

# Stroke in the Female: Role of Biological Sex and Estrogen

*Stephanie J. Murphy, Louise D. McCullough, and Joanne M. Smith*

## Abstract

Women are protected from stroke relative to men until the years of menopause. Because stroke is the leading cause of serious, long-term disability in the United States, modeling sex-specific mechanisms and outcomes in animals is vital to research. Important research questions are focused on the effects of hormone replacement therapy, age, reproductive status, and identification of sex-specific risk factors. Available research relevant to stroke in the female has almost exclusively utilized rodent models. Gender-linked stroke outcomes are more detectable in experimental studies than in clinical trials and observational studies. Various estrogens have been extensively studied as neuroprotective agents in women, animals, and a variety of in vitro models of neural injury and degeneration. Most data in animal and cell models are based on 17 $\beta$ -estradiol and suggest that this steroid is neuroprotective in injury from ischemia/reperfusion. However, current evidence for the clinical benefits of hormone replacement therapy is unclear. Future research in this area will need to expand into stroke models utilizing higher order, gyrencephalic animals such as nonhuman primates if we are to improve extrapolation to the human scenario and to direct and enhance the design of ongoing and future clinical studies and trials.

**Key Words:** cerebral ischemia; estradiol; estrogen; hormone replacement therapy; neuroprotection; stroke

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<sup>1</sup>Abbreviations used in this article: CNS, central nervous system; CVD, cardiovascular diseases; HERS, Heart and Estrogen-Progestin Replacement Study; HRT, hormone replacement therapy; SAH, subarachnoid hemorrhage; SERMs, selective estrogen receptor modulators; TIA, transient ischemic attack; WEST, Women's Estrogen for Stroke Trial; WHI, Women's Health Initiative.

## Introduction

Women typically experience lower rates of vascular disease and atherosclerosis-related ischemic stroke than males. This epidemiological advantage is lost by the perimenopausal years, emphasizing that female reproductive hormones play some role in this sex difference. Aging women sustain a large burden for stroke, an observation frequently overlooked in the lay public's view of breast cancer as the main killer of women. It must also be emphasized that in both sexes, stroke is a major public health problem throughout the world. Stroke accounts for approximately one of every 14 deaths in the United States (AHA 2002), making it the third leading cause of death after heart disease and all forms of cancer.

Over the past few years, exploring sex-specific differences in brain injury and the role of sex steroids in these differences has become a fascinating area of research. Recent emphasis into women's health issues has refocused scientists to view hormonal cycles, menopausal physiology, and hormone replacement therapy (HRT<sup>1</sup>) as important to disease outcome. Historically, animal models of vascular and neurological disease have studied males solely. Use of males was justified as a means of reducing experimental variability caused by female hormone cycling and was based on the assumption that mechanisms of cell injury or treatment effects observed in males would also apply to females. In recent years, it has been recognized that disease conditions and responses to therapy may be different between the sexes, and women must be incorporated into clinical trials and human research. Reversing the "male-only" approach in experimental, animal-based studies has been slower to change. Much remains to be explored about stroke in women, such as the effects of age and reproductive status, gender-specific risk factors, and the importance of background sex steroid availability.

## Animal Models of Stroke

The presence of intact cerebral vasculature is essential to the study of abnormal brain perfusion. Because of the complexity of the brain and its response to injury, in vitro systems alone cannot thoroughly evaluate cerebral ischemia and its consequences. Preclinical and translational research into the causes, pathogenesis, and therapeutic management of stroke

therefore utilize animal models in addition to other techniques such as tissue culture and brain slices.

Human stroke is a diverse disease in terms of causes, manifestations, and anatomical sites of ischemia. Three types of stroke are generally seen in clinical patients: ischemic, intracerebral hemorrhage, and subarachnoid hemorrhage. The most common type of stroke is ischemic stroke, which accounts for 88% of stroke cases (AHA 2002). Most animal stroke models are based on this type (Table 1). A clot or other blockage within an artery leading to the brain causes ischemic stroke. An intracerebral hemorrhage is a type of stroke caused by the sudden rupture of an artery within the brain. Blood is then released into the brain, compressing brain structures. This type accounts for 9% of stroke cases (AHA 2002). Subarachnoid hemorrhage (SAH<sup>1</sup>) is also a type of stroke caused by the sudden rupture of an artery, but it differs from an intracerebral hemorrhage in that the location of the rupture leads to blood filling the space surrounding the brain rather than inside it.

About 3% of clinical stroke cases fall into this category (AHA 2002).

