit (Roche, Penzberg, Germany) following the manufacturer's instruction. Briefly, after washing with 0.1 % PBS-Triton-X100, the slides were permeabilized with 10 µg/ml proteinase K in 10 mM Tris/HCl (pH 7.4) for 15 min and incubated with TUNEL reaction mixture including enzyme solution (TdT) and Tetramethylrhodamine (TMR)-labeled
TUNEL-positive nucleotides in a humidified chamber for 1 h at 37 °C. Slides for negative control were incubated with the label solution without terminal transferase for TUNEL. Samples were analyzed with a LSM510 Meta confocal microscope. For quantitative analyses, the number of surviving neurons, and TUNEL-positive cells per 250 µm length of medial CA1 pyramidal cell layer was counted bilaterally in 4-5 sections per animal to provide a single value for each animal. A Mean ± SE was calculated from the data in each group (n = 6-8 animals) and statistical analysis performed as described below.

**DAB Staining.** For DAB staining, sections were incubated with 10% normal goat/horse serum in PBS containing 0.1% Triton-X100 and 0.3% H₂O₂ for 1 h at room temperature to block nonspecific surfaces. Sections were then incubated with the primary antibodies overnight at 4°C in PBS containing 0.1% Triton-X100. The antibodies used were as follows: mouse anti-4-Hydroxy-2-nonenal (4-HNE) (1:500, Genox Corp, Baltimore, MD), mouse anti-8-Hydroxy-2’-deoxyguanosine (8-OHdG) (1:100, Genox Corp) and anti-NOX2/gp91phox (1:1000, Abcam, Cambridge, MA). Afterwards, sections were washed with the same buffer, followed by incubation with secondary biotinylated horse anti-mouse or goat anti-rabbit antibodies (Vector Laboratories, Inc., Burlingame, CA) at a dilution of 1:200 in PBS containing 0.1% Triton X-100 for 1 h at room temperature. Sections were then washed, followed by incubation with ABC reagents for 1 h at room temperature in the same buffer. Sections were rinsed in the same buffer and incubated with DAB reagent according to the manufacturer’s instructions (Vector Laboratories, Inc.) for 2-10 min. Following DAB incubation, sections were washed briefly with distilled water and dehydrated in graded alcohols, cleared in xylene and mounted using xylene-based mounting medium. Images were captured on an Axiophot-2 visible/fluorescence microscope using an AxioVision4Ac software system (Carl Zeiss, Germany).
**Double/triple Immunofluorescence 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 appropriate primary antibodies overnight at 4°C in the same buffer. The following primary antibodies were used in different combinations: anti-NeuN (1:500, Millipore), anti-P47phox, anti-GFAP, and anti-NOX2 (1:1000, Abcam). After primary antibody incubation, sections were washed for 4 x 10 min at room temperature, followed by incubation with Alexa Fluor594/647 donkey anti-mouse or rabbit, Alexa-Fluor488/594 donkey anti-rabbit or mouse, or Alexa-Fluor488/594 donkey anti-goat secondary antibody (1:500; Invitrogen Corporation, Carlsbad, CA) for 1h at room temperature. Sections were then washed with PBS containing 0.1% Triton X-100 for 4 x 10 min, followed by 3 x 5 min with PBS and briefly with water, and then mounted with water-based mounting medium containing anti-fading agents (Biomeda, Fischer Scientific, Pittsburgh, PA). A simultaneous examination of negative controls (omission of primary antibody) confirmed the absence of nonspecific immunofluorescent staining, cross-immunostaining, or fluorescence bleed-through.

**Confocal Microscopy and Image Analysis.** All the double and triple-labeled images were captured on an LSM510 Meta confocal laser microscope (Carl Zeiss, Germany) using either a 5X or 40X oil immersion Neofluor objective (NA, 1.3) with the image size set at 1024 x 1024 pixels. The following excitation/emission laser 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 Fluor594 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.
2.2.3 Tissue Collection and Molecular Techniques

**Brain Homogenates and Subcellular Fractionations.** For brain tissue preparation, rats were sacrificed under anesthesia at the indicated time points. Whole brains were removed and the hippocampal CA1 region microdissected from both sides of the hippocampal fissure and immediately frozen in liquid nitrogen. Total protein and crude membrane fractions were extracted as follows. Briefly, tissue samples were gently homogenized using a glass homogenizer in 1.2 ml ice-cold buffer A containing 1.5 ml of 10 mM HEPES (pH 7.4), 0.5 mM MgCl_2, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 5 mM dithiothreitol (DTT), 10 mM β-phosphoglycerol, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM 4-nitrophenyl phosphate (PNPP) and protease inhibitor mixture (Sigma, St. Louis, MO). Two hundred microliters of the above homogenates were separated and sonicated as total protein lysates (T). The remaining homogenates were centrifuged at 1,000 x g at 4°C for 10 min to get supernatant (S1) fractions and nuclear fractions (P1). S1 fractions were centrifuged at 13,000 x g for 20 min at 4°C (results in S2 fractions, and P2, mitochondria/microsome fractions), and further centrifuged at 100,000 x g for 30 min to obtain the cytosolic fractions (S3) and the plasma membrane-enriched fractions (P3). The pellet membranes fractions were re-suspended in buffer A containing 0.1% Triton X-100 for 10 sec by sonication. The protein concentrations were determined by the Modified Lowry Protein Assay (Pierce, Rockford, ILL). All the samples were stored at -80°C until use.

**In situ Detection of Superoxide Production.** The production of superoxide (O_2^-) radicals was investigated using hydroethidine (HEt) (Molecular Probes, Invitrogen) as described previously by our group and others [124,125]. In the present study, HEt (1mg/ml in 200 μl PBS) was administered intravenously 30 min before ischemia. Animals were anesthetized using isoflurane 3h after ischemia and transcardially perfused with cold PBS and 4% PFA. Sham non-ischemic control animals were also treated with HEt solution as O_2^- production control. Fluorescent
intensity of the oxidized HEt was measured on a confocal laser microscope using an excitation wavelength of 543 nm and the emission was recorded at wavelengths between 560 and 590 nm. The images were examined using LSM 510 image software and the optical intensity of HEt signals in each recorded medial CA1 objective field was measured with the Image J analysis software (Version 1.30v; NIH, Bethesda, MD). The mean intensity was determined from four fields in each animal, and values (4-5 rats in each group) were expressed as fold changes versus sham control.

**NADPH Oxidase Activity Assay.** NADPH oxidase activity was determined based on superoxide-induced lucigenin photoemissions. For assaying NADPH oxidase enzymatic activity, 50 µg membrane fractions were used. Enzyme assays were carried out in a final volume of 1 ml containing 50 mM Krebs-Ringer Phosphate buffer, pH 7.0, 1 mM EGTA, 150 mM sucrose, 0.5 mM lucigenin, 0.1 mM NADPH, and tissue homogenate. Enzyme reactions were initiated with the addition of NADPH. No enzymatic activity could be detected in the absence of NADPH. Photoemissions, expressed in terms of relative light units (RLU), were measured every min for 5 min using a luminometer. Assays were carried out in the dark at room temperature with appropriate controls. The rate of NADPH consumption was monitored by measuring the means values in absorbance (340 nm) and NADPH oxidase activity was normalized by the amount of protein and the change in optical density (OD). Activity was calculated as OD/µg protein/min.

**Superoxide Production Assay.** Superoxide production was measured from the indicated supernatant (S2) fractions using a LumiMax Superoxide Anion Detection kit (Stratagene, La Jolla, CA) following the manufacturer’s protocol. Briefly, 50 µg sample protein were suspended in 100 µl of superoxide anion (SOA) assay medium, and then mixed with 100 µl of reagent mixture containing 0.2 mM luminol, 0.25 mM enhancer in SOA assay medium. Light emissions at 30-sec intervals were recorded by a standard luminometer. Values were standardized to the
amount of protein and photons of light counted were expressed as RLU per µg protein. Mean ± SE were calculated from the data collected in each group for graphical depiction expressed as fold changes vs. sham control group. Statistical analyses of the data were performed as described below.

**Statistical Analysis.** Statistical analysis was performed using either one-way analysis of variance (ANOVA) or two-way ANOVA analysis, followed by Student-Newman-Keuls post-hoc tests to determine group differences. When groups were compared to a control group (e.g. sham), Dunnett's test was adopted for post-hoc analyses after ANOVA. When only two groups were compared, a Student’s t-test was used. Statistical significance was accepted at the 95% confidence level (P < 0.05). Data was expressed as mean ± standard error (SE).

2.3 Results

2.3.1 17β-Estradiol Protects the Hippocampus CA1 Region from GCI-induced Delayed Neuronal Cell Death

*Figure 2-1* shows the neuroprotective effect of 17β-Estradiol (17β-E₂) upon the hippocampal CA1 region following GCI. As shown in *Figure 2-1A*, staining for NeuN (a neuronal marker) and Fluoro Jade B (a neuronal degeneration marker) revealed that GCI (Pla) induced a profound loss of NeuN staining with an elevation of Fluoro Jade B staining in the hippocampus CA1 region at 7d after GCI reperfusion as compared to Sham control. *Figure 2-1B(a)* shows quantification of the number of “surviving” neurons (cells positive for NeuN but negative for Fluoro Jade B) in the CA1 region from all animals, which confirms that 17β-E₂ exerts a robust neuroprotective effect against cerebral ischemia. Additionally, staining for TUNEL, a marker for DNA fragmentation, revealed that GCI (Pla) significantly increased TUNEL staining and the number of TUNEL-positive cells in the CA1 region as compared to Sham control, with 17β-E₂ significantly attenuating this effect (*Figure 2-1B(b)*). Furthermore,
17β-E2 neuroprotection appeared to be mediated by estrogen receptors (ER) as intracerebroventricular (icv) administration of the ER antagonist, ICI182,780, reversed 17β-E2 effects upon NeuN and Fluoro Jade B staining (Figure 2-1A), number of surviving neurons (Figure 2-1B(a)), and number of TUNEL-positive cells in the CA1 region (Figures 2-1A&B(b)).

2.3.2 17β-Estradiol Profoundly Attenuates Neuronal NADPH Oxidase Activation, Superoxide Anion (O$_2^-$) Production and Oxidative Damage after Ischemia

Since reactive oxygen species (ROS) can play a major role in damaging neurons following GCI reperfusion, we next examined whether 17β-E2 exerts an antioxidant effect through regulation of NADPH oxidase activation and O$_2^-$ production in the hippocampal CA1 region at different times after GCI. As shown in Figure 2-2A&B, NADPH oxidase activity and O$_2^-$ production in the CA1 were significantly elevated in Pla versus Sham control as early as 30 min after reperfusion, with peak NADPH oxidase activity and O$_2^-$ levels observed at 3h (~ 6-7 fold increase vs. Sham), followed by a sharp fall from 6h-24h. Intriguingly, low dose 17β-E2 replacement profoundly attenuated NADPH oxidase activation and O$_2^-$ production in the hippocampal CA1 region following reperfusion (Figure 2-2A), an effect blocked by the ER antagonist ICI180,782 (Fig 2-2B). O$_2^-$ production was also assessed using the in situ oxidized HEt method, in which HEt, a marker of O$_2^-$ production, is selectively taken up by cells and oxidized by O$_2^-$ into ethidium, which provides a red fluorescence signal. As shown in Figure 2-2C(a&b), assessment of oxidized HEt signal in the CA1 region at 3h after reperfusion revealed a robust induction of O$_2^-$ in the Pla group, as compared to Sham controls. 17β-E2 markedly attenuated the induction of O$_2^-$ in the CA1, an effect blocked by the ER antagonist, ICI182,780. Examination of oxidative damage markers revealed that in agreement with reduction of NADPH oxidase activity and O$_2^-$ by 17β-E2 following GCI, 17β-E2 also markedly attenuated oxidative damage in the CA1 region at 24h after GCI as measured by immunostaining for 4-HNE, a marker of lipid oxidative damage, as well as 8-OHdG, a marker of DNA oxidative damage (Figure 2D).
2.3.3 NOX2 Localizes to Hippocampal Neurons and its Inhibition is Neuroprotective

After Ischemic Injury

The NOX2 isoform is a major isoform of NADPH oxidase and has been shown to be highly expressed in many brain regions, including the hippocampus [64]. We thus examined whether NOX2 is localized in neurons or astrocytes in the hippocampal CA1 region following GCI. Triple immunostaining in the CA1 region at 3h after GCI in Pla animals for the neuronal marker NeuN, NOX2, and the glia marker, GFAP revealed that NOX2 is predominantly localized in neurons, and is found in the membrane and cytoplasmic compartments (Figure 2-3A). P47phox, which forms a complex with NOX2 leading to NADPH oxidase activation, was shown to also exhibit a predominant neuronal localization. To determine whether NADPH oxidase plays a major role in O$_2^-$ production and oxidative damage in the CA1 following GCI, we utilized the NADPH oxidase competitive antagonist, gp91ds-tat, which is a nine amino acid peptide sequence of the p47Phox docking site on NOX2 that prevents p47phox from forming a complex with NOX2 [126]. The tat component of the inhibitor is derived from the coat of the HIV virus and has been implicated to penetrate the cell while functioning to promote inhibitor localization at the plasma membrane [127]. Figure 2-3B shows gp91ds-tat effect upon NADPH oxidase activation and O$_2^-$ production following GCI. Gp91ds-tat, but not the scrambled peptide control (Scr), markedly abolished the enhanced NADPH oxidase activation and O$_2^-$ production in the CA1 region at 3h after reperfusion. Gp91ds-tat also significantly increased the number of surviving neurons in the CA1 region at 7d after GCI as compared to Pla or the scrambled peptide control-treated animals as illustrated in Figure 2-3C, suggesting that activation of neuronal NADPH oxidase and enhanced O$_2^-$ production plays an important role in inducing neuronal death in the hippocampal CA1 region following GCI.
2.4 Discussion and Significance

It is well known that ischemic reperfusion can induce significant damage to the brain due to the enhanced generation of ROS, such as the highly reactive $O_2^-$ ion [10,128,129,130,131], which can give rise to other damaging ROS such as hydroxyl ion and peroxynitrite [132]. The current study provides evidence that the membrane enzyme, NADPH oxidase, plays a major role in the generation of $O_2^-$ in the hippocampal CA1 region following ischemic reperfusion, and that inhibition of NADPH oxidase activation by either a competitive NADPH oxidase inhibitor (gp91ds-tat) or by $17\beta$-E2 treatment profoundly protects the hippocampal CA1 region from oxidative damage and neuronal cell death. The source of NADPH oxidase activity and $O_2^-$ generation following ischemic reperfusion appears to be primarily neuronal, as enhancement of oxidized HEt, an *in situ* marker of $O_2^-$ production, occurred predominantly in neurons. Furthermore, both NOX2 and p47phox were highly colocalized in neurons, but not in glia following GCI.

