

# Neuroprotective Effects of Estradiol in Middle-Aged Female Rats\*

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## ABSTRACT

Estrogen replacement therapy in postmenopausal women ameliorates cognitive dysfunction and decreases the risk and/or severity of neurodegenerative conditions such as Alzheimer's disease and stroke. Furthermore, estradiol exerts neuroprotective effects in a variety of *in vitro* and *in vivo* models of brain injury. We have previously shown that physiological levels of estradiol attenuate ischemic brain injury in young female rats. However, neurodegenerative events occur more frequently in elderly women who are chronically hypoestrogenic. Therefore, we investigated whether aging rats remain responsive to the neuroprotective actions of estradiol. Young (3–4 months) and middle-aged (9–12 months) rats were ovariectomized and treated for 1 week with estradiol before middle cerebral artery occlusion

(MCAO). Regional cerebral blood flow was monitored in some animals at the time of injury. Brains were collected 24 h after MCAO and infarct volume was analyzed. Our data demonstrate that in both young and aging rats, low and high physiological doses of estradiol decrease ischemic injury by almost 50%, compared with oil-treated controls. Additionally, our data suggest that estradiol acts in both age groups via blood flow-independent mechanisms, as basal and postinjury blood flow was equivalent between estradiol- and oil-treated young and aging rats. These data demonstrate that replacement with physiological levels of estradiol protects against stroke-related injury in young and aging female rats and strongly suggest that older animals remain responsive to the protective actions of estradiol. (*Endocrinology* 142: 43–48, 2001)

IT IS WELL established that estradiol plays a critical reproductive role in the brain during fetal and neonatal development and during adulthood. More recently, it has become increasingly clear that estradiol also plays an important nonreproductive neurotrophic and neuroprotective role (for review see Refs. 2 and 3). During development estradiol stimulates neurite outgrowth and arborization of neuritic branches in organotypic cultures (4) and in dispersed neuronal cell cultures (5, 6). In addition, it protects the developing brain against injury in several experimental models (for review see Ref. 3). In the adult, estradiol stimulates the number of functional dendritic spines in the CA1 region of the hippocampus (7, 8) and stimulates synaptogenesis (9). It also protects against brain injury in the adult: estradiol replacement in ovariectomized (OVX) rats significantly decreases ischemic injury (10–13) and injury induced by other neurotoxic stimuli (14). Moreover, ischemia-induced brain injury is less extensive on proestrus when estradiol is high, than on other days of the estrous cycle (15) and females exhibit less cell death compared with males (11, 16).

Neurodegenerative conditions such as Alzheimer's disease and cerebrovascular stroke occur more frequently in older postmenopausal than in young women; therefore, it is

important to assess whether estrogen continues to exert protective actions in the aging brain. The goals of this study were to determine whether 1) physiological levels of estradiol decrease the extent of brain injury; 2) middle-aged rats remain responsive to these modest levels of estradiol; and 3) the ability of estradiol to protect is mediated by hormone-induced differences in cerebral blood flow.

## Materials and Methods

### Animals and experimental treatments

Young (3–4 months, 250–300 g BW) and middle-aged (9–12 months, 350–400 g BW) female, Sprague Dawley rats were maintained in a 14-h light, 10-h dark cycle with access to food and water *ad libitum*. All procedures are in accordance with the NIH Guide and have been approved by the University of Kentucky, Medical Center IACUC Committee. Rats were ovariectomized to eliminate endogenous ovarian estradiol ( $n = 8$ –14/experimental group) under Metaphane anesthesia. Immediately after ovariectomy, a SILASTIC brand (Konigsberg Instruments, Pasadena, CA) capsule, containing vehicle (sesame oil, Sigma St. Louis, MO) or 180  $\mu\text{g/ml}$  or 1 mg/ml of 17 $\beta$ -estradiol (Sigma), was implanted sc into young and middle-aged rats. SILASTIC brand capsules were made by injecting vehicle or estradiol into tubing that was 0.062/0.125 inches inner/outer diameter and that was capped with 5 mm of wooden applicator sticks (Fisher Scientific, Pittsburgh, PA). Capsules were stored in a vial containing oil or the same concentration of estradiol as was in the capsule until they were used. Young rats received a 30-mm capsule (volume 0.070 ml); whereas middle-aged rats received a 40-mm capsule (volume 0.105 ml). The two doses of estradiol replacement (180  $\mu\text{g/ml}$  and 1 mg/ml) delivered through SILASTIC brand capsules produce serum levels of estradiol that are equivalent to basal or proestrous levels observed in the rat estrous cycle, respectively (17). Approximately half (15 of 38) of the young rats were included in a previously published study (12). Both the young and middle-aged rats were collected over the same time interval to ensure that hormone replacement, surgical methods, and analysis of infarct size and cerebral blood flow were consistent in all animals.