Many animal stroke models have been developed and characterized (Table 1), but no one model alone may fully mimic human stroke because of the heterogeneity of human clinical disease. Nonetheless, experimental animal models of stroke allow investigators to recreate specific aspects of human stroke carefully and to study pathophysiological and neuroprotective mechanisms as well as therapeutic responses under controlled conditions and in ways that cannot be performed easily or at all in clinical patients and in human subjects. For example, experimental stroke models allow investigators to have a controlled animal preparation for focusing on selected causal factors or mechanisms in contrast to human stroke, with its multiple contributing factors and possible confounding variables. More rigorous histopathological, biochemical, and physiological measurements can also be performed in animals. Finally, animal models allow investigators to study immediate and early

**Table 1 Animal stroke models<sup>a</sup>**

| Stroke model  | Animal species   |
|---|--|
| Focal cerebral ischemia (transient or permanent)            |  |
| • Middle cerebral artery occlusion (MCAO)                   | Nonhuman primate, dog, cat, rabbit, guinea pig, rat, mouse |
| • MCAO + ipsilateral common carotid artery occlusion (CCAO) | Dog, rat   |
| • MCAO + bilateral CCAO                                     | Rat  |
| • Spontaneous brain infarction                              | Spontaneously hypertensive rats                            |
| Global cerebral ischemia                                    |  |
| Complete (transient)  |  |
| • Decapitation  | Rat  |
| • Aortic and vena caval occlusion                           | Dog  |
| • Neck tourniquet or cuff inflation                         | Nonhuman primate, dog, cat, rat                            |
| • Cephalic artery occlusion (neck, thorax)                  | Nonhuman primate, cat                                      |
| • Cardiac arrest +/- cardiopulmonary resuscitation (CPR)    | Nonhuman primate, dog, sheep, pig, rat, mouse              |
| • Bilateral CCAO  | Gerbil   |
| Incomplete (transient or permanent)                         |  |
| • Hemorrhage/hypotension                                    | Cat, pig   |
| • Hypoxia-ischemia  | Dog, cat, sheep, pig, rat, mouse                           |
| • Intracranial hypertension +/- unilateral CCAO             | Rat  |
| • 2-Vessel occlusion (2-VO) +/- hypotension                 | Rat, mouse   |
| • 4-Vessel occlusion (4-VO)                                 | Rat  |
| • Unilateral CCAO   | Gerbil   |
| Multifocal cerebral ischemia                                |  |
| • Autologous or heterologous blood clot embolization        | Rat  |
| • Microsphere embolization                                  | Rabbit, rat  |
| • Photochemical-initiated multifocal embolization           | Rat  |
| Intracerebral hemorrhage                                    |  |
| • Intracerebral autologous blood infusion                   | Nonhuman primate, dog, cat, pig, rabbit, rat               |
| • Bacterial collagenase                                     | Pig, rat   |
| • Ischemia-reperfusion hemorrhage                           | Nonhuman primate   |
| Subarachnoid hemorrhage and vasospasm                       |  |
| • Intracranial arteries                                     | Nonhuman primate, dog, cat, pig, rabbit, rat, mouse        |
| • Extracranial arteries                                     | Dog, rabbit, rat   |

<sup>a</sup>Based on information from the following references (see text): Andaluz et al. 2002; Ashwal and Pearce 2001; De Lecinana et al. 2001; Ginsberg and Busto 1989; Lin et al. 2003; Macrae 1992; McAuley 1995; Megyesi et al. 2002; Traystman 2003.

ischemic events, events that can be difficult to examine in human patients because of the variable time delays in early recognition of a stroke and initial therapeutic intervention.

The identification and characterization of genetic and molecular components of stroke will also be critical to our future understanding, diagnosis, and treatment of this disease. The ongoing work in both human and selected animal genome projects has already led to the identification of candidate genes for stroke susceptibility (for review, see Carr et al. 2002; Schwarz et al. 2002) as well as sex differences in molecular mechanisms of ischemic damage (McCullough and Hurn 2003). Animal stroke models will therefore be essential for allowing investigators to examine what genes and proteins are affected in response to both stroke and estrogen therapy in both males and females. The use of genetically engineered mice, such as estrogen receptor subtype knockouts (Dubal et al. 2001; Sampei et al. 2000a), will be particularly useful in further understanding the complexities of ischemic pathophysiology with respect to gender and exogenous estrogen.

Both Old World (i.e., baboon, macaque) and New World (i.e., marmoset, squirrel monkey) monkeys have been used in stroke research since the 1930s (Fukada and del Zoppo 2003; Peterson and Evans 1937). However, there was no emphasis on sex differences in these early studies. To date, research on sex differences and sex hormone influences in stroke has utilized rodent models almost exclusively and has not yet been examined in nonhuman primates. Still, the nonhuman primate model could provide a useful bridge between experimental studies in rodents and clinical trials in humans. For example, Old World monkeys such as macaques and baboons have menstrual cycles and hormonal responses analogous to human females (Bellino and Wise 2003) and can be invaluable in stroke studies of gender differences and the perimenopausal state. Because considerable evidence indicates that cerebral ischemia triggers inflammation (e.g., Del Zoppo et al. 2001; Iadecola and Alexander 2001), there is also much interest in examining the contribution of inflammation to ischemic brain injury and worsening of neurological outcome as well as how sex steroids may influence these inflammatory processes. Therefore, another advantage in utilizing nonhuman primate models in evaluating stroke in women is that the immune system of these animals more closely resembles that of humans than other animal species, including rodents. In the future, nonhuman primate models may therefore play an important role in therapeutic development as the next step after rodents and other nonrodent models in testing promising procedures and medications in a system very close to the human in which both physiological and cognitive function can be evaluated during stroke and recovery. In an effort to contribute toward that next step, we review below the knowledge obtained to date from human clinical trials and observational studies as well as experimental animal stroke studies on gender differences and the effects of estrogen administration on stroke.