Our study suggests that the NOX2 isoform of NADPH oxidase has a critical role in the enhanced NADPH oxidase activation and $O_2^-$ production following GCI, as NOX2 and p47phox were highly colocalized in CA1 neurons at 3h post-reperfusion, the time of peak NADPH oxidase activity and $O_2^-$ production. Furthermore, administration of the competitive NOX2 NADPH oxidase inhibitor, gp91ds-tat, dramatically attenuated NADPH oxidase activation and $O_2^-$ production following reperfusion and strongly attenuated neuronal cell death. Additional work by others suggests that NOX2 also has a role in oxidative damage in the cerebral cortex, as cortical infarct damage was reduced following MCAO in NOX2 knockout mice, or after administration of the NADPH oxidase inhibitor, apocynin [76,133,134]. Thus, NOX2 NADPH oxidase appears to play a major role in ischemic ROS generation and oxidative damage in the brain.
Our study also showed that 17β-E₂ profoundly attenuates neuronal NADPH oxidase activation and O₂⁻ production in the CA1 region following GCI reperfusion. This effect appeared specific for the ischemic situation, as 17β-E₂-treated sham animals did not show reduced NADPH oxidase activity or O₂⁻ levels as compared to vehicle-treated sham controls.

NOX2 NADPH oxidase has also recently been demonstrated to have a significant role in Alzheimer’s disease pathology [135,136], and thus our observed 17β-E₂ mechanism of strongly inhibiting NADPH oxidase activation and ROS generation could also have relevance to 17β-E₂ beneficial effects in Alzheimer’s disease. This possibility awaits further study. We should add that the effects of 17β-E₂ on NADPH oxidase were shown to be ER-mediated as they were blocked by acute treatment with the ER antagonist, ICI182,780. The ability of acute ICI182,780 treatment to block 17β-E₂ neuroprotection, in which 17β-E₂ was administered for one week prior to GCI, suggests that 17β-E₂ actions immediately after ischemia are critically important for the neuroprotective effect of 17β-E₂. Importantly, work by others in the Brann laboratory revealed that the antioxidant and neuroprotective effect of 17β-E₂ in our study appeared to be mediated primarily by ERα, as antisense oligonucleotide knockdown of ERα but not ERβ resulted in a loss of NADPH oxidase and O₂⁻ regulatory effects and neuroprotection by 17β-E₂. In particular, based on the results of our study, 17β-E₂ action to modulate activation of pro-survival factors (e.g. Akt) to counteract oxidative pro-death pathways (e.g. NADPH oxidase/ROS) early after cerebral ischemia appears key to the ability of 17β-E₂ to exert neuroprotection in the hippocampal CA1 region, while ICI182,780 blocked these key 17β-E₂ actions. Interestingly, the Brann laboratory also showed that Estrogen Dendrimer Conjugate (EDC), an 17β-E₂ conjugate that cannot enter the nucleus, strongly attenuated NADPH oxidase activation and exerted neuroprotection in GCI, suggesting that an extranuclear ER is involved in 17β-E₂ antioxidant and neuroprotective effects [29].
In conclusion, the current study advances the field by elucidating a novel and potent ERα-mediated action of 17β-E₂ in the brain to suppress membrane NADPH oxidase activation and O₂⁻ generation in hippocampal CA1 neurons following ischemia. The proposed studies have the potential to significantly advance our understanding of 17β-E₂ signaling in the female brain and further elucidate the mechanisms underlying 17β-E₂ neuroprotection in order to provide effective therapeutic targets against the neurodegenerative diseases accompanying menopausal women.
**Figure 2-1:** 17β-Estradiol (17β-E₂) attenuates apoptotic neuronal cell death in hippocampal CA1 region at 7d after global cerebral ischemia in an estrogen receptor-dependent manner.

(A) Hippocampal sections from sham, placebo (Pla), 17β-E₂ and 17β-E₂ plus the estrogen receptor antagonist ICI 182,780 treated female ovariectomized animals were double stained with NeuN (red) and Fluoro-Jade B (green), or separately stained with TUNEL (red, right column). Cells that stained positive for NeuN staining and negatively for Fluoro-Jade B staining were identified as “surviving neurons”. In contrast, NeuN/Fluoro-Jade B double stained (yellow) cells represent CA1 pyramidal neurons undergoing degeneration. (B) Quantitative summary of data (means ± SE, n = 6-8 animals per group) shows the number of surviving neurons per 250 µm length of medial CA1. Scale bar = 50 µm, magnification 40X. *P <0.01 vs. Pla group, †P <0.05 vs. 17β-E₂ treatment group.
Figure 2-2: 17β-E2 attenuates NADPH oxidase activity, superoxide production and oxidative damage in hippocampal CA1 after global cerebral ischemia. (A) Time course of NADPH oxidase activity (a) and superoxide production (b) in hippocampal CA1 region from sham, placebo (Pla) and 17β-E2 treated rats. Values are means ± SE of 4-5 rats in each group and expressed as fold changed vs. sham + Pla group. *P <0.05 vs. sham; **P <0.05 vs. Pla at the same timepoint. There was no difference between Pla + sham and 17β-E2 + sham groups. (B) Effects of ICI182,780 on NADPH oxidase activity (a) and superoxide production (b) in CA1 at 3h after reperfusion. *P <0.05 vs. vehicle group. (C) Representative pictures of oxidized HEt staining taken from medial CA1 region 3h after reperfusion. Strong oxidized HEt staining was detected in Pla and 17β-E2 + ICI treated brains after ischemia, while weak oxidized HEt signals were detected in sham and 17β-E2 groups (a). 17β-E2 significantly attenuated superoxide generation compared to Pla, which was reversed by ICI182,780 (b). *P < 0.01, vs. sham and 17β-E2. (D) Effect of *P <0.05 vs. sham on oxidative damage markers for lipid peroxidation (4-HNE) and DNA damage (8-OHdG) 1d after ischemia. Note that 17β-E2 strongly decreased 4-HNE and 8-OHdG staining. Scale bar in all sections = 50 µm, magnification 40X. HEt: hydroethidine; ICI: ICI182,780.
Figure 2-3: Critical role NOX2-containing NADPH oxidase in superoxide production and ischemic neuronal damage in hippocampal CA1. (A) Confocal images show co-localization of NADPH oxidase isoforms NOX2 and p47phox with neuronal marker NeuN but not with astrocytic marker GFAP in hippocampus CA1 region at 3h after reperfusion. (B) Effect of gp91ds-tat peptide on NADPH oxidase activity and superoxide production from hippocampal CA1 samples 3h after ischemia, indicating NOX2-containing NADPH oxidase is the major source of superoxide anions produced in the CA1 following ischemic reperfusion. (C) Representative photographs of double staining with NeuN (red) and Fluoro-Jade B (green) show that administration of gp91ds-tat peptide exerts profound neuroprotection in CA1 as determined at 7d after reperfusion. CA1 cells positively stained with NeuN but not Fluoro-Jade B were identified as “surviving neurons” and counted per 250 µm length of medial CA1 from 5-6 rats in each group. †Represents $P <0.05$ vs. Pla and Scr treatment groups. Scr: gp91 scrambled-tat.
3.1 Introduction

3.1.1 Free Radical Production Promotes Ischemic Damage

Ischemic stroke is a significant clinical problem of complex neuropathology, and is the third leading cause of death in the United States of America. Abundant evidence suggests that oxidative stress contributes significantly to the neuronal cell death that occurs following ischemic stroke. For instance, following reperfusion of the brain, there is a rapid increase of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which has been shown to result in increased lipid peroxidation, protein nitrosylation, DNA damage and blood brain barrier dysfunction, thereby contributing to neuronal cell death [69,76,134]. It has been previously suggested that mitochondria are a primary source of ROS following ischemic reperfusion [137,138]. Recently however, there is emerging evidence that a membrane enzyme, NADPH oxidase, contributes significantly to ROS generation following ischemic reperfusion [61,111,139]. Activation of NADPH oxidase leads to generation of the superoxide ion (O$_2^-$), a ROS which can be converted to the highly reactive hydroxyl radical and to peroxynitrite, a highly damaging RNS [69,139]. To date, five NADPH oxidase enzyme (NOX) isoforms have been identified (NOX 1-5), and localization studies have shown that of the five NOX enzymes, the NOX2 and NOX4 isoforms are highly localized in the hippocampus CA1 and cerebral cortex [64,65]. NOX2 appears particularly important in oxidative stress-induced neuronal cell death following stroke, as evidenced by a dramatic reduction in infarct size in NOX2 knockout animals [68], and in NOX2 inhibitor-treated animals [29].
3.1.2 Rac1 GTPase: Evidence for a Role in Regulation of NADPH Activation

NOX2 activity requires Ras-Related C3 Botulinum Toxin Substrate 1 (Rac1), a Rho GTPase enzyme which functions as an allosteric regulator, converting between GDP (inactive) and GTP (active) forms to produce O$_2^-$. Physiologically, Rac functions to regulate the cell cycle, cell adhesion and actin cytoskeletal structure. Upon cellular stress, however, Rac activation induces NOX2 complex formation and the production of O$_2^-$ free radicals [140]. Previous studies from our own laboratory using GCI in the male rat have found an early elevation of Rac1 activity (30min. and 6h) following reperfusion within the hippocampus CA1 region, while antisense oligonucleotides (AS-ODN) inhibition and administration of a Rac-1 specific pharmacological inhibitor found profound neuroprotection [111]. Along the same lines, studies of recombinant adenoviral expression of a dominant negative Rac1 in a model of hepatic ischemia suppressed free radical production and revealed that Rac1 inhibition is protective against ischemic reperfusion-induced necrosis and apoptosis [141]. Although the Rac family also consists of other isoforms (Rac2, Rac3) expressed in the brain, [109,110] our studies will focus on Rac1 since it is ubiquitously distributed and present in the adult rat hippocampus [142,143,144]. Additionally, the Rac2 isoform is known to be highly expressed in the myloid cells of the immune system [145], whereas the Rac3 isoform is primarily present in the developing brain [146].

The factors that regulate brain NOX2 NADPH oxidase activation are not well understood. Studies in immune cells have provided evidence that the Rho GTPase, Rac1, plays an important role in NOX2 activation in immune cells. Upon cellular stress, Rac dissociates from its cytosolic complex with guanosine diphosphate (GDP) dissociation inhibitor (GDI). The inactive Rac-GDP is then exchanged for GTP through the action of guanine nucleotide exchange factors (GEFs), Tiam1 and Trio, which are located on the membrane. Once active, Rac-GTP associates with the membrane and interacts with the tetratricopeptide repeat (TPR) site on p67phox subunit [147] and with membrane bound NOX2, resulting in NADPH oxidase complex activation.
Surprisingly, there is not much known on the role of Rac1 GTPase in the ischemic brain. Work from our group has shown that administration of the Rac GTPase inhibitor (NSC23766) decreases activation of the proapoptotic signaling kinase, JNK in the hippocampus CA1 region following ischemic stroke reperfusion and reduces apoptosis [111]. Furthermore, the hormone 17β-Estradiol (17β-E2) was shown to suppress Rac1 GTPase activation in the hippocampus following ischemic stroke as a component of its neuroprotective actions (see Chapter 2). It is currently unknown whether Rac1 GTPase plays an important role in NADPH oxidase activation, ROS generation and oxidative stress in the hippocampus following stroke, and whether inhibition of Rac GTPase activation might preserve cognitive function following cerebral ischemia. The studies in the current aim will address these important questions.

**Specific Aim #2. To elucidate the signaling mechanisms underlying 17β-E2-mediated regulation of NOX2 activation and O₂⁻ generation in the brain after cerebral ischemia.**

The primary objective of the present aim was to decipher the mechanisms responsible for the observed 17β-E2-mediated neuroprotection in the CA1 after stroke (see Chapter 2), with a specific focus on Rac1 and its potential role in NADPH oxidase activation and O₂⁻ production. The male rat will provide us with a baseline model, where Rac1 role in free radical generation will be explored by NSC 23766, a selective Rac1 inhibitor administration to disrupt Rac1 signaling.