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### *In vivo cerebral ischemia*

One week after ovariectomy and estradiol or vehicle treatment, rats were anesthetized with a mixture of ketamine/acepromazine (80.0/0.52 mg/kg, ip). Body temperature was monitored with a rectal probe and maintained at 36.5–37.5 C with a heating pad throughout surgery and recovery. Cerebral ischemia was induced via insertion of a 4/0 (young rats) or 4/0 or 3/0 (middle-aged rats) black monofilament suture to occlude the middle cerebral artery (n = 8–14/experimental group). Rats underwent permanent cerebral ischemia using methods described in detail in our previous publication (12). Briefly, the right middle cerebral artery (MCA) was occluded via insertion of a poly-L-lysine coated monofilament suture (Ethicon) from the right external carotid artery, through the right internal carotid artery to the base of the MCA. In a preliminary experiment, insertion of 4/0 and 3/0 sutures was tested to assess whether equivalent occlusion of the MCA in middle-aged rats necessitated the use of the larger diameter suture. The data show that a 3/0 suture (larger diameter) was necessary for successful MCA occlusion in middle-aged rats (Fig. 1).

### *Histologic preparation*

Rats were euthanized using an overdose of ketamine/acepromazine. Brains were collected 24 h after the onset of ischemia and sectioned into 1 mm coronal slices using a brain matrix. As previously described (12), alternate slices were stained in 2% triphenyltetrazolium chloride (TTC) and then fixed in 10% buffered formalin. We analyzed total, cortical, and striatal infarct volumes using coronal sections that span the brain via computer-assisted imaging (NIH version 1.60). The total and regional areas of injury present on each coronal section (bregma points +4.2 mm, +2.2 mm, +0.2 mm, –1.8 mm, –3.8 mm) were clearly demarcated by TTC staining, and measured using NIH Image. Then, for each brain, the areas of injury from the five coronal sections were integrated to determine total, cortical, and striatal infarct volumes (mm<sup>3</sup>).

### *Blood flow measurements*

One week after ovariectomy and treatment, rats (n = 3/experimental group) were anesthetized with a mixture of ketamine and acepromazine (80.0/0.05 mg/kg ip). Through a craniotomy, a laser Doppler probe (0.8 mm diameter) was positioned over the surface of the right parietal cortex (approximately 5 mm posterior to bregma and 4 mm lateral to the midline). The probe was positioned over an area distant from large pial vessels to obtain low, stable readings representative of cortical perfusion (18). The blood flow measurements were taken from an area that corresponds to the region affected by cerebral ischemia, as ischemic injury spans the brain from approximately 4.2 mm anterior to bregma to 5.8 mm posterior to bregma. Baseline measurements were obtained once a minute for 10 min. Ten minutes after the onset of ischemia, measurements were obtained once a minute for 35 min. Data are expressed as averages over 5 min.