## Gender Differences in Stroke

### Clinical Trials and Observational Studies

Although many aspects of stroke such as clinical presentation and management are similar in both sexes, gender differences exist in incidence, recurrence, functional outcomes, pathophysiology, and mortality rates. The largest and still ongoing study to address the issue of gender and perimenopausal status in stroke is the Framingham Heart Study (<http://www.nhlbi.nih.gov/about/framingham/index.html>). This longitudinal cohort study was initiated in 1946 with the overall objective of identifying common contributing factors to cardiovascular diseases (CVD<sup>1</sup>) such as stroke by following its development over three generations in participants who had not yet developed clinical signs of CVD or suffered a heart attack or stroke. Women were included in this study since its inception because clinicians recognized that CVD, like stroke, occurred later and with lower frequency in women than in men, and they wanted to study why women benefited from this relative protection (Murabito 1995). Gender effects in the Framingham Study are most evident in participants within the 45- to 54-yr age cohort, but equalize in the 55- to 64-yr-old group (Wolf 1990). Differences between the sexes in risk profiles for stroke and other manifestations of CVD have also been described (Hurn and Macrae 2000; Paganini-Hill 2001; Stokes et al. 1987).

On the whole, stroke incidence appears to be higher in men than in women (Stegmayr et al. 1997; Sudlow and Warlow 1997) but increases with age in both sexes (Pren-cipe et al. 1997). Recent age-adjusted stroke incidence rates would also suggest a higher first stroke event for both Caucasian and African American males compared with females (AHA 2002). However, the American Heart Association (2002) reports that overall, approximately 40,000 more women than men have a stroke each year. This finding is thought to be due to the greater average life expectancy for women. In terms of occurrence, stroke occurs later in life than myocardial infarction in men, whereas in women, stroke and myocardial infarction incidences are similar (Kannel et al. 1983). In study participants younger than 60 yr of age, no clear gender difference was seen for incidence in brain infarction (Kannel 1971). However, in persons aged  $\geq 85$  yr, women were observed to have a higher stroke incidence (Barker and Mullooly 1997). Conversely, Giroud and colleagues (1991) reported a higher incidence of cortical infarction in men versus women even well beyond menopause. With respect to perimenopausal status, postmenopausal women up to age 55 were observed to have an increased risk of such CVD as stroke compared with premenopausal women of the same age (Kannel et al. 1976). With respect to recurrence of stroke in men and women, cumulative 5-yr recurrence rates in brain infarction for men were almost twice that for women (Sacco et al. 1982).

The question of whether functional outcome after stroke is gender independent is still under exploration. For ex-

ample, depression appears greater in women after stroke (Kotila et al. 1998). Several studies of geriatric stroke patients evaluated outcome measures such as motor function, cognitive function, activities of daily living function, and nursing home residency. Based on these outcome measures in geriatric patients, women appeared to be more functionally impaired by stroke than men and were found to be institutionalized more often than men (Di Carlo et al. 2003; Holroyd-Leduc et al. 2000; Kelly-Hayes et al. 1988; Leibson et al. 1998; Wyller et al. 1997). However, most of these studies did not specifically address differences in social and cultural variables such as marital status and the availability of a family caregiver as a possible explanation for this apparent difference in functional outcomes between older men and women.

In terms of pathophysiology, some variable gender differences in cerebrovascular occlusive disease distribution have been described. Caplan and colleagues (1986) reported that in men, extracranial large artery disease predominated, whereas intracranial medium-sized artery disease was more common in women. Conversely, Wolf and Kannel (1986) observed no significant differences in stroke type. In young women ranging in age from 15 to 44 yr, stroke was either predominantly embolic in origin (Carolei et al. 1993) or was caused by a SAH (Petitti et al. 1996, 1997). Of all of the stroke subtypes, SAH was more commonly observed in women than men (Davis 1994). Interestingly, in longitudinal studies, women sustain fewer strokes of all types than age-matched men (Kurtzke 1985).

Sex differences in stroke mortality are also under examination but may vary between stroke subtypes. For example, age-specific mortality for ischemic stroke was lower for women than for men <64 yr but was higher among older women aged  $\geq 65$  yr (AHA 2002; Ayala et al. 2002). Mortality for intracerebral hemorrhagic stroke in women was lower than or similar to those among men at all ages studied, but women had a higher risk of death from SAH, with gender differences increasing with age (AHA 2002; Ayala et al. 2002). Other studies, after adjusting for age but not stroke subtype, find that men appear to have a higher mortality rate (Modan and Wagener 1992; Tuomilehto et al. 1996). In contrast, the American Heart Association (2002) reported that in 2000, 38.6% of deaths from stroke were male and 61.4% were female. In a more recent study, older women were found to have a higher 28-day case-fatality rate as well as a higher in-hospital and 3-mo mortality (Di Carlo et al. 2003).