**3.2 Materials and Methods**

**3.2.1 Induction of Global Cerebral Ischemia**

**Animals, Treatments and Surgical Protocol.** The experimental model of global cerebral ischemia (GCI) has been widely used to study delayed neuronal cell death of sensitive hippocampus CA1 pyramidal neurons, which occurs 2-4 days after the onset of reperfusion [148]. In the current study, three-month old adult male Sprague–Dawley rats were used and GCI was induced by four-
vessel occlusion, as described previously in Chapter 2 of the dissertation [122,149]. In some studies, ovariectomized female rats supplemented with 17β-E2 minipumps were utilized as previously described (See Chapter 2). Treatment groups included sham non-ischemic animals, vehicle-treated controls and placebo (ischemic) treatment group. All procedures were approved by the Medical College of Georgia institutional committee for care and use of animals (#07-04-932) and were in accordance with National Institutes of Health guidelines.

**Intracerebroventricular Administration.** To investigate the role of Rac1 GTPase in response to ischemic injury, 25 µg of the Rac inhibitor, NSC23766 (Tocris Bioscience, Ellisville, Missouri, USA), were dissolved in 5 µL saline and administered bilaterally into the lateral cerebral ventricles of rats 15 min before ischemia for a total dose of 50 µg. Vehicle-treated control animals received bilateral saline injections. The 50 µg dose of the inhibitor was chosen based on a previous dose response curve performed in our lab which showed it to be the most optimal and effective dose in inhibiting Rac GTPase activation in the hippocampal CA1 region following intracerebroventricular (i.c.v) injection, and which did not display any significant behavioral side effects [111]. To investigate the role of NOX2 NADPH oxidase, we utilized a specific competitive NOX2 inhibitor, gp91ds-tat, which is a 9 amino acid peptide sequence of the p47Phox docking site on NOX2 that prevents p47phox from forming a complex with NOX2 [111]. A scrambled gp91ds-tat peptide was used as a control. Gp91ds-tat and the scrambled peptide control were administered bilaterally via i.c.v injection in the lateral ventricles at a dose of 100 ng in 5 µL. The dose of gp91ds-tat was chosen based on previous studies by our laboratory showing its effectiveness and lack of side effects [29]. For i.c.v. injections, the rats were placed on ear bars of a stereotaxic instrument under anesthesia. Drug infusion was performed using a steppermotorized microsyringe (Stoelting, Wood Dale, IL, USA) at a rate of 1 µL/min through a pre-implanted cannula in the cerebral ventricle (from the bregma: anteroposterior, ± 0.8 mm; lateral, 1.5 mm; depth, 3.5 mm).
3.2.2 Immunohistochemistry and Histological Analysis

Histological Analysis. Histological examination of the ischemic brain was performed by neuronal-specific nuclear protein (NeuN) and the neuronal degeneration marker (Fluoro-Jade B) in coronal sections of the hippocampal CA1 region, as described previously in Chapter 2 of the dissertation [116].

TUNEL Staining. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end (TUNEL) staining was performed on the free-floating coronal sections using the In Situ Cell Death Detection kit (Roche, Basel, Switzerland) following the protocol described in Chapter 2 of the dissertation.

DAB Staining. For DAB staining, sections were incubated as specified in Chapter 2 of the dissertation. The following primary antibodies were incubated overnight at 4°C in PBS containing 0.1% Triton X-100. The antibodies used were as follows: mouse anti-4-hydroxy-2-nonenal (4-HNE) (1:500; Genox, Baltimore, MD USA), mouse anti-8-hydroxy-2’-deoxyguanosine (8-OHdG) (1:100; Genox), rabbit anti-p-H2A.X (1:200; Cell Signaling Technology, Danvers, MA USA). Afterwards, sections were washed with the same buffer, followed by incubation with secondary biotinylated goat anti-mouse or goat anti-rabbit antibodies (Vector Laboratories, Burlingame, CA USA) at a dilution of 1:200 in PBS containing 0.1% Triton X-100 for 1h at room temperature.

Confocal Microscopy and Image Analysis. Images were captured on an LSM510 Meta confocal laser microscope (Carl Zeiss) using either a 5X or 40X oil-immersion Neofluor objective (1.3 numerical aperture) with the image size set at 1024 X 1024 pixels, as previously described [124].
3.2.3 *Molecular Assays*

**Brain Homogenates.** For brain tissue preparation, rats were sacrificed under isoflurane anesthesia at 30 min, 3h, 6h and 24h after GCI. The hippocampal CA1 region was micro-dissected from both sides of the hippocampal fissure. Total and membrane tissue lysates were generated, as described previously in *Chapter 2* of the dissertation.

**In Situ Detection of Superoxide Production.** The production of superoxide (O$_2^-$) free radicals was investigated using hydroethidine (HEt) (Invitrogen, Carlsbad, CA USA) as described previously by our group and others [29,124,125]. A detailed description is found in *Chapter 2* of the dissertation.

**NADPH Oxidase Activity and Superoxide Production Assays.** NADPH oxidase activity was determined based on superoxide-induced lucigenin photoemissions, while superoxide production was measured from total fractions using a LumiMax Superoxide Anion Detection kit (Stratagene, La Jolla, CA USA) following the protocol of the manufacturer, as described previously by our laboratory [29] and in *Chapter 2* of this dissertation. A mean ± SE was calculated from the data collected in each group for graphical depiction, expressed as fold change vs. sham control group. Statistical analysis of the data was performed as described below.

**Rac1 GTP Binding Assay.** Rac1 activation assays were performed using PAK1-PBD color agarose beads (Cell Biolabs, Inc., San Diego CA). Briefly, 400 μg samples were mixed with 20 μl PAK1-PBD agarose beads, and incubated for 1h at 4°C. The reaction was terminated by addition of MgCl$_2$. The agarose beads were collected by spinning at 12,000 x g for 1 min at 4°C and the supernatants were removed. Precipitated complexes were washed three times with magnesium-containing TBS buffer and boiled in sample buffer. Proteins were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and detected by immunoblotting using an anti-Rac1-specific antibody.
Western Blotting. Western blotting was performed as described in detail previously [116]. The antibodies used were as follows: β-Actin (1:2000, Sigma, St Louis, MO), Rac1 (1:1000, BD Transduction Laboratories Inc) and pan-Cadherin (1:200, Santa Cruz Biotechnology). The membrane was then washed with T-TBS to remove unbound antibody, followed by incubation with 2° HRP-conjugated or Alexa Fluor 680 goat anti-rabbit/mouse IgG for 1-2h at room temperature. Bound proteins were visualized using the Odyssey Imaging System (LI-COR Bioscience, Lincoln, NB) and semi-quantitative analyses of the bands were performed with the Image J analysis software (Version 1.30v; NIH, USA). Band densities for the indicated proteins were normalized and expressed relative to total proteins, Actin or Cadherin as indicated in the figures. Normalized means were then expressed as fold changes of the corresponding value for control (sham operated) animals. A Mean ± SE was calculated from the data from all the animals for graphical presentation and statistical comparison.

Statistical Evaluation. Four to five animals were used per group. All values were expressed as the means ± SE. Statistical analysis of the results was carried out by One-Way Analysis of Variance (ANOVA), followed by the Student-Newman–Keuls test. Statistical significance was accepted at the 95% confidence level. Differences of p<0.05 were considered significant.

3.3 Results

3.3.1 Rac1 GTPase Activation Contributes Significantly to Delayed Neuronal Cell Degeneration in the Hippocampus CA1 Region Following GCI

Figure 3-1 shows the effects of administration of a Rac GTPase inhibitor, NSC23766, on neuronal cell survival in the hippocampal CA1 region. NSC23766 pretreatment significantly protected against delayed neuronal cell degeneration and death in the hippocampal CA1 region at 7d after GCI. Representative photomicrographs of stained hippocampal CA1 sections revealed that GCI induced a significant loss of hippocampal CA1 neurons at 7d following ischemic...
reperfusion, as indicated by a significant decrease in NeuN-positive stained cells in saline-treated animals following GCI as compared to sham controls (*Figure 3-1A*). In addition, there was a significant increase in staining for Fluoro-Jade B (a neurodegeneration marker) and TUNEL (a method for detecting apoptosis) in the hippocampal CA1 region in saline-treated (ischemic) animals as compared to sham controls, which suggests there is increased neuronal degeneration and apoptosis following ischemic reperfusion. Merged, colocalized images of the hippocampus CA1 further demonstrate that the Fluoro-Jade B and TUNEL staining is predominantly neuron-specific. Intriguingly, i.c.v administration of the Rac GTPase inhibitor (NSC23766) strongly protected the hippocampal CA1 neurons from neuronal degeneration and apoptotic cell death, as evidenced by preserved NeuN neuronal staining and strongly attenuated Fluoro-Jade B and TUNEL staining in NSC23766-treated animals as compared to saline controls (*Figure 3-1A*). In *Figure 3-1B*, quantification of staining results from all animals is provided (expressed in terms of neuronal density - e.g. number of hippocampal CA1 cells per 250 µm). Surviving neurons within the hippocampus CA1 are defined as NeuN-positive and Fluoro-Jade B- and TUNEL-negative stained cells. The results demonstrate a profound reduction in neuronal density in vehicle-treated ischemic animals as compared to sham (non-ischemic) animals. NSC23766 treatment markedly attenuated hippocampal CA1 neuronal death following GCI, suggesting a critical role for Rac GTPase activation in delayed neuronal cell degeneration and death in the hippocampal CA1 region following GCI.

### 3.3.2 Temporal Pattern of NADPH Oxidase Activation and Superoxide (O$_2^-$) Production in Hippocampus CA1 of the Male Rat after Ischemic Injury

Rac1 GTPase activation has been suggested to be critical for NADPH oxidase function in immune cells and may have a similar role in the brain. To explore this possibility, we first examined the temporal pattern of NADPH oxidase activation and O$_2^-$ production in the male rat hippocampal CA1 region following GCI. As shown in *Figure 3-2A*, NADPH oxidase activation
in the hippocampal CA1 region increased rapidly following ischemic reperfusion, with an elevation observed as early as 30 min post-reperfusion, and peak NADPH oxidase activation levels observed at 3h post-reperfusion (~3 fold increase versus sham controls). NADPH oxidase activation levels subsequently fell at 6h and 24h after reperfusion, but mean levels were still higher than sham controls. Similarly, in Figure 3-2B, O$_2^-$ levels showed a similar rapid elevation at 30 min following GCI reperfusion, with peak levels observed at 3h post-reperfusion (~4-fold increase as compared to sham controls). Thereafter, O$_2^-$ levels fell slightly at 6h post-reperfusion, and by 24h post-reperfusion, O$_2^-$ levels, while still moderately elevated, were no longer statistically different from sham controls.

3.3.3 Rac GTPase Activation is Critical for Enhanced NADPH Oxidase Function and the Induction of Superoxide Anion after Ischemia

We next examined the role of Rac GTPase activation in enhanced NADPH oxidase activation and O$_2^-$ production in the hippocampal CA1 region following GCI by determining the effect of inhibiting Rac GTPase activation via administration of NSC23766. NADPH oxidase activation and O$_2^-$ production were examined at 3h following ischemia-reperfusion (e.g. the time-point of peak NADPH oxidase activation and O$_2^-$ production following GCI), and in situ O$_2^-$ production was also assessed using the in situ oxidized hydroethidine (HEt) method, in which HEt, a marker of O$_2^-$ production, is selectively taken up by cells and oxidized by O$_2^-$ into ethidium, which provides a red fluorescence signal. As shown in Figure 3-3A&B, treatment with NSC23766 significantly attenuated the GCI-induced elevation of NADPH oxidase activation and free radical production in the hippocampal CA1 region at 3h after reperfusion. In addition, “in situ” O$_2^-$ production, as measured by oxidized HEt staining, confirmed increased O$_2^-$ production in the hippocampal CA1 region following GCI, which was strongly attenuated by the Rac inhibitor, NSC23766 (Figure 3-3C).
3.3.4 Inhibition of Rac GTPase Activation Attenuates Induction of Oxidative Stress Damage Following Ischemic Insult

We next examined the effect of Rac GTPase inhibition upon oxidative stress damage in the hippocampal CA1 region following GCI. Immunostaining for the oxidative stress markers, 4-HNE (4-hydroxynonenal), 8-OHdG (8-hydroxydeoxyguanosine), and p-H2AX (phospho-histone) was conducted at 24h after GCI to assess lipid peroxidation, DNA damage and oxidative histone phosphorylation, respectively. The results show robust increases in immunostaining intensity for all three oxidative stress markers in the saline-injected (ischemia) group as compared to sham controls (Figure 3-4). Rac GTPase inhibition through administration of NSC23766 resulted in an almost complete attenuation of the oxidative stress damage as indicated by a strong attenuation of 4-HNE, 8-OHdG and p-H2AX immunostaining in the hippocampal CA1 region as compared to saline (ischemia) controls.