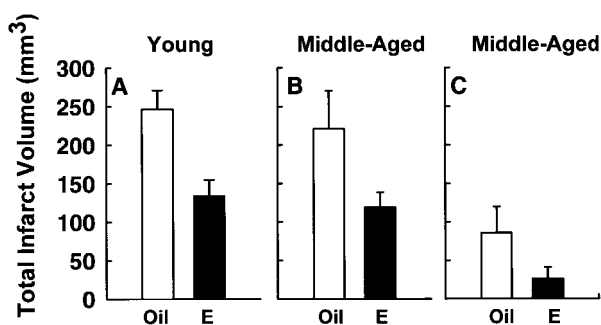


FIG. 1. Ischemic injury is equivalent in young and middle-aged rats. A, Cerebral ischemia induced by a 4/0 monofilament suture in young rats (n = 9–10/group) produced ischemic injury that is equivalent to (B) cerebral ischemia induced by a 3/0 monofilament suture in middle-aged rats (n = 4/group). C, The 4/0 monofilament suture produced a significantly smaller infarct in middle-aged females (n = 3/group), compared with young females. Values represent mean  $\pm$  SE.

### *17 $\beta$ -estradiol RIA*

Trunk blood was collected at the time rats were killed, and sera were obtained after centrifugation of the blood. Serum samples were frozen until the time of assay. Sera (n = 7–11/experimental group) were extracted in anhydrous ethyl ether and RIA was conducted for 17 $\beta$ -estradiol concentrations using a double-antibody commercial kit (ICN Biomedicals, Irvine, CA).

### *Data analysis*

All data are expressed as mean  $\pm$  SE. Infarct volumes and serum estradiol concentrations were analyzed with one-way ANOVA. Post hoc analyses were carried out with the Newman-Keuls test. Baseline laser Doppler measurements were analyzed using the Student's *t* test. Ischemic laser Doppler measurements were analyzed using repeated measures ANOVA. All differences were considered significant at *P* < 0.05.

## Results

In a preliminary experiment, we tested whether a larger diameter suture was required in middle-aged compared with young rats to effectively occlude the MCA. The data show that a 4/0 suture (smaller diameter) produced a significantly smaller infarct in ovariectomized middle-aged rats compared with young rats (Fig. 1, A–C). Whereas a 3/0 suture (larger diameter) produced similar infarct volumes in middle-aged as a 4/0 suture produced in young ovariectomized rats. We concluded that the larger diameter suture was necessary for successful MCA occlusion in middle-aged rats (Fig. 1, A–C). Therefore, we used a 4/0 suture in young rats and a 3/0 suture in middle-aged rats in all experiments to test whether aging influences the ability of estradiol to protect against ischemic stroke injury.

We found that estradiol dramatically decreased ischemic brain injury in both young and middle-aged female rats, compared with respective oil-treated controls. Figure 2 is a composite of representative coronal brain sections from oil- and estradiol-treated, young and middle-aged rats following cerebral ischemia. Our data clearly show that both doses of estradiol replacement significantly reduced overall infarct volume as compared with oil-treated controls in both young (Fig. 3A) and middle-aged (Fig. 3B) rats. The protection afforded by both low and high physiological estradiol pretreatment was identical in both age groups.

To determine whether estradiol's neuroprotective effects in young and middle-aged rats were region specific, we analyzed treatment effects in the cerebral cortex (Fig. 4, A and B) and in the striatum (Fig. 4, C and D). Estradiol pretreatment dramatically and equivalently reduced the cortical infarct in both age groups. In contrast, estradiol did not decrease the extent of brain injury in the striatum in young or middle-aged rats.

We assessed whether estradiol protects the cerebral cortex of young or middle-aged rats by increasing basal cerebral blood flow, or by attenuating the extent of ischemic flow following MCAO. Table 1 shows that basal regional cerebral blood flow was not affected by the presence of estradiol. Because baseline blood flow was not affected by hormone treatment or age, we expressed the extent of occlusion as a percentage of baseline. The results show that estradiol did not protect against injury by affecting the extent of decreased blood flow in young (Fig. 5A) or middle-aged (Fig. 5B) rats.

FIG. 2. Representative brain sections from an oil- and estradiol-treated, young and middle-aged rat after permanent cerebral ischemia. Infarcted tissue is white, whereas live tissue is darkly stained by TTC. In the absence of estradiol, brain injury was extensive in (A) young and (C) middle-aged rats. Physiological estradiol pretreatment (180  $\mu\text{g}/\text{ml}$ ) reduced the extent of infarct in both (B) young and (D) middle-aged rats. The volume of infarct included significant portions of the cerebral cortex and striatum.