## Animal Studies

At the time of this writing, no published studies in nonhuman primate and other nonrodent stroke models have specifically examined gender and ischemic outcome in brain (Curry 2001). However, experimental studies have established that young adult female rodents sustain smaller injury than males after global/forebrain (Hall et al. 1991; Hurn and

Macrae 2000; Payan and Conard 1977) or focal ischemia (Alkayed et al. 1998; Hurn and Macrae 2000; Hurn et al. 2002; Li et al. 1996; Wise et al. 2001; Zhang et al. 1998). One study in a rat transient focal ischemia model showed no gender differences (Vergouwen et al. 2000). This overall neuroprotective effect of female gender on ischemic injury is still evident even in the presence of specific stroke risk factors such as diabetes (Toung et al. 2000; Vannucci et al. 2001) and hypertension (Alkayed et al. 1998; Cai et al. 1998; Carswell et al. 1999; Okamoto et al. 1974; Sadoshima et al. 1988). Based on evaluations of other outcomes such as length of survival (Sadoshima et al. 1988) or incidence/mortality rates in spontaneous (Yamori et al. 1976) or induced stroke (Berry et al. 1975; Payan and Conrad 1977), researchers have reported that female rodents have greater survival times and lower rates of stroke incidence/mortality than male animals. Results from studies that use genetically engineered mice to examine molecular mechanisms of ischemic damage also suggest that outcome can be sex dependent, even when the manipulated gene is not linked to reproduction (Sampei et al. 2000b).

In female rats, this gender-specific effect may be further influenced by the estrous cycle stage, with smaller infarct volumes in proestrus (high endogenous estradiol levels) animals compared with metestrus (low endogenous estradiol levels) (Carswell et al. 2000). Such differences may be due to the postischemic changes in cortical infarction, neutrophil accumulation, and antioxidant enzyme activities that have been shown to be inversely correlated with circulating estrogen levels in normal, cycling female rats (Liao et al. 2001). Sex differences in stroke sensitivity can also be abolished by ovariectomy (Alkayed et al. 1998; Dubal et al. 1998; Fukuda et al. 2000; Rusa et al. 1999; Santizo et al. 2002; Simpkins et al. 1997; Wang et al. 1999; Zhang et al. 1998) or by declining estrogen levels during reproductive senescence (Alkayed et al. 2000; Yamori et al. 1976).

Most recently, investigators have begun to look at functional outcomes in experimental stroke by evaluating a battery of behavioral, cognitive, and sensorimotor tests (for review, see DeVries et al. 2001; Hattori et al. 2000). Differences in behavioral tasks that exist between genders in mice have already been recognized (Voikar et al. 2001). However, little work has been done in examining possible gender differences as well as the effects of exogenous estrogen in these functional measures as it applies to animal models of cerebral ischemia.

## Conclusions

Overall, sex-linked stroke outcomes are much more evident in experimental studies than in clinical trials and observational studies. This difference is due largely to the diversity of ischemic disease in humans as well as the heterogeneity of stroke patients versus the more controlled and homogeneous conditions of experimental stroke. However, after adjusting for age and other stroke risk factors, it is clear that

premenopausal women have a lower stroke burden compared with men and postmenopausal women.

## Estrogen Administration

### Clinical Trials and Observational Studies

The Heart and Estrogen-Progestin Replacement Study (HERS<sup>1</sup>) was the first randomized, blinded trial on the effect of combined HRT (estrogen and medroxyprogesterone acetate) on coronary disease progression. After 4 yr of combined HRT therapy, the HERS found no reduction in risk for coronary events, stroke, or transient ischemic attack (TIA<sup>1</sup>) but did observe a three-fold increase in venous thromboembolism (Hulley et al. 1998). An important observation in the HERS was that patients receiving combined HRT sustained an early increased risk of cardiovascular events that was offset by a lower event rate in subsequent years. It was assumed that this observation was due to an early prothrombotic risk followed by protection, and that prolonged follow-up would demonstrate an overall beneficial effect for combined HRT. However, the release of the 6.8-yr follow-up on the HERS cohort (HERS II) in 2002 (Grady et al. 2002) revealed no benefit of prolonged HRT treatment on cardiovascular or cerebrovascular endpoints.

The objective of the HERS study was to investigate the effects of combined HRT on coronary disease progression, with stroke and TIA as secondary endpoints. In contrast, the Women's Estrogen for Stroke Trial (WEST<sup>1</sup>) was the first randomized trial designed to examine stroke recurrence as the primary endpoint (Viscoli et al. 2001). This trial, like the HERS, also evaluated secondary prevention and enrolled older women with pre-existing cerebrovascular disease (TIA or stroke 90 days before randomization). The WEST found no benefit on total stroke incidence and a surprising increase in fatal stroke among women who were assigned to therapy of estradiol alone. Furthermore, the WEST did not demonstrate a beneficial effect of 17 $\beta$ -estradiol for secondary prevention of stroke and ischemic injury. The unexpected findings of HERS, HERS II, and WEST are not easily reconciled with earlier epidemiological data suggesting that estrogen would be protective.