3.3.5 Rac1 Activation in the Female Rat is Critical for NADPH Oxidase Activation and is Significantly Attenuated by 17β-Estradiol

Finally, we also examined the role of Rac1 in NADPH oxidase activation and superoxide elevation in the hippocampal CA1 region in the female rat after GCI, and determined whether 17β-E2 regulates Rac1 activation. Similar to our observation in the male rat, administration of the Rac1 inhibitor, NSC23766 (Rac1-I) icv 30 min prior to GCI, markedly attenuated NADPH oxidase activation and O₂⁻ production in the female rat hippocampal CA1 region at 3h after reperfusion (NADPH oxidase activity: Veh 7.70 ± 1.32 vs. NSC23766 3.32 ± 1.25 fold increase vs. sham, p < 0.05; Superoxide levels: Veh 6.21 ± 0.88 vs. NSC23766 2.14 ± 0.50 fold increase vs. sham, p < 0.05). Furthermore, as shown in Figure 3-5A, the Rac1 inhibitor was neuroprotective in the female rat, as demonstrated by an increased number of surviving neurons in the CA1 as compared to a Vehicle control (Veh 7.60 ± 1.50 vs. NSC23766 38.20 ± 8.27 surviving neurons, p < 0.05). We next examined Rac1-GTP binding, which is critical for Rac1
activation \((Figure \ 3-5B)\). Our studies showed that Rac1-GTP binding is significantly elevated in \(Pla\) animals at 3h after reperfusion as compared to sham controls, suggesting that Rac1 activation is markedly increased after GCI. Importantly, \(17\beta\)-E\(_2\) markedly attenuated Rac1-GTP binding, while having no effect upon total Rac1 protein levels, suggesting that \(17\beta\)-E\(_2\) attenuates Rac1 activation following GCI. Activated Rac1 is known to move to the membrane to facilitate formation of the active NOX2 complex after an appropriate stimulus. We thus examined membrane levels of Rac1 in the hippocampal CA1 region after GCI and determined the effect of \(17\beta\)-E\(_2\). Our results showed that membrane levels of Rac1 were increased in the \(Pla\) group animals at 3h after reperfusion as compared to \(Sham\) controls, and \(17\beta\)-E\(_2\) treatment prevented this effect \((Figure \ 3-5C)\).

### 3.4 Discussion and Significance

The current study adds to growing evidence of an important role of Rac1 GTPase in oxidative stress and neuronal cell death following cerebral ischemia. Previous work by our group demonstrated that Rac1 GTPase activation increases from 10 min to 72h in the hippocampal CA1 region following GCI in rats, with peak levels from 3h-6h post reperfusion \([29,111]\). The elevation of Rac GTPase activation appears to contribute to the pathology following ischemic stress as evidenced by our finding that administration of a Rac GTPase inhibitor, NSC23766 or Rac1 antisense oligonucleotides \([111]\) attenuated apoptotic neuronal cell death following GCI. In addition, the current study revealed that neuronal degeneration in the hippocampal CA1 region was also markedly inhibited by the pretreatment with the Rac GTPase inhibitor following GCI, suggesting an important role for Rac GTPase activation in delayed neuronal degeneration following ischemic reperfusion injury. We also examined post-treatment administration of NSC23766 (15 min. after ischemia; 25 \(\mu\)g bilaterally i.c.v) and while it significantly enhanced survival of hippocampal neurons \((29.25\pm4.48)\) as compared to saline-treated controls \((4.25\pm1.25)\), the effect was not as robust as that observed with pretreatment (data not shown).
Our study also provides insight into how Rac1 GTPase activation contributes to cerebral ischemia pathology, by demonstrating that Rac1 GTPase activation is critical for induction of oxidative stress in the hippocampal CA1 region following ischemic reperfusion injury. Inhibition of Rac GTPase activation markedly decreased oxidative neuronal damage to hippocampal CA1 region lipids, proteins, and DNA after cerebral ischemia. The decrease in oxidative stress following Rac GTPase inhibition is likely due to a correlated significant attenuation of \( \text{O}_2^- \) generation, which was observed following ischemic reperfusion. Interestingly, targeted inhibition of Rac1 GTPase via a dominant negative approach has been shown to similarly exert anti-oxidative stress effects against ischemia/reperfusion injury in the liver [150]. Furthermore, deletion of Rac1 GTPase in mouse rod photoreceptors via a Cre/Lox approach has been shown to strongly protect rod photoreceptors against photo-oxidative stress [151]. Rac1 is also expressed in endothelium of vascular cells, and targeted deletion of endothelial Rac1 has been demonstrated to reduce focal cerebral ischemia-induced edema in mice [152]. Additionally, Kahles and colleagues have demonstrated that in \textit{vivo} Rac1 inhibition via administration of the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, atorvastatin, prevented ischemia/reperfusion-induced blood brain barrier disruption [134]. These findings, coupled with the results of the current study, support a critical role for Rac1 GTPase activation in the induction of oxidative stress in both neural and non-neural tissues.

Our results further suggest that Rac1 GTPase contributes to induction of oxidative stress in the hippocampus by facilitating activation of NADPH oxidase, a key membrane enzyme that generates \( \text{O}_2^- \) ions [29,61,153]. NSC23766 strongly attenuated NADPH oxidase activation and \( \text{O}_2^- \) generation in the hippocampal CA1 following GCI, as well as oxidized HEt staining, which is a marker of \textit{in situ} \( \text{O}_2^- \) generation. Moreover, studies in phagocytic cells have shown that Rac1 GTPase plays a key role in activation of the NOX2 NADPH oxidase isoform by a direct binding and recruiting of the p67phox subunit to the membrane [147,154]. Rac1 GTPase may also be
involved in activation of NOX1 and NOX3 isoforms, but it does not appear to be involved in activation of the NOX4, NOX 5 or Duox isoforms [154]. Work by our lab and others has provided evidence that the NOX2 isoform is a major contributor to $O_2^-$ production and oxidative stress neuronal damage in the hippocampus and cerebral cortex following cerebral ischemia, as evidenced by studies using a competitive NOX2 inhibitor and NOX2 knockout mice [29,68]. Based on these observations, it is proposed that Rac1 GTPase likely regulates $O_2^-$ generation and oxidative stress in the hippocampus following GCI through a regulatory action on the NOX2 isoform in the brain. However, a regulatory effect by Rac1 GTPase on NOX1 and NOX3 in the brain cannot be entirely excluded and deserves further study. Mechanistically speaking, NSC23766 has been shown to inhibit Rac1 binding and activation by preventing interaction of the Rac-specific guanine exchange factors (GEFs), Trio and Tiam1, with Rac1 [155]. Thus, it is postulated that regulation of Tiam1 and Trio following cerebral ischemia contributes to the enhanced Rac1 GTPase activation following ischemia/reperfusion. Additional studies are needed to address this interesting possibility.

Furthermore, our studies provide evidence that Rac1 activation increases in females following GCI, similar to males, and is critical for NADPH oxidase activation and superoxide production in the female hippocampal CA1 region following ischemic reperfusion. Of significant interest, our study showed that 17β-E2 strongly attenuated Rac1 activation in the female rat hippocampal CA1 region following GCI. Since Rac1 activation has been shown to be critical for activation of NOX2, the results suggest that 17β-E2 attenuation of NOX2 activation may be due to 17β-E2 attenuation of Rac1 activation. The mechanism whereby 17β-E2 regulates Rac1 activation may involve regulation of phosphorylation of Rac1 at Ser71. Previous work by our group and others showed that Akt can phosphorylate Rac1 at Ser71 and that this effect is correlated with reduced Rac1 activation [140]. Work by others in the Brann lab has shown that 17β-E2 indeed enhances phosphorylation of Rac1 at Ser71 at 3h after GCI,
and the effect was correlated with a significant attenuation of Rac1 activation [111]. Furthermore, administration of an Akt inhibitor markedly attenuated the ability of 17β-E2 to enhance phosphorylation of Rac1 and reduce Rac1 activation after GCI [156]. Studies presented in Chapter 3 of this dissertation suggest 17β-E2 suppression of NADPH oxidase activation and O$_2^-$ generation is due, in part, to 17β-E2 attenuation of Rac1 activation. This finding is consistent with previous studies showing that Rac1 activation is critical for NOX2 complex formation and activation [29,134].

Additional work performed by our collaborators in China reveals a functionally important role for Rac GTPase activation in the pathology of cerebral ischemia. Studies using the Morris Water Maze revealed the preservation of spatial learning and memory in Rac GTPase inhibitor-treated animals (injected with NSC23766) as compared to vehicle-treated controls at 7-9 days post ischemic reperfusion. This suggests that rac1 activation has a critical role in the pathology and cognitive functional impairment that follows cerebral ischemia.

Finally, it is likely that Rac1 GTPase contributes to multiple pathological events and signaling pathways, which collectively facilitate neuronal damage and cognitive dysfunction following cerebral ischemia. For instance, previous work by our group has shown that Rac1 GTPase also has an important role in activation of the proapoptotic JNK signaling pathway following cerebral ischemia, as Rac1 binds to a scaffold complex of POSH and MLK3 and facilitates activation of JNK [111,140]. Thus, in addition to enhancing NADPH oxidase activation and oxidative stress following ischemic reperfusion, Rac1 also enhances proapoptotic JNK signaling, which collectively can enhance oxidative damage and apoptotic neuronal cell death in the hippocampal CA1 region, leading to hippocampal dysfunction and cognitive decline.

In conclusion, the current study provides evidence of an important role of Rac1 GTPase in ischemic reperfusion injury-induced NADPH oxidase activation, O$_2^-$ production and oxidative stress in the hippocampal CA1 region following GCI, and thereby contributes significantly to the
delayed neuronal cell death and to a negative functional cognitive outcome following cerebral ischemia. The studies also demonstrate that 17β-E2 markedly attenuates Rac1 activation, which may be a key mechanism underlying its ability to suppress NADPH oxidase action and ROS production following ischemic reperfusion. Studies that target Rac1 GTPase for inhibition may thus have efficacy in the preservation of neuronal survival and cognitive function following stroke in humans, and therefore should be further explored.
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B

![Bar chart showing neuronal density comparison between Sham, Saline, and NSC23766 treatments.](chart)

*Significant difference.

(Cells/250 μm CA1)
**Figure 3-1:** Rac GTPase activation contributes significantly to neuronal cell death in the hippocampus CA1 region following global cerebral ischemia (GCI). (A) NSC23766 significantly attenuated cerebral ischemia-induced Rac1 activity at 3h after ischemia. *Represents p<0.05 (n=4-5 animals) vs. reperfusion at 3 hours (R3h) and saline treatment groups. (B) Representative hippocampal CA1 sections from sham, saline–treated (ischemia) and NSC23766-treated (ischemia) male rats were labeled with NeuN (Blue), TUNEL (Red) and FJadeB (Green) staining at 7d following GCI. Merged images represent CA1 neurons undergoing degeneration, indicated by white staining. (C) Quantification showed that NSC23766 was strongly neuroprotective of the CA1 region. Data was obtained from five independent animals and a typical experiment is presented. Results are expressed as neuronal density with mean ± SE. *p<0.05 vs. sham control and NSC23766.
Figure 3-2: Temporal expression of NADPH oxidase activity and superoxide production in the hippocampal CA1 region following GCI. Homogenates taken from the hippocampus CA1 at 30min, 3h, 6h and 24h after reperfusion were subjected to NADPH oxidase activity (A) and superoxide production (B) assays to assess temporal expression following ischemia-reperfusion. Data is expressed as optical density (OD) and represented as fold vs. sham from four to five animals. *p<0.05 vs. sham control.
Figure 3-3: Rac GTPase activation is critical for enhanced NADPH oxidase activity and superoxide production in the hippocampal CA1 region following GCI. NSC23766 pre-treatment reduces NADPH oxidase activity (A) and superoxide production (B) at 3h post-reperfusion in the hippocampus CA1 after GCI. Data for experiments (A) and (B) was measured as optical density (OD) and expressed as fold difference in comparison to sham from four to five animals. *p<0.05 vs. sham, #p<0.05 vs. saline control. HET staining measuring endogenous superoxide production in CA1 coronal brain sections obtained from four to five animals; magnification 40X (C).
Figure 3-4: Rac GTPase activation is critical for enhanced oxidative damage in the hippocampus CA1 following GCI. DAB staining of representative coronal CA1 sections at 24h post-reperfusion show NSC23766 ability to attenuate staining for oxidative stress markers for lipid peroxidation (4-HNE), DNA damage (8-OHdG) and histone phosphorylation (p-H2A.X). (Four to five animals per treatment group, magnification used was 20X).
Figure 3-5: Critical role of Rac1 in ischemic neuronal damage in the female rat CA1 region, and the effects of 17β-E2 in regulating Rac1 activation, membrane localization and interaction with NOX2. (A) Pretreatment with Rac1 inhibitor before ischemia resulted in significant neuroprotection as evidenced by an increased number of surviving neurons in the medial CA1 region. NeuN (red), Fluoro-Jade B (green). (B) Activated Rac (Rac1-GTP) was extracted from samples at 3h reperfusion using GST-PAK and visualized by blotting with anti-Rac1 antibody. Total Rac1 and actin before extraction were determined as loading controls. (C) Western blot analyses of membrane-bound Rac1 protein expression after 3h reperfusion. Membrane localized Rac1 was significantly increased in the CA1 region by ischemia as compared with sham rats, an effect that was significantly inhibited by 17β-E2 treatment. Total Rac1 protein was not changed by ischemia or 17β-E2 treatment. In B and C, values are means ± SE of 5–6 rats in each group; *P <0.05 vs. sham or 17β-E2 groups. Rac1 (M) – Membrane; Rac1 (T) – Total.
4.1 Introduction

4.1.1 Pro-apoptotic Factor p53 Promotes Neuronal Cell Death in Models of Cerebral Ischemia

Over the past decade, evidence has emerged supporting a neuroprotective role of 17β-Estradiol (17β-E2) in neurodegenerative diseases [10,18,35,113,114,115], although the mechanism for such broad-based neuroprotection remains to be elucidated. Stroke continues to be the 3rd leading cause of death and disability in the United States, with post-menopausal women representing the largest demographic associated with increased risk of strokes and worse outcomes as compared to aged-matched men [119,120]. Following menopause, 17β-E2 levels in the serum fall to ~0.1% of levels observed in young normally cycling women. The fall in 17β-E2 at menopause has been suggested to potentially contribute to the increased risk of stroke and worse outcome following stroke in postmenopausal women [157]. In support of this possibility, numerous basic science studies have demonstrated that 17β-E2 is neuroprotective in various models of stroke, and models of other neurodegenerative disorders such as Alzheimer’s disease [26,158,159].

Work from our laboratory and others have demonstrated that 17β-E2 exerts potent neuroprotection in the hippocampal CA1 region following global cerebral ischemia (GCI), a region critical for cognitive function, learning and memory [31,32,116]. An important question is how does 17β-E2 protect the vulnerable hippocampus CA1 region from delayed neuronal cell
death? Our previous work implicated attenuation of NADPH oxidase activation and Reactive Oxygen Species (ROS) production as a critical mechanism for 17β-E2 neuroprotection in the hippocampus [29]. We propose that NADPH oxidase-induced ROS regulate downstream apoptotic factors as a mechanism of inducing delayed neuronal cell death in the hippocampus. In support of this suggestion, we demonstrated that administration of an NADPH oxidase inhibitor significantly attenuated apoptotic cell death in the hippocampal CA1 region following GCI [29]. Based on these findings, we hypothesize that 17β-E2 may regulate the expression, activity and/or stability of the pro-apoptotic factor, p53. We further hypothesize that NADPH oxidase activation may be a critical upstream factor in the regulation of p53 activation and stability following GCI.

Previous work has implicated p53 in delayed neuronal cell death following GCI. For instance, evidence from p53 knock-out mice studies revealed reduced neuronal cell death after GCI as compared to WT-controls [86]. Along the same lines, Pifithrin-alpha (PFTα), a p53 specific pharmacological inhibitor, successfully blocked p53 nuclear transport and DNA binding, while increasing the number of surviving neurons [88] and promoting functional recovery in stroked animals [160]. Upon ischemic cellular stress, p53 levels dramatically increase, triggering transcriptional dependent and independent apoptotic pathways leading to mitochondrial permeabilization and neuronal cell death.

4.1.2 BH3 Family Members Puma and Noxa Contribute to Ischemic Damage

Current evidence suggests that p53 encodes sequence-specific transcription factors controlling the expression of genes whose products mediate apoptosis such as the p53-regulated BH3 family members Puma and Noxa proteins. Puma has been implicated as a critical mediator of p53-induced delayed neuronal cell death effects. Although both Puma and Noxa have been shown to mediate neuronal cell death, only Puma deficiency was significantly protective against apoptosis, stressing the importance of this factor in the apoptotic cascade [103,104,161]. Recently available knock-out (KO) mice lacking Puma and Noxa genes could not successfully produce the
p53 null phenotype. Instead, each gene only captures an aspect of the pleiotropic p53 action at best [162,163]. With respect to ischemic strokes, Puma-deficient mice exposed to the Middle Cerebral Artery Occlusion (MCAO) model demonstrated only a 50% decrease in cortical volume, suggesting that other compensatory mechanisms besides Puma gene expression may contribute to the p53 apoptotic phenotype [102]. However, work by Chan and coworkers [101] demonstrated that Puma is upregulated in the hippocampal CA1 region following GCI, and that Puma upregulation was inhibited by PFTα. Furthermore, the inhibition of Puma upregulation by the p53 inhibitor was correlated with significant neuroprotection.

4.1.3 *Post-translational Acetylation of P53 Activates its Pro-Death Function*

P53 activity and stability can be regulated by several post-translational modifications, including acetylation, methylation and phosphorylation. Recent work has shown that p53 can be acetylated at the Lysine$^{373}$ residue on the C-terminal of the p53 protein, which leads to enhanced p53 pro-apoptotic function due to increasing the stability and transcriptional activity of p53 [84,90,91]. Acetylation of p53 is controlled by two classes of enzymes known as histone acetyltransferases (HATs), which add an acetyl group to lysine residues, and histone deacetylases (HDACs), which remove the acetyl group [81]. HATs include proteins such as p300/CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF), known to acetylate (stabilize) p53 on the carboxyl terminus of p53, at Lysines$^{373}$, Lysine$^{382}$ and at Lysine$^{320}$, respectively. Interestingly, Mdm2, an E3 ubiquitin ligase, has been shown to inhibit the ability of HATs such as p300 to acetylate p53, leading to destabilization and ubiquitination/degradation of p53 [164]. It is known that there is a delicate balance and an inverse relationship between p53 acetylation (activation) and degradation, as decreased ubiquitinated p53 levels have been shown to be correlated with increased p53 stabilization in neurons [80]. With respect to HDACs, the NAD$^+$ dependent HDAC deacetylase, Sirtuin1 (Sirt1), has been recently demonstrated to deacetylate p53 in cancer cells [165]. Evidence supporting Sirt1 pro-survival effects within the hippocampus...
comes from studies in which administration of a Sirt1 activator, resveratrol, rescued the rat hippocampus from ischemia-induced neuronal cell death in a Sirt1-mediated pathway [166] that involved the deacetylation of Lysine^{373} and Lysine^{382} on the p53 protein [166,167,168]. The relationship between Sirt1 and downstream p53 was also demonstrated in Sirt1-deficient mice studies, where p53 hyperacetylation and enhanced developmental defects were observed in the Sirt1-deficient mice [169]. However, contrary to these results, treatment with the Sirt1 inhibitor, Niacinamide, was shown to attenuate CA1 neuronal cell death following a 10 minute GCI insult in rats, while neuronal specific Sirt1 overexpressing transgenic mice lost the protection after focal cerebral ischemia and experienced a reference memory deficit [170]. Thus, these results suggest that Sirt1 may actually promote stroke damage [171,172]. Since Sirt1 also can deacetylate histone proteins [173,174], and HDAC inhibitors have been shown to be neuroprotective in stroke [97,98,175], the neuroprotective effects of the Sirt1 inhibitor in stroke could be due to deacetylase actions on histone proteins rather than p53.

Finally, with respect to potential 17β-E2 regulation of p53, studies in breast cancer cells have shown that 17β-E2 can enhance ERα binding affinity to p53, leading to a downregulation of its transcriptional activation [92,93]. In addition, other studies reported that 17β-E2 could attenuate apoptosis via an inhibition of resveratrol-induced acetylation of p53 in cancer cells [94]. It is unknown whether p53 acetylation changes in the brain following GCI. Also unknown is whether 17β-E2 can regulate p53 acetylation and thereby regulate its activity and stability as a component of its neuroprotective actions in stroke. Also unknown is whether NADPH oxidase could contribute to changes in p53 acetylation in neurons following GCI. The current studies were designed to address these deficits in our knowledge in these important areas.

**Specific Aim #3. To determine whether 17β-E2 affects downstream pro-apoptotic factors p53 and PUMA levels or activation and establish the underlying regulatory mechanism.** We will investigate the ability of 17β-E2 to attenuate post-translational p53 acetylation and
interactions between the upstream mediator, O$_2^-$, and downstream p53, potentially serving as a future target for stroke therapy.

4.2 Materials and Methods

4.2.1 Induction of Global Cerebral Ischemia

**Animals and Surgical Protocol.** Adult Sprague-Dawley female rats (3 months of age) were bilaterally ovariectomized (OVX) and immediately implanted with placebo (PLA) or 17β-estradiol (17β-E$_2$) Alzet minipumps (0.025mg; 14-21 day release), producing a low physiological Diestrous I dose of 10 pg/ml of 17β-E$_2$ in the blood for 7 days (7d) prior to the induction of stroke [116]. In some animals, long-term 17β-E$_2$ deprivation was performed in which the animals were ovariectomized and 10 weeks later (10W), placebo or 17β-E$_2$ minipumps were implanted as previously described [29]. A 4-vessel occlusion (4-VO) model of global cerebral ischemia (GCI) was performed one week later, which damages the highly susceptible hippocampus CA1 region. All animals (except sham controls) underwent GCI, performed as described previously in Chapter 2, Materials and Methods section of the dissertation.

**Intracerebroventricular Drug Administration.** To determine whether crosstalk occurs between NADPH oxidase-induced membrane superoxide (O$_2^-$) production and downstream pro-apoptotic factors, Gp91ds-Tat, a competitive NOX2 inhibitor and its scrambled peptide control were used at the doses specified. To examine whether the Sirtuin1 (Sirt1) enzyme is critical for neuronal survival, we administered 3.94 µg of the Sirt1 inhibitor, Sirtinol, dissolved in 1mM DMSO and 10% DMSO injections diluted with saline to vehicle-treated controls, based on the most effective dose specified in the literature [166]. Intracerebroventricular drug infusions into the lateral ventricles of placebo treated female rats 30 min before induction of GCI were conducted at the specified reperfusion timepoints documented in the corresponding figure legends. (Refer to Chapter 2 for detailed protocol information).
4.2.2 Immunohistochemistry and Histological Analysis

**Histological Analysis.** Histological examination of the ischemic brain was performed as previously described in *Chapter 2* of the dissertation, with sections obtained at specific timepoints reported in the appropriate figure legends. Briefly, frozen sections were collected and cut at (20 µm) in the coronal plane of the dorsal hippocampus, with every fifth section collected and utilized for staining. Images were captured on an LSM510 Meta confocal laser microscope (Carl Zeiss, Thornwood, NY) as described by our laboratory [124].

**DAB Staining.** For DAB staining, sections were incubated as specified in *Chapter 2* of the dissertation. The goat anti-Noxa (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-Acetyl p53 specifically recognizing p53 acetylation at Lysine by p300 (1:200, Millipore, Billerica, MA) primary antibodies were incubated overnight at 4° Celsius (C) in PBS containing 0.1% Triton X-100. Afterwards, sections were washed with the same buffer, followed by incubation with a secondary biotinylated anti-goat or rabbit antibodies (Molecular Probes, Eugene, OR, USA) at a dilution of 1:500 in PBS containing 0.1% Triton X-100 for 1h at room temperature. Images were captured on an Axiophot-2 visible microscope using an AxioVision4Ac software system (Carl Zeiss, Germany) at a magnification of 10X or 40X, respectively.

**Cresyl Violet Staining.** Sections were stained with cresyl violet nissl stain to measure neuronal survival and then examined with a light microscope at the reperfusion timepoint mentioned in the figure legend. The neuronal density of CA1 neurons per 1 mm length of the medial CA1 pyramidal cell layer was counted bilaterally in four sections per animal. Cell counts from the right and left hippocampus on each of the four sections were averaged to provide mean value.

**Double Immunofluorescence Staining.** Coronal sections were incubated under the protocol specified throughout the dissertation (For additional information regarding methodology, refer to *Chapter 2*). The following primary antibodies were used in different combinations: rabbit anti-
acetyl p53 specifically recognizing p53 acetylation at Lysine^{373} by p300, mouse anti-NeuN (1:200, Millipore, Billerica, MA) and rabbit anti-Puma α/β (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). After primary antibody incubation, sections were washed and incubated with Alexa Fluor594/647 donkey anti-rabbit or Alexa-Fluor488/594 donkey anti-mouse secondary antibodies (1:500; Invitrogen Corporation, Carlsbad, CA), respectively. A simultaneous examination of negative controls (omission of primary antibody) confirmed the absence of nonspecific immunofluorescent staining, cross-immunostaining, or fluorescence bleed-through.

**Confocal Microscopy and Image Analysis.** Images were captured on an LSM510 Meta confocal laser microscope (Carl Zeiss) using a 40X oil-immersion Neofluor objective (1.3 numerical aperture), with the image size set at 1024 X 1024 pixels, as previously published [124] and as described in Chapter 2.

4.2.3 Molecular Assays using Hippocampal CA1 Homogenates

**Brain Homogenates and Subcellular Fractionations.** For brain tissue preparation, rats were sacrificed under isoflurane anesthesia at reported reperfusion timepoints after GCI, as mentioned in the corresponding figure legends. The protocol for obtaining total tissue lysates in the following study was described in Chapter 2 of the dissertation.

**Co-Immunoprecipitation.** For Co-immunoprecipitation (Co-IP), total fractions (each containing 200 µg of protein) obtained at ischemic reperfusion timepoints described in the figure legends, were diluted 4-fold with 500 µL of HEPES buffer containing 50 mM HEPES (pH7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 1 mM each of EGTA, EDTA, PMSF and Na₃VO₄. The supernatant was incubated with 5 µg mouse anti-p53 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and mixed for 4h at 4º C. After the addition of protein A/G-sepharose, the mixture was incubated at 4º C for additional 2h. Samples were washed three times with HEPES
buffer and eluted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer then boiled for 5 min.