Recent data from the Women's Health Initiative (WHI<sup>1</sup>) (<http://www.nhlbi.nih.gov/whi/>) has brought many fundamental issues to light about the utility and safety of chronic estrogen use in women. The WHI, one of the largest preventative studies of its kind, was initiated in 1991 with the overall goal of identifying major causes of death and disability in postmenopausal women through prevention/intervention protocols and risk factor identification. HRT is one intervention under examination in WHI clinical trials (Rossouw et al. 2002). Two parallel HRT trials were originally designed. In one trial, women with a prior hysterectomy were randomized to placebo or unopposed estrogen. The other trial examined women with an intact uterus who were randomized to placebo or estrogen plus progestin

therapy in acknowledgment of the increased risk of endometrial cancer with unopposed estrogen therapy. The primary outcome of these trials was incidence of coronary heart disease, and the primary adverse outcome was invasive breast cancer. Secondary endpoints included the effect of HRT on stroke, pulmonary embolism, endometrial cancer, colorectal cancer, hip fracture, and death (Rossouw et al. 2002).

The combined estrogen plus progestin HRT trial, which was to have continued until 2005, was terminated in July 2002 based on recommendations by the WHI Data and Safety Monitoring Board. The Board found that overall risks from use of combined HRT outweighed the benefits. In addition to an increased risk of breast cancer, other adverse effects included an increased stroke risk, with eight more strokes (41%) per year for every 10,000 women in the combined HRT group (Rossouw et al. 2002). When stroke subtype is considered, combined HRT increased the risk of ischemic stroke in generally healthy postmenopausal women, but excess risk for all strokes attributed to combined HRT appeared to be present in all subgroups examined (Wasserthcil-Smoller et al. 2003). Other outcomes also suggested an overall negative effect on health, including increases in cardiovascular events and pulmonary embolism. The treatment trial for unopposed estrogen continues.

Since the early 1970s, observational studies have found lower risks of chronic heart disease and stroke in women taking postmenopausal estrogens, suggesting that estrogen is vasoprotective (for review, see Langer 2002). Observational studies for stroke prevention are not as clearly positive; in most of the reports, no increased risk or small benefits in the prevention of fatal strokes are described (Paganini-Hill 2001). The results from more recent clinical trials emphasize the existence of unanticipated and paradoxical effects of estrogen as it is currently administered in women. In light of the WHI, estrogen's neuroprotective properties and potential benefit in central nervous system (CNS<sup>1</sup>) ischemic injury must be reassessed. Because the largest burden for stroke is in postmenopausal women, there is great and continued interest in HRT as a means of preventing or treating cerebrovascular disease.

### Animal Studies

The effects of exogenous estrogen administration or replacement on cerebral ischemic injury size have been studied intensely in rodent models of experimental stroke (Table 2). In fact, almost all of our understanding of the neuroprotection of estrogen originates in rodent data. Very few studies are available in higher order, gyrencephalic animals such as nonhuman primates.

Almost universally, estrogen reduced rodent brain injury after an ischemic insult (Table 2; for reviews, see Dhandapani and Brann 2002b; Green and Simpkins 2000; Hurn and Macrae 2000; Murphy et al. 2003; Wise and Dubal 2000). In both permanent and transient focal cerebral

**Table 2 Effect of exogenous estrogen treatment or exposure in rodent experimental stroke models**

| <b><i>Transient Focal Ischemia Models</i></b> |   |   |                          |
|---|---|---|--------------------------|
| Rodent species                                | Estrogen effect on stroke injury  | Proposed mechanism(s) of estrogen effect  | Reference (see text)     |
| Wistar RSF rats <sup>a</sup>                  | Reduced cortical and striatal infarct size  | Blood flow-independent mechanism at end-ischemia demonstrated via <sup>14</sup> [C]iodoantipyrine autoradiography                             | Alkayed et al. 2000      |
| Charles River intact and castrated male rats  | Reduced ischemic area (frontal and parietal cortex + basal ganglia)   | No mechanism examined or tested   | Hawk et al. 1998         |
| Wistar male rats                              | Reduced total hemispheric and striatal infarct size   | Blood flow-enhancing, or flow-preserving, mechanism during early reperfusion demonstrated via <sup>14</sup> [C]iodoantipyrine autoradiography | McCullough et al. 2001   |
| Wistar OVX female rats                        | Chronic estrogen replacement reduced cortical and striatal injury. No effect with acute single-injection estrogen exposure. | Blood flow-independent mechanism at end-ischemia demonstrated via <sup>14</sup> [C]iodoantipyrine autoradiography                             | Rusa et al. 1999         |
| Sprague-Dawley OVX female rats                | Selectively protected cortical tissue from ischemia damage  | CBF-independent mechanism during occlusion and early reperfusion demonstrated via digital laser perfusion monitor                             | Shi et al. 2001          |
| OVX female CD rats                            | Reduced ischemic area (frontal and parietal cortex + basal ganglia)   | No mechanism examined or tested   | Simpkins et al. 1997     |
| Wistar intact male rats                       | Acute and chronic treatment equally reduced cortical and striatal injury  | No mechanism examined or tested   | Toung et al. 1998        |
| Wistar castrated male rats                    | Reduced cortical and striatal injury  | No mechanism examined or tested   | Toung et al. 1998        |
| Wistar OVX rats                               | Acute or chronic treatment did not alter cortical ischemic volume   | No mechanism examined or tested   | Vergouwen et al. 2000    |
| Sprague-Dawley OVX female rats                | Reduced ischemic area (frontal and parietal cortex + basal ganglia)   | No mechanism examined or tested   | Zhang et al. 1998        |
| <b><i>Permanent Focal Ischemia Models</i></b> |   |   |                          |
| Sprague-Dawley OVX female rats                | Increased infarct volume  | Antioxidant mechanism currently under investigation via 4-hydroxynonenal immunohistochemistry   | Carswell and Macrae 2003 |
| Male NMRI mice                                | Reduced brain infarct area  | Receptor-independent antioxidative mechanism demonstrated in vitro in primary culture of chick embryonic neurons                              | Culmsee et al. 1999      |
| Sprague-Dawley OVX female rats                | Chronic estradiol replacement decreased cortical infarct volume. No effect when given acutely at onset of ischemia.         | Blood flow-independent mechanism demonstrated during ischemia via laser-Doppler flowmetry   | Dubal et al. 1998        |