**Western Blots.** Western blotting was performed on total lysates collected post-reperfusion, as described in the relevant figure legends. The antibodies used were as follows: Rabbit anti-Acetyl p53 specifically recognizing p53 acetylation at Lysine$^{373}$ by p300, (1:150, Millipore, Billerica, MA), Rabbit anti-Actin (1:300, Sigma-Aldrich, St. Louis, MO), Rabbit anti-p53K372me1 (1:300, Diagenode, Denville, NJ, USA), rabbit anti-Mdm2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-p53 (1:200, Cell Signaling Technology, Inc., Boston, MA), rabbit anti-Puma α/β (1:150, Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-Ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then washed with T-TBS to remove unbound antibody, followed by incubation with 2° HRP-conjugated or Alexa Fluor 680 goat anti-rabbit/mouse IgG and Alexa Fluor 680 donkey anti-rabbit/goat IgG for 1-2h at room temperature. Bound proteins were visualized using the Odyssey Imaging System (LI-COR Bioscience, Lincoln, NB) and semi-quantitative analysis of the bands was performed with the Image J analysis software (Version 1.30v; NIH, USA). Band densities for the indicated proteins were normalized and expressed relative to total protein, actin, as indicated in the figures. Normalized means were then expressed as fold changes of the corresponding value for control (sham operated) animals. A Mean ± SE was calculated from the data from all the animals for graphical presentation and statistical comparison. Refer to Chapter 2 of the dissertation for additional details.

**Real Time-PCR.** Total RNA from the female rat hippocampus CA1 was isolated following ischemic reperfusion as described in the corresponding figure legends and LightCycler RNA Amplification Kit SYBR Green I (Roche Applied Science, IN) was utilized. A negative control, which consisted of pooled total RNA run in the RT-PCR without RT added, was also included.
Five hundred nanograms of the RT reactions were used for the PCR, with the following primer sets to identify p53 transcription: forward primer 5’- TCT CCC CAG CAA AAG AAA AA - 3’ and reverse primer 5’ – CTT CGG GTA GCT GGA GTG AG – 3’. PCR reactions were conducted under the following conditions: 35 cycles at 95° C for 5 min, followed by 65° C for 30 min and 72° C for 3 min at an annealing temperature of 65° C and 4mM of MgCl₂. A total of 5 µL of the PCR product was electrophoresed, visualized with ethidium bromide, and photographed for documentation. A standard curve was generated and results were quantified accordingly in pg/mL.

Statistical Evaluation. Four to five animals were used per treatment group. All values were expressed as the means ± SE. Statistical analysis of the results was carried out by One-Way Analysis of Variance (ANOVA), followed by the Student-Newman–Keuls post-hoc test to determine group differences. When groups were compared to a control group (e.g. sham), Dunnett's test was adopted for post-hoc analyses after ANOVA. When only two groups were compared, a Student’s t-test was used. Statistical significance was accepted at the 95% confidence level (P < 0.05). Data was expressed as mean ± standard error (SE).

4.3 Results

4.3.1 17β-Estradiol Enhances Deacetylation and Inactivation of p53 Following Global Cerebral Ischemia

Figure 4-1 shows the effects of 17β-Estradiol (17β-E₂) upon the hippocampal CA1 region following GCI. Temporal expression of total lysates for p53 (A) and Acetyl p53 (B) was measured by Western blotting at the 3h, 6h and 1d reperfusion timepoints. It is important to note that the Acetyl p53 antibody targets Lysine³⁷³ residue of p53, well known to induce a pro-death cascade of p53 actions [91,176]. Placebo-treated (ischemic) rats revealed significantly higher levels of p53 at 6h and 1d as compared to sham controls. In addition, placebo-treated animals had
significantly higher Acetyl p53 protein levels at 3h, 6h and 1d as compared to sham controls. 17β-E₂ treatment significantly attenuated ischemia-induced p53 elevation and acetylation at all reperfusion timepoints, particularly at 6h and 1d as compared to placebo-treated controls. To determine in which cell types activated p53 was expressed, we performed double immunohistochemistry for the neuronal marker, NeuN (red), and Acetyl p53 (green) (C). The results show that Acetyl p53 was primarily localized in neurons, and that 17β-E₂ robustly attenuated p53 neuronal acetylation as compared to placebo-treated controls.

4.3.2 17β-Estradiol Downregulates BH3 Family Members Puma and Noxa After Cerebral Ischemia

We next examined expression of the downstream p53 transcriptional target proteins, Puma and Noxa, as an indirect measure of p53 transcriptional activation. To determine whether Puma exhibits neuronal localization and is regulated by 17β-E₂, we double stained coronal hippocampal CA1 sections from the 1d timepoint samples for the neuronal marker, NeuN, (red) and the BH3 pro-apoptotic factor, Puma (green). Representative images revealed Puma predominantly localized in pyramidal neurons of the hippocampus CA1 region in all treatment groups studied. We also observed robust Puma expression in placebo (ischemic) animals as compared to sham controls, and 17β-E₂ treatment strongly attenuated Puma levels almost back to baseline sham levels, as depicted in Figure 4-2(A). Quantitative Western blot analysis of total Puma expression using an anti-Puma antibody to detect α/β protein subunits at 1d ischemic reperfusion (B) revealed a similar pattern for Puma elevation in ischemic rats and a significant reduction upon 17β-E₂ administration by more than three folds, thus confirming the confocal data in panel (A). In panel (C), we examined an additional p53-induced BH3 family pro-apoptotic protein, Noxa, via DAB immunostaining. Our results demonstrate a similar pattern for Noxa upregulation in ischemic animals, and 17β-E₂ treatment markedly attenuating the ischemia induced Noxa upregulation.
4.3.3 The Ubiquitin-Proteasome System Underlies Estrogen-Induced Neuroprotective Mechanism for p53 Degradation

The aforementioned data demonstrates that 17β-E2 can attenuate p53 acetylation (i.e. activation) and reduce p53 levels in the hippocampal CA1 region following GCI. However, the mechanisms by which 17β-E2 can deacetylate p53 and reduce p53 levels remain unclear. One possibility is that 17β-E2 can reduce gene expression of p53 and thereby reduce p53 protein levels. Another possibility is that deacetylation of p53 at Lysine373 decreases stability of p53 and leads to its ubiquitination and degradation, as has been demonstrated in studies in cancer cells [79,177]. Figure 4-3(A) illustrates the results of examination of p53 gene expression in the hippocampal CA1 region at 6h and 1d after GCI using standard real time (RT)-PCR technique. As shown in Figure 4-3(A), 17β-E2 treatment did not significantly affect p53 gene expression in the hippocampal CA1 region at any timepoint examined. We thus turned to examine the second possibility – e.g. that the enhanced deacetylation of p53 at Lysine373 by 17β-E2 causes the p53 protein to become unstable, and leads to degradation of p53 via a ubiquitin/proteasomal mechanism. To examine this possibility, we Co-immunoprecipitated (Co-IP) p53 and immunoblotted for ubiquitin at the 1d reperfusion timepoint using total hippocampal CA1 region samples. The results show that 17β-E2 treatment significantly increases p53 ubiquitination as compared to placebo-treated or sham control animals (Figure 4-3B). We next examined p53 interaction with the E3 ubiquitin ligase, Mdm2, as it has been implicated to directly bind and induce p53 cytoplasmic translocation and degradation via the ubiquitin-proteasome system. Co-IPs were performed examining p53-Mdm2 interactions at 6h reperfusion (Fig 4-3 C). As shown in Fig 4-3 C, the placebo group had a pattern for a decrease of p53-Mdm2 interaction at 6h after reperfusion that correlated with an increase of p53 protein levels. In contrast, 17β-E2-treated animals had a significant elevation of p53-Mdm2 interaction at 6h after reperfusion, which correlated with a significant decrease in p53 protein levels (Panel C).
4.3.4  NOX2 NADPH Oxidase Inhibition Attenuates Crosstalk Signaling Between Superoxide and p53 at the Onset of Ischemic Reperfusion

Previous work by our laboratory revealed that 17β-E₂ can attenuate NOX2 NADPH oxidase-induced superoxide (O₂⁻) production, and that administration of a competitive NOX2 inhibitor, Gp91ds-Tat, was strongly neuroprotective against GCI-induced neuronal damage in the hippocampal CA1 region [29]. We proposed that NADPH oxidase-induced ROS may lie upstream of p53 and could modulate p53 acetylation following GCI. To address this possibility, we used intracerebroventricular (i.c.v.) injections of Gp91ds-Tat and a scrambled tat control peptide (Sc-Tat) that does not exert inhibitory effects on NADPH oxidase. When compared to scrambled-tat control peptide-treated animals, Gp91ds-Tat-treated animals had a significant attenuation of GCI-induced elevation of p53, acetylated p53 and Puma protein levels in the hippocampal CA1 region at 3h after GCI reperfusion, thus providing novel supportive evidence for NADPH oxidase-induced ROS regulation of p53 expression and post-transcriptional modification, as well as downstream Puma elevation following GCI (Figure 4-4).

4.3.5  The Sirtuin1 Inhibitor, Sirtinol, Promotes Neuronal Survival within the Hippocampal CA1 Region Following Cerebral Ischemia

In Figure 4-5, we examined whether the class III histone deacetylase (HDAC), Sirtuin1 (Sirt1), plays a key role in the mechanisms underlying 17β-E₂-induced neuroprotection of the hippocampus. We measured neuronal cell viability at 7 days after reperfusion using the nissl stain, Cresyl Violet (CV) (A). Our experimental paradigm consisted of DMSO versus Sirtinol i.c.v. injections in 17β-E₂-treated animals as compared to ischemic (PLA) controls. The results demonstrate an apparent reduction in neuronal survivability of ischemic (PLA-treated) animals. However, 17β-E₂+DMSO treatment showed some neuroprotection, while 17β-E₂+Sirtinol treatment revealed intact, healthy CA1 neurons and robust CV staining, indicating that 17β-E₂-
treatment given in conjunction with Sirt1 inhibition is beneficial to neuronal survival in our GCI experimental paradigm. Quantification of the results is provided in panel (B).

4.3.6 Estrogen Downregulates p53 Methylation and Prevents p53 Function Following Global Cerebral Ischemia

We next turned our attention to another important post-translational modification – p53 methylation, which renders p53 active and stabilizes the protein to sufficiently trigger neuronal cell death. Using a methylated p53 antibody, p53K372me1, recognizing the pro-apoptotic Lysine\(^{372}\) residue on the p53 protein, we assessed \(17\beta\)-E\(_2\) regulation at 1d reperfusion. The results showed that methylation of p53 at Lysine\(^{372}\) in the hippocampal CA1 region is significantly increased in placebo-treated ischemic animals as compared to sham non-ischemic animals (Figure 4-6). In addition, \(17\beta\)-E\(_2\) treatment markedly decreased methylated p53 levels, as compared to the placebo-treated group. The findings suggest that \(17\beta\)-E\(_2\) may regulate p53 activity and stability via multiple post-translational mechanisms.

4.3.7 Long-term Estrogen Deprivation Promotes p53 Acetylation and Activation in the Nucleus of Hippocampal CA1 Cells After Global Cerebral Ischemia

Previous work by our laboratory demonstrated that \(17\beta\)-E\(_2\) neuroprotection is lost if it is preceded by a long period of \(17\beta\)-E\(_2\) deprivation (10 week (10W) ovariectomy) [29]. We thus wanted to examine whether the loss of \(17\beta\)-E\(_2\) neuroprotection after long-term \(17\beta\)-E\(_2\) deprivation could be correlated and potentially due to a loss of ability of \(17\beta\)-E\(_2\) to enhance deacetylation of p53 in the hippocampal CA1 region following GCI. We therefore compared animals treated immediately with \(17\beta\)-E\(_2\) to those in which \(17\beta\)-E\(_2\) treatment was initiated after a long term, 10W period of \(17\beta\)-E\(_2\) deprivation (treated after 10 weeks ovariectomy) (Figure 4-7). The results showed that immediate \(17\beta\)-E\(_2\)-treatment caused a marked reduction in p53 acetylation and nuclear translocation in the hippocampal CA1 region, while in contrast this effect was completely
lost in the long-term 17β-E2-deprived animals that received 17β-E2 treatment 10W after ovariectomy. Our findings reveal a loss in sensitivity of the hippocampal CA1 region to 17β-E2 signaling actions in long-term 17β-E2 deprived animals, which may underlie the loss of neuroprotection by 17β-E2 observed previously.

4.4 Discussion and Significance

The current study advances the field by demonstrating, for the first time, that p53 undergoes enhanced acetylation on Lysine\(^{373}\) in the hippocampal CA1 region following global cerebral ischemia. Acetylation of p53 at Lysine\(^{373}\) has been shown to enhance stability and pro-apoptotic activity of p53 [84,90,91]. Thus, our study suggests that post-translational modification of p53 by acetylation may be an important novel regulatory mechanism for enhancing p53 stability and activation following cerebral ischemia, leading to enhanced apoptotic cell death.

Our study also shows, for the first time, that 17β-E2 acts in the hippocampus to enhance deacetylation of p53 following GCI. 17β-E2 also prevented the induction of important p53 transcriptional targets, Puma and Noxa, which collectively may contribute to the profound neuroprotective effect of 17β-E2. Additionally, our data demonstrates that 17β-E2-treatment induces an increased interaction of p53 with the E3 ubiquitin ligase, Mdm2, which is correlated with enhanced ubiquitination and a decrease of p53 protein levels. Finally, we present strong evidence for crosstalk between ROS and downstream p53 expression and activation, which, to our knowledge, has never been documented in the stroked brain.

Throughout our studies, we primarily focused on the 1d reperfusion timepoint to examine p53 modifications, as work by other groups have implicated it to be prominent for ischemia-induced p53 apoptosis [85,178]. Our observations were further confirmed in MCF7 breast cancer cell studies showing direct interactions of the ER with p53, demonstrating the downregulation of its transcriptional activity [92,93] as well as the ability of 17β-E2 to regulate p53 acetylation state, thus blocking the initiation of the apoptotic pathway under stressful conditions [94]. Particularly
with respect to GCI, we found that 17β-E2 regulation of the pro-death Lysine\textsuperscript{373} on the C-terminal of the p53 protein, deacetylated p53 and prevented its nuclear translocation and therefore activation, thus preventing the neuronal apoptotic cascade, an effect which was reversed after a long period of 10 week (10W) 17β-E2 deprivation. The observed elevation of acetylated p53 in the nucleus of 10W 17β-E2-deprived rats in all treatment groups suggested the loss in 17β-E2 sensitivity within the hippocampus CA1 and supported a critical period for 17β-E2 neuroprotection following ischemia, as was previously published by our group [29]. Our unexpected finding of an elevation in nuclear acetylated p53 levels in 10W 17β-E2-deprived shams could be explained by other studies conducted in our laboratory, which support our observations and reveal an elevation in NOX2 activation levels in 10W shams (unpublished data), pointing to a possible crosstalk mechanism between NOX2-induced O$_2^-$ production and downstream p53. Furthermore, since the majority of strokes naturally accompany the aging process, we examined p53 acetylation in 3 month (young) and 24 month (old) female animals. We saw a robust, ischemia-induced increase in acetylated p53 staining in old as compared to young rats, indicative of p53 activation levels and that 17β-E2 administration was ineffective in reversing the damage and providing neuroprotection in old rats (unpublished data). Our preliminary observations further support the critical period hypothesis of 17β-E2 actions [6,10,179] and that elevated levels of p53 are damaging during the aging process [180].

Several other pro-death (Lysine\textsuperscript{317}, Lysine\textsuperscript{382}) and pro-survival (Lysine\textsuperscript{320}) acetylation sites have been implicated in the literature to regulate p53 function and have different biological consequences [90,91]. A significant caveat of our studies is that we only examined one pro-death acetylation site (Lysine\textsuperscript{373}) using the acetylated- Lysine\textsuperscript{373}-specific antibody. It is possible that acetylation at the other known acetylation sites could change after cerebral ischemia and/or 17β-E2 treatment as well, but further studies will be needed to address this issue. In addition to acetylation, other post-translational modifications can also influence p53 function and could be
regulated by 17β-E2. For instance, studies in breast cancer cells revealed that 17β-E2 inhibited resveratrol-stimulated phosphorylation of Serines 15, 20 and 392 of p53 and acetylation of p53 in a concentration-and time-dependent manner, and decreased apoptosis [94]. To our knowledge, no one has examined whether 17β-E2 exerts a similar regulatory effect upon phosphorylation of p53 in the brain following cerebral ischemia. Further studies are thus needed to address this issue. Finally, it has been demonstrated that p53 can be methylated at Lysine372, which leads to enhanced stability and activity of p53 [181]. Intriguingly, we found that 17β-E2 treatment significantly attenuated methylation of p53 at Lysine372 following global cerebral ischemia, which may contribute to decreasing stability and activity of p53 and the overall neuroprotective effect of 17β-E2. Our results are in agreement with the breast cancer literature, where 17β-E2 similarly was found to reduce p53 methylation at Lysine372 [182,183].

Our study also demonstrated the ability of 17β-E2 to regulate the p53-induced BH3 family members, Puma and Noxa. We showed that 17β-E2 neuroprotective properties prevented the amplification of these factors involved in the neuronal apoptotic pathway at the critical 1d ischemic reperfusion timepoint. Work from transient GCI studies measuring Puma expression in the hippocampus CA1 [101] and in the cortex of MCAO ischemic animals [101] similarly confirms Puma elevation at 24h following ischemic reperfusion [101], while the breast cancer literature provides support for 17β-E2 downregulation of Puma transcriptional activity [184].

It is currently unknown as to how 17β-E2 induces deacetylation of p53 following GCI. P53 acetylation can be controlled by histone acetyltransferases (HATs), which add an acetyl group to lysine residues on p53, and by histone deacetylases (HDACs), which remove the acetyl group from p53. Thus, 17β-E2-induced-deacetylation of p53 could be due to either a decrease of HAT expression and activity and/or an increase of HDAC expression or activity. In preliminary studies, we examined whether 17β-E2 regulates expression of Sirt1, an HDAC known to modulate acetylation of p53. The studies showed no effect on Sirt1 mRNA levels (unpublished
observation). Likewise, we tested a Sirt1 inhibitor, Sirtinol, to see if it would block the neuroprotective action of 17β-E2 in GCI. The preliminary study found that Sirtinol did not block 17β-E2-induced neuroprotection, and in fact, enhanced neuronal cell survival (unpublished observation). In agreement with our preliminary findings, administration of Nicotinamide, another Sirt1 inhibitor, was shown to attenuate CA1 neuronal cell death following GCI insult in rats [172] and to protect the cortex of mice from ischemic strokes [171]. There are several possible explanations for these unexpected findings: 1) Sirtinol is a non-specific inhibitor which may also act on the Sirtuin 2 isoform of the enzyme [185] and 2) HDAC inhibitors such as Sirtinol may also achieve their beneficial effects by targeting DNA histones, a concept which is currently being tested as a potential therapeutic in stroke clinical trials and so far was shown as neuroprotective [96,97,186].

Lastly, we turned our attention to the p53 degradation and ubiquitination system since 17β-E2 treatment effectively reduced p53 protein levels without changing its gene expression, indicative of a degradation mechanism for p53 reduction. We further elucidated whether 17β-E2 influences the expression and interactions of p53 with the E3 ubiquitin ligase, Mdm2, which facilitates protein degradation via ubiquitination. Interestingly, p53-Mdm2 interaction was enhanced by 17β-E2 at 6h after cerebral ischemia, and this correlated with a decrease in p53 protein. Similar observations have been reported in breast cancer cells, where 17β-E2 was also shown to regulate p53 levels via an Mdm2-mediated mechanism [184].

Our data also suggests that NADPH oxidase activation is critical for the regulation of p53 levels and acetylation state, as well as levels of the downstream target gene, Puma. Blocking O$_2^-$ production from the membrane NOX2 NADPH oxidase complex via administration of the competitive Gp91ds-Tat inhibitor resulted in the attenuation of cerebral ischemia-induced p53 levels, acetylated p53 levels, and Puma expression. Our suggestion that NADPH oxidase activation regulates p53 expression and activity is consistent with results in cancer cells and
endothelial cells demonstrating that NOX2 upregulation led to induction of p53 and enhanced apoptosis, while knockdown or inhibition of NOX2 reversed these effects [106,187].

In conclusion, the current study sheds light on a novel post-translational deacetylation regulatory mechanism of 17β-E2 in cerebral ischemia, modulating p53 activation and attenuating the expression of pro-apoptotic Puma, thereby providing neuroprotection against oxidative stress cell death in the hippocampus CA1. The proposed studies have the potential to significantly advance our understanding of 17β-E2 signaling in the female brain and may be applied to other neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease, thus facilitating the design of future therapeutic drug targets clinically utilized in the treatment of menopausal women.
A.

Fold change vs. sham

B.

Fold change vs. sham

C.

NeuN  Acetyl p53  Merged

Sham  

PLA  

E2  

p53  

Actin  

Acetyl p53  

Actin
Figure 4-1: Estrogen (17β-E₂) Deacetylates p53 in the Hippocampal CA1 Region as a Neuroprotective Mechanism against Global Cerebral Ischemia (GCI). (A & B) Homogenates obtained from the hippocampus CA1 at 3h, 6h and 24h after reperfusion were subjected to Western blot analyses to examine the temporal expression of p53 (A) and Acetyl p53 (B). Actin bands represent loading control. Data is expressed as fold differences as compared to sham from four to five animals. *p<0.05 vs. Sham, #p<0.05 vs. PLA control. (C) Representative hippocampal CA1 sections were double labeled with the neuronal marker NeuN (red) and Acetyl p53 (green) at 1d following ischemic reperfusion. Merged images represent acetylated p53 in pyramidal CA1 neurons of the hippocampus. Magnification 40X.
**Figure 4-2**: Estrogen (17β-E₂) Attenuates Downstream Pro-apoptotic BH3 Family Members, Puma and Noxa, in the CA1 Following Ischemic Injury. (A) Hippocampal CA1 sections were double stained for NeuN neuronal marker (red) and Puma (green) at 1d following global cerebral ischemia. Merged images depict Puma neuronal localization in the CA1 region of the hippocampus. Magnification 40X. (B) Western blot using anti-Puma antibody shows Puma protein expression at 1d following ischemic reperfusion. Actin bands represent loading control. Data is expressed as fold differences in comparison to sham from four to five animals. *p<0.05 vs. PLA. (C) Effect of 17β-E₂ treatment on Noxa protein expression at 1d reperfusion timepoint following cerebral ischemia. Representative photomicrographs of DAB staining obtained at a magnification of 10X.
Figure 4-3: Estrogen (17β-E₂) Promotes p53 Degradation by Increasing p53-Mdm2 Interactions. (A) P53 gene expression remains unchanged in the hippocampus CA1 at 6h and 24h following cerebral ischemia as measured by RT-PCR. (B) 17β-E₂ increases p53-ubiquitin interactions and promotes p53 degradation at 1d after GCI reperfusion. (C) 17β-E₂ increases p53-Mdm2 binding by three folds as compared to placebo-treated control levels, facilitating p53 degradation in pyramidal neurons at 6h after cerebral ischemic injury. P53 protein expression level serves as Co-IP control. *p<0.05 vs. Sham & PLA.
Figure 4-4: Superoxide-p53 Crosstalk is Attenuated by NOX2 Inhibitor Treatment at the Onset of Ischemic Reperfusion in the Hippocampal CA1 Region. Inhibition of superoxide (O$_2^-$) production by membrane enzyme NADPH Oxidase using the competitive NOX2 inhibitor, Gp91ds-Tat, reduces p53 activation and expression, while diminishing Puma protein levels, as compared to the scrambled tat (Sc-Tat) control group. CA1 total lysates were collected at 3h at reperfusion onset and probed with appropriate antibodies to detect protein expression in Western blot. Actin was used as a loading control. Data is represented as fold differences in comparison to sham from four to five animals. *$p<0.05$ vs. Sham, #$p<0.05$ vs. PLA.
A. SHAM  PLA  DMSO + E2  SIRTINOL + E2

B. Folds Change vs. Sham

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* p < 0.05
# p < 0.01
^ p < 0.001
**Figure 4-5: Sirtuin1 Inhibition Is Neuroprotective against Global Cerebral Ischemia.**

(A) Cresyl violet staining measuring neuronal survival of hippocampal CA1 cells 7d after ischemic reperfusion. Intracerebroventricular injections of 17β-E$_2$–treated rats with the Sirtuin1 (Sirt1) inhibitor, Sirtinol, promote neuronal survival as compared to PLA and DMSO-treated controls. Magnification = 40X, n=5-6 animals per treatment group. (B) Quantification of staining from panel (A). Data is represented as fold differences in comparison to sham from four to five animals. *$p<0.05$ vs. Sham, $^\#p<0.05$ vs. Sham, PLA & SIRTINOL+E2, $^\wedge p<0.05$ vs. PLA & DMSO+E2.
Fold Change vs. Sham

Sham | PLA | E2

Treatment Groups

p53K372me1
Actin
Figure 4-6: Estrogen (17β-E₂) Reduces p53 Methylation and Destabilizes p53 after Global Cerebral Ischemia. Western blot detecting methylated p53 expression at 1d reperfusion using anti-p53K372me1 antibody. 17β-E₂ treatment attenuated methylated p53 levels as compared to placebo-treated animals. Actin was used as a loading control. Data is represented as fold differences in comparison to sham from four to five animals. *p<0.05 vs. Sham, "p<0.05 vs. PLA.
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Figure 4-7: Long-term Estrogen (17β-E₂) Deprivation Facilitates p53 Activation via Acetylation and Nuclear Translocation in the Hippocampus CA1 after Global Cerebral Ischemia. Representative DAB images of immediate (7d) and 10 week (10W) deprived 17β-E₂-treated animals at 1d following ischemic reperfusion. Nuclear acetyl p53 staining labels activated p53 in CA1 neurons. Note that 17β-E₂ neuroprotection is lost during a long period of 17β-E₂ deprivation and that p53 acetylation readily occurs in the nucleus of 10W 17β-E₂-deprived animals as compared to 7d 17β-E₂-treated animals. Magnification 40X.
INTEGRATED DISCUSSION

There is abundant evidence in the scientific literature that 17β-E2 can exert neuroprotective actions in the brain. Although a large body of 17β-E2 literature exists, many questions are still left unanswered: What provides the 17β-E2 molecule with its remarkable neuroprotective properties? How does 17β-E2 protect neurons following an ischemic event? Is there a “critical period” for the protective effects of 17β-E2? The current study focused on increasing our understanding as to how 17β-E2 elicits its potent neuroprotective effects following ischemic injury. We hypothesized a mechanism whereby 17β-E2 neuroprotection is achieved by attenuating membrane ischemia-induced NOX2 activation of O₂⁻ production. We further postulated 17β-E2 actions to involve the deacetylation (inactivation) of downstream pro-apoptotic factor, p53, and that the ischemia-induced pro-apoptotic pathways mediate O₂⁻-p53 crosstalk. Our studies have advanced the field by providing insight into a novel regulatory mechanism for 17β-E2–mediated suppression of membrane oxidative stress via an extranuclear estrogen receptor (ER) mechanism, as well as 17β-E2–mediated inactivation of the critical GTPase enzyme, Rac1 following cerebral ischemia. We also provide strong evidence, for the first time, supporting 17β-E2 post-translational deacetylation (inactivation) of the pro-apoptotic factor, p53 and reduction of downstream transcriptional factor, Puma. Lastly, we also document ischemia-induced crosstalk signaling between O₂⁻ and p53 following cerebral ischemia.