**Table 2 Continued**

| <b><i>Permanent Focal Ischemia Models (continued)</i></b>                   |  |  |                             |
|---|--|--|-----------------------------|
| <b>Rodent species</b>   | <b>Estrogen effect on stroke injury</b>  | <b>Proposed mechanism(s) of estrogen effect</b>  | <b>Reference (see text)</b> |
| Sprague-Dawley OVX young (3-4 mo) and middle-aged (9- to 12-mo) female rats | Chronic estradiol replacement decreased total and cortical infarct volume  | Blood flow-independent mechanism demonstrated during ischemia via laser-Doppler flowmetry  | Dubal and Wise 2001         |
| SHR OVX female rats   | Reduced infarct volume as compared to vehicle-treated OVX rats   | Blood flow-independent mechanism demonstrated during ischemia via laser-Doppler flowmetry  | Fukuda et al. 2000          |
| Sprague-Dawley male rats  | Systemic administration reduced infarct size   | Recovery of autonomic function demonstrated, suggesting estrogen prevents or reverses stroke-induced autonomic dysfunction   | Saleh et al. 2001a          |
| Sprague-Dawley male rats  | Local microinjection administration reduced infarct size   | Did not attenuate ischemia-induced autonomic function, suggesting that extracortical CNS sites may mediate estrogen's effects on autonomic sites   | Saleh et al. 2001b          |
| Sprague-Dawley OVX young (3-4 mo) and middle-aged (9-11 mo) female rats     | Chronic estradiol replacement decreased total and cortical infarct volume  | No mechanism examined or tested  | Wise and Dubal 2000         |
| <b><i>Transient Global Ischemia Models</i></b>                              |  |  |                             |
| Male Mongolian gerbils  | Highest ICV dose and IP dose significantly prevented damage in hippocampal CA1 pyramidal cells   | Inhibition of hypoxia-induced increase in intracellular calcium demonstrated in vitro in hippocampal slices. Findings suggest neuroprotection through inhibition of calcium release from intracellular calcium stores and through inhibition of calcium influx from the extracellular space. | Chen et al. 1998            |
| Male Mongolian gerbils  | Significantly reduced delayed neuronal death in hippocampal CA1 pyramidal cells  | Attenuation of the ischemia-induced increase in extracellular excitatory amino acids demonstrated via in vivo microdialysis  | Chen et al. 2001            |
| Wistar OVX female rats  | Significant relationship between hippocampal cell loss and estradiol level; no protective effect   | No mechanism examined or tested  | Harukuni et al. 2001        |
| Sprague-Dawley OVX female rats  | Significantly higher live cell counts and lower caspase-3 active peptide positivity in hippocampal CA1 neurons. Improved only hippocampal blood flow as demonstrated by hydrogen clearance method. | Neuroprotective mechanism appears to involve improvement of perfusion and inhibition of caspase-3 activity, a key effector of the apoptotic process  | He et al. 2002              |