The three PhD thesis aims presented in this dissertation are intertwined by the common theme of 17β-E2 neuroprotective mechanisms following global cerebral ischemia (GCI). Briefly, our major findings as well as their relevance to the literature are detailed as follows: in Aim1, we
elucidated 17β-E2 ability to protect the brain via an extranuclear ERα-mediated action to suppress membrane NADPH oxidase (NOX2) activation and O$_2^-$ generation in hippocampal CA1 neurons following 1d ischemic reperfusion (Chapter 2 of the dissertation). Until recently, it was believed that the majority of post-ischemic, intracellular O$_2^-$ source of oxidative stress was from the mitochondria [188,189]. Our study implicates an important role for the membrane-associated NOX2 NADPH oxidase enzyme as well in O$_2^-$ generation following cerebral ischemia. Our findings are supported by Oxygen Glucose Deprivation (OGD) studies confirming membrane neuronal NADPH oxidase as another main source of O$_2^-$ production during ischemic reoxygenation [61]. Our study also demonstrated, for the first time, that 17β-E2 profoundly attenuates NADPH oxidase activation and O$_2^-$ generation in the hippocampus following cerebral ischemia. Since NADPH oxidase activation has been implicated in many neurodegenerative disorders in addition to stroke, 17β-E2 regulation of NADPH oxidase activation may provide a mechanism for the purported broad-based neuroprotective action of 17β-E2 in multiple neurodegenerative disorders.

In **Aim2**, we continued to explore 17β-E2 regulation of the membrane NOX2 enzyme in the generation of oxidative stress in the female rat after GCI. Interestingly, we found that activation of the critical NOX2 activator enzymatic subunit, Rac1, is significantly attenuated by 17β-E2, providing a potential mechanistic explanation of how 17β-E2 may attenuate the activation of NADPH oxidase in the brain. Studies of the vasculature reaffirm Rac1 transcriptional downregulation by 17β-E2 under a physiological state and thus are in agreement with our results [190]. Logically, we then decided to study Rac1 GTPase activation in the male rat and discovered that it plays an important role in ischemic reperfusion injury, and that its pharmacological inactivation prevents neuronal cell death largely by diminishing NOX2-induced oxidative stress. Previously in the literature, Rac1 enhancement of cerebral ischemic damage has been noted [111,134], yet the consequences of its inhibition and functional role have not been thoroughly described (Refer to Chapter 3 of the dissertation for a detailed discussion).
We next turned our attention to examine downstream mediators of ischemic neuronal cell death, well-known to amplify the oxidative stress response and contribute to enhanced ischemic damage. Our findings in *Aim3* revealed, for the first time, that p53 undergoes enhanced acetylation at Lysine\(^{373}\) in the hippocampal CA1 region following cerebral ischemia. Acetylation of p53 at Lysine\(^{373}\) has been shown to enhance stability and proapoptotic activity of p53 \([84,90,91]\). Thus, our study suggests that post-translational modification of p53 by acetylation may be an important novel regulatory mechanism for enhancing p53 stability and activation following cerebral ischemia, leading to enhanced apoptotic cell death. Our study also showed, for the first time, that 17β-E\(_2\) acts in the hippocampus to enhance deacetylation of p53 following cerebral ischemia, while preventing the induction of important p53 pro-apoptotic transcriptional targets, Puma and Noxa. Our data also demonstrated that 17β-E\(_2\) treatment induces an increased interaction of p53 with the E3 ubiquitin ligase, Mdm2, which is correlated with enhanced ubiquitination and a decrease in p53 protein levels. Since p53 is a major pro-apoptotic signal implicated in neuronal cell death following cerebral ischemia and other neurodegenerative disorders, our finding of 17β-E\(_2\) regulation of p53 acetylation and degradation could represent an important mechanism underlying 17β-E\(_2\) neuroprotective actions in the brain. Additionally, for the first time to our knowledge, we also report a crosstalk signaling mechanism at early reperfusion onset after GCI, suggesting O\(_2^-\)-p53 crosstalk (*Chapter 4 of the dissertation*). The breast cancer literature provides substantial evidence supporting 17β-E\(_2\)-mediated transcriptional downregulation of p53 \([92,93]\), as well as the regulation of p53 apoptotic state through the control of its acetylation levels \([94]\). However, it is important to note that the latter studies are not reflective of the molecular changes brought about by cellular stress to the brain, as in the case of GCI, and that 17β-E\(_2\) post-translational deacetylation and attenuation of p53 activation in the ischemic brain has never been documented before. A clear gap of knowledge also exists between O\(_2^-\)-p53 crosstalk signaling, particularly with respect to neurodegenerative disease, as most of the previously attained evidence was gathered from studies of different tissue types throughout the
body. The reader is referred to Figure 6-1 for a visual summary of the Aims 1-3 presented throughout the PhD dissertation.

Interestingly, our results may be further extrapolated to other brain regions. For instance, the cortex, a brain area which controls higher executive function, is extremely susceptible to Middle Cerebral Artery Occlusion (MCAO). 17β-E2 pre-treatment has been implicated to be remarkably protective in females exposed to the MCAO model by preventing neuronal cell death through various molecular pathways [158,191,192]. Specifically relevant to our work, 17β-E2 pre-treatment at a physiologically low dose was shown to diminish MCAO-induced $O_2^-$ production at reperfusion onset in the female rat cortex [10]. The mechanisms for this 17β-E2–induced decrease in $O_2^-$ production are unknown, but it is possible that it could be due to regulation of NOX2 NADPH oxidase, as NOX2 is present in the cortex and has been implicated to play a critical role in ischemic neuronal cell death following MCAO, similar to its role in the hippocampus [76,193]. It would be interesting to conduct future studies investigating paralleling molecular pathways of 17β-E2 neuroprotection in various brain regions.

Many neurodegenerative disorders are associated with the excessive generation of Reactive Oxidant Species (ROS). Particularly in Alzheimer’s disease (AD), oxidative stress has been implicated to play a pivotal role in the progression of disease pathology. The literature supports a strong relationship between stroke and AD, suggesting the convergence of molecular pathways [194,195]. In fact, findings from our own laboratory have shown tau hyperphosphorylation (a principal component in AD neurofibrillary tangle formation) strongly induced after GCI and that 17β-E2 inhibits its upregulation by suppressing the activation of the JNK/c-Jun/Dkk1 signaling pathway in the hippocampus CA1 region [116]. We have also observed 17β-E2 ability to reduce Amyloid Precursor Protein (APP) levels, a cleaved product responsible for amyloid plaque generation in the AD brain, in the CA3 region of the hippocampus following GCI (unpublished findings). Other laboratories have also confirmed the powerful, antioxidant effects of 17β-E2 in attenuating ROS generation in vivo and in vitro models of AD
[196,197,198]. Intriguingly, APP-overexpressing mice lacking the catalytic subunit Nox2 of NADPH oxidase do not develop oxidative stress, cerebrovascular dysfunction, or behavioral deficits, indicating that NOX2 NADPH oxidase has a critical role in the pathology of Alzheimer’s disease [135]. Future work thus should examine 17β-E2 regulation of NOX2 activation and O$_2^-$ production in AD neuropathology and the contributions of p53 to the AD apoptotic cascade.
SUMMARY

In summary, our studies provide a contextual framework for 17β-E2 broad-based antioxidant and neuroprotective signaling in the ischemic brain. We have contributed an important piece to the puzzle by unveiling the remarkable potential of the 17β-E2 steroid hormone to utilize several paralleling pathways to alleviate the oxidative stress which accompanies ischemia. Our results are reflective of the benefits of a physiologically low dose of 17β-E2 in the hippocampus CA1 after global cerebral ischemia and implicate its neuroprotective properties through the activation of pro-survival molecular signaling pathways. Our findings are innovative in that they highlight the previously unrecognized and significant contribution of a membranous source of O$_2^-$ production to ischemic damage and 17β-E2 regulation thereof. Moreover, we elucidate a novel level of 17β-E2 post-translational regulation of the key apoptotic factor in cerebral ischemia and report undocumented crosstalk signaling between membrane O$_2^-$ and downstream p53 in the amplification of the neuronal cell death cascade. We recognize the limitations of our study design and molecular techniques, and consequently propose additional future studies to confirm our results in NOX2 knockout and p53 null mice, while investigating the impact of aging on 17β-E2 signaling in older animals following cerebral ischemia.

Clinical studies of post-menopausal women hormone replacement therapy (HRT) and stroke outcome remain controversial. One of the most clinically powerful set of studies conducted measuring stroke risk was the Women’s Health Initiative (WHI), which has failed to find a beneficial cardiovascular and neurological effect of HRT in post-menopausal women, but rather found an increase in stroke incidence [12]. The surprising findings of the WHI left the 17β-E2 field experts perplexed on whether to prescribe it as therapy for the relief of menopausal symptoms (Chapter 1 of dissertation contains a thorough discussion of the issue). The WHI study only
addressed stroke risk and not outcome, which is the subject of our studies. The WHI studies have been criticized due to the average age of subjects being 63 years of age, far past the menopause. It has led to the suggestion that there may be a “critical period” for estrogen replacement benefit – e.g. replacement therapy must be initiated prior to the menopause to be beneficial. There are a number of clinical studies underway, so far reporting positive results and addressing this important issue [58,199]. Our findings throughout the dissertation strongly support a profound neuroprotective role in experimental cerebral ischemia for 17β-E2. Additionally, we provide substantial basic science support for the “critical period hypothesis” of 17β-E2 neuroprotection, stating that the beneficial effects of 17β-E2 actions are only preserved during the years preceding menopausal transition. Our findings showed that 17β-E2 actions to decrease acetylation of p53 were lost if a prolonged period of estrogen deprivation preceded the 17β-E2 administration. This suggests a desensitization of the hippocampus occurs following prolonged period of estrogen deprivation, such as occurs following menopause. Other data from our laboratory has shown the loss of 17β-E2 neuroprotective ability in the hippocampus CA1 following a 10 week period of long term 17β-E2 deprivation, while 17β-E2 beneficial effects were preserved in the uterus (data outside scope of dissertation). [29]. These observations further suggest that there may be tissue-dependent changes in sensitivity to 17β-E2 actions following prolonged periods of hypoestrogenicity, and that a critical period for estrogen benefit may exist in the brain.

Taken altogether, the results of this work advance our understanding of how 17β-E2 exerts its neuroprotective actions in the brain in cerebral ischemia by demonstrating a potent attenuation of membrane NADPH oxidase activation, ROS generation and downstream activation and stability of the pro-apoptotic p53 signaling pathway. The studies may also help explain how 17β-E2 exerts broad-based neuroprotection in a variety of neurodegenerative disorders in addition to cerebral ischemia, based on the fact that NADPH oxidase activation has been implicated as a common mechanism contributing to the pathology in a variety of neurodegenerative disorders.
NOX2 activation, superoxide production and oxidative stress damage is strongly increased in the hippocampus following GCI, and 17β-E₂ markedly attenuates these effects to exert neuroprotection.

Rac1 activation, which is critical for NOX2 activation, increases following GCI, and 17β-E₂ prevents this increase, and thereby attenuates NADPH oxidase activation.

Summary: 17β-E₂

- Decreased oxidative stress
- Decreased apoptosis
- Neuroprotection

Acetylation/stabilization of p53 increases following GCI. 17β-E₂ attenuates p53 acetylation and increases degradation of p53 via a Mdm2-mediated mechanism.
**AIM1.** The goal was to establish a temporal pattern for NOX2 activation, O$_2^-$ production and oxidative stress following ischemia and determine the regulatory and neuroprotective role of 17β-E$_2$. **AIM2.** The objective was to elucidate the signaling mechanisms underlying 17β-E$_2$-mediated suppression of NOX2 activation and O$_2^-$ generation in the brain after cerebral ischemia. **AIM3.** The purpose of our studies was to determine whether 17β-E$_2$ can post-translationally reduce p53 activation and Puma induction by enhancing Sirt-1 deacetylase expression to inhibit this pro-death pathway.