**Table 2 Continued**

| <b><i>Transient Global Ischemia Models (continued)</i></b> |  |   |                             |
|--|--|---|-----------------------------|
| <b>Rodent species</b>                                      | <b>Estrogen effect on stroke injury</b>  | <b>Proposed mechanism(s) of estrogen effect</b>   | <b>Reference (see text)</b> |
| C57BL/6J OVX female mice and apoE-deficient OVX mice       | Significant reduction in neuronal damage in caudate-putamen and in CA1 pyramidal cell layer seen in C57BL/6J mice. No significant effect on neuronal damage in caudate-putamen and CA1 pyramidal cell layer seen in apoE-deficient mice. | The neuroprotective effect of estrogen appears to be mediated by or dependent on endogenous apoE, a lipid transport protein.  | Horsburgh et al. 2002       |
| Male Mongolian gerbils                                     | Significant neuroprotection against ischemia-induced hippocampal neuronal death  | In postischemic CA1 neurons, attenuation of ischemia-induced increases in activated caspase-3 and blocking of the increase in the proapoptotic neurotrophin receptor p75 <sup>NTR</sup> demonstrated. Findings suggest that estrogen intervenes at the level of apoptotic signaling cascades to prevent apoptotic neuronal death. | Jover et al. 2002           |
| Sprague-Dawley OVX female rats                             | Low doses of estrogen reduced hippocampal and striatal neuronal damage   | Enhancement of brain Ca <sup>2+</sup> -dependent NOS activity and expression, especially neuronal NOS, demonstrated. Findings suggest that this mechanism allows estrogen to promote intra-ischemic cerebrovasodilation.  | Pelligrino et al. 1998      |
| Male gerbils   | Higher doses prevented hippocampal neuronal loss at early stages after ischemia  | Antioxidative mechanism examined in vitro in primary culture of embryonic rat cerebrocortical neurons but considered unlikely at same dose in vivo  | Sudo et al. 1997            |
| Sprague-Dawley OVX female rats                             | Reduced ischemic damage in the hippocampus and striatum  | Perfusion-independent effects demonstrated via laser Doppler flowmetry, radiolabeled microspheres, and intransischemic EEG power changes  | Wang et al. 1999            |
| <b><i>Subarachnoid Hemorrhage Model</i></b>                |  |   |                             |
| Sprague-Dawley OVX female rats                             | Reduced secondary ischemic lesion volume and mortality; no effect on clot volume   | Cortical and subcortical blood flow-enhancing, or flow-preserving, mechanism demonstrated 30 min after subarachnoid hemorrhage via laser Doppler flowmetry and hydrogen clearance method, respectively  | Yang et al. 2001            |

<sup>a</sup>apoE, apolipoprotein E; CBF, cerebral blood flow; CNS, central nervous system; EEG, electroencephalographic; ICV, intracerebroventricularly; IP, intraperitoneally; NOS, nitric oxide synthase; OVX, ovariectomized; RSF, reproductively senescent female; SHR, spontaneously hypertensive rats.

ischemia models (Table 2), estrogen appears to have a neuroprotective effect in estrogen-deficient rodents (males, ovariectomized females, and reproductively senescent females). Gender differences in the outcome of hemorrhagic stroke and the influence of estrogen are unclear, although one experimental study has demonstrated that estrogen can reduce mortality and secondary ischemic damage in a rat model of SAH (Yang et al. 2001). The therapeutic range of “neuroprotective” steroid doses is limited in these studies. Because there are few studies of long-term estrogen exposure, the effect of treatment duration remains unclear. The effective dose and duration may differ between sexes, suggesting that neuroprotective mechanisms are not necessarily identical in male and female animals.

Most studies have evaluated 17 $\beta$ -estradiol, and few data have been reported with the less potent estrogens such as estriol. A single study has demonstrated a deleterious effect of estrogen in a rat model of transient forebrain ischemia (Harukuni et al. 2001), but no mechanism of injury was tested. A more recent study also showed that estrogen worsened stroke outcome in a rat model of permanent focal ischemia (Carswell and Macrae 2003). Moreover, in ovariectomized female rats rendered diabetic before transient forebrain ischemia, the neuroprotective benefits of estrogen replacement therapy were not evident (Santizo et al. 2002).

Consequently, little is known about what distinguishes the neuroprotectant estrogen from a proinjury estrogen. Most estrogens are vasoactive and have potent effects on endothelium and vascular smooth muscle cells of brain blood vessels (Murphy et al. manuscript in preparation; Rubanyi et al. 2002), as well as neurons and glia within brain parenchyma (Dhandapani and Brann 2002a,b; Hurn and Brass 2003). For example, 17 $\beta$ -estradiol can increase cerebral blood flow during and after vascular occlusion and ameliorate postischemic hyperemia (Hurn et al. 1995; McCullough et al. 2001; Watanabe et al. 2001). Therefore, it will be important in future research to evaluate systematically and quantitatively vaso- versus neuroprotection if we are to understand estrogen’s action in brain fully.

## Conclusions

Estrogen has been extensively studied as a neuroprotective agent in women, animals, and a variety of in vitro models of neural injury and degeneration. Most of the data from animal and cell models suggest that estrogen can benefit an ischemic brain and reduce cell death. However, the total evidence for the clinical benefit of HRT as an ischemic neuroprotectant is arguably small (see Nelson et al. 2002 for review). It is not immediately obvious why HRT was ineffective and potentially deleterious in women in recent clinical trials. One factor is that both the HERS and WEST included women with known vascular disease and who began treatment at an advanced age, well beyond meno-

pause. The WHI data are believed to address this question in part because the study evaluated effects in healthy postmenopausal women. Although oriented toward primary prevention, 7.7% of women participants in the WHI had documented vascular disease. Furthermore, many women were enrolled despite relative contraindications to HRT, such as smoking, previous stroke, or venous thromboembolism (Rossouw et al. 2002). It is believed that such subjects more likely represent the “average patient” considering HRT, than if such variables were excluded. Earlier observational trials may have preselected a healthier, lower risk group of women who may have been able to derive benefit from HRT.

It must also be emphasized that estrogen’s mechanisms of protection for brain and the cerebral vasculature are quite complex (McCullough and Hurn 2003) (Table 2). There is evidence implicating, and refuting, the importance of nuclear hormone receptor signaling mechanisms to gender differences and to the anti-ischemic activity of 17 $\beta$ -estradiol (Dubal et al. 2001; Sampei et al. 2000a; Sawada et al. 2000; Simpkins et al. 1997). However, it is also clear that rapid receptor-mediated and receptor-independent intracellular signaling is relevant in neuroprotection and does not involve gene transcription (Falkenstein et al. 2000; Linford et al. 2000). These actions involve putative membrane estrogen receptors, kinase cascades, and intracellular signaling that activate ion channels, neurotransmitter receptors, and enzymes such as nitric oxide synthase. Such mechanisms may be critical to estrogen’s protection in experimental stroke. Finally, many estrogens have potent, concentration-dependent lipid antioxidant activity, such as 2-hydroxy estrone, 2-hydroxy estradiol, phenolic estrone and 17 $\beta$ -estradiol (Kume-Kick and Rice 1998; Mooradian 1993). In summary, it is likely that the estrogens act at multiple sites in injured brain and utilize both receptor-dependent and receptor-independent, as well as non-cell-type-specific, signaling processes.

## Future Research—Prospects and Needs

Current experimental data on gender differences and the effects of exogenous estrogen have been derived almost exclusively from rodent stroke models. The discrepancy between observational studies, large randomized clinical trials, and preclinical data may be due in part both to the need for improved design of clinical trials and to the rodent species bias in animal stroke models addressing these research questions. It is necessary for future research in this area to expand into stroke models utilizing higher order, gyrencephalic animals such as nonhuman primates if we are to extrapolate better to the human scenario and to improve the direction and design of ongoing and future clinical studies and trials. Further study of the mechanisms underlying gender differences and leading to the increased stroke incidence observed in postmenopausal women is also warranted in both rodent and nonrodent models. The duration of the es-

trogen-deficient state is clearly an important issue. Rodent studies suggest that the timing of administration is critical and may be one possible explanation for the lack of benefit seen in clinical trials. Likewise, observations on parity have not been reported or examined.

Previous and ongoing clinical and experimental inquiries have focused heavily on estrogen, but the effects of progesterone alone or its metabolites in both ischemic and nonischemic brain injury (i.e., degenerative, traumatic) have gained greater scrutiny in the past few years (for review, see Roof and Hall 2000; Stein 2001; Stein and Hoffman 2003; Stein et al. 1999; Vink et al. 2001). However, most of the clinical literature addressing the issue of female sex hormones and cerebrovascular/CNS disorders has focused on combined HRT in postmenopausal women. This emphasis on combined hormone scenarios makes it difficult to separate out the individual as well as interactive roles of estrogen and progestins.

Although combined estrogen/progestin compounds are the most commonly prescribed hormone regimen in the United States, it is not known whether progestins interact with estrogen and diminish its neuroprotective effects. Experimental data suggest that progesterone increases subcortical damage after vascular occlusion in animals (Murphy et al. 2000), and progestins like medroxyprogesterone acetate can reverse the beneficial effect of estrogen seen on atherosclerotic plaque formation in nonhuman primates (Adams et al. 1997; Williams et al. 1994). However, recent clinical results argue against this hypothesis. The Estrogen Replacement and Atherosclerosis trial utilized estrogen with or without a progestin and found no benefit in coronary disease progression as measured angiographically in either treatment group (Herrington et al. 2000). Still, progestin type, formulation, and route and timing of administration are important factors to consider when determining whether progestins alone increase susceptibility or protection in cerebrovascular disease or ischemic brain injury. The role of medroxyprogesterone acetate in the suspended combined HRT WHI trial has yet to be elucidated. Progestins that might be detrimental, neutral, or beneficial when administered solely might prove to be antagonistic, neutral, or synergistic when combined with estrogen.

Interest in selective estrogen receptor modulators (SERMs<sup>1</sup>) as an alternative form of HRT has increased as researchers have sought to maximize estrogen's benefits and minimize its disadvantages. SERMs represent a class of drugs with mixed estrogen agonistic and antagonistic activity in different tissues. An ideal SERM would theoretically function as an antagonist in breast and uterus and as an agonist in bone, brain, and the cardiovascular system. Of the SERMs available today, none can be considered "ideal." Only a few SERMs are currently approved for clinical uses such as the treatment of primary and metastatic breast cancer, the prevention and treatment of postmenopausal osteoporosis, and the treatment of infertility in anovulatory women. The effects and mechanism(s) of action of SERMs in brain are currently under investigation, but few studies

have addressed the potential neuroprotective benefits of SERMs following ischemic brain injury (for review, see Dhandapani and Brann 2002b; Littleton-Kearney et al. 2002; Murphy et al. 2003).

## Summary

The widening gap between clinical trial results and experimental laboratory-based data suggest that our understanding of the cerebral ischemic pathophysiology and of estrogen's role as a cerebroprotectant is incomplete. Continuing and future studies are still needed to optimize combined or estrogen alone HRT options, alternative therapies such as SERMs, and the prevention/management of cerebrovascular/CNS disorders.

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