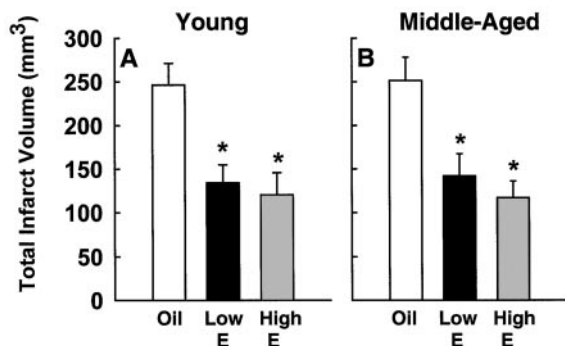
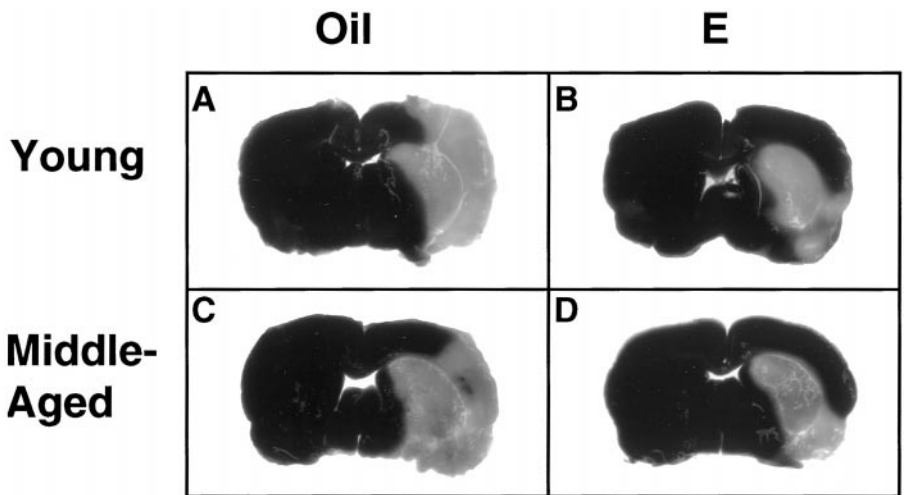


FIG. 3. Estradiol protects against total ischemic brain injury in young and middle-aged rats. Low and high physiological levels of estradiol decreased total injury in (A) young (Low E:  $P < 0.01$ ; High E:  $P < 0.03$ ) and (B) middle-aged (Low E:  $P < 0.03$ ; High E:  $P < 0.01$ ) rats. Values represent mean  $\pm$  SE.

Figure 6 shows serum estradiol concentrations produced by insertion of a SILASTIC brand capsule containing either 180  $\mu\text{g}/\text{ml}$  or 1 mg/ml concentrations of estradiol in young (Fig. 6A) and middle-aged (Fig. 6B) rats. The low concentration of estradiol replacement (180  $\mu\text{g}/\text{ml}$ ) produced equivalent levels of estradiol in both age groups. The higher concentration of estradiol replacement (1 mg/ml) also produced equivalent levels of estradiol in young and middle-aged rats. Low and high physiological estradiol replacement in young and middle-aged rats produced serum  $17\beta$ -estradiol levels that are equivalent to basal and proestrous levels circulating in the rat estrous cycle, respectively (17).

### Discussion

The results of this study clearly establish that estradiol is a potent protective factor in the aging brain. Our study focuses attention on the neuroprotective effects of physiological estradiol in the brain during the middle-aged period of life, when reproductive cycles become irregular and constant estrous becomes more common. We demonstrate three important findings. First, pretreatment with low or high physiological concentrations of estradiol following ovariectomy exerts striking and equivalent neuroprotection against stroke injury induced by permanent MCAO in young and middle-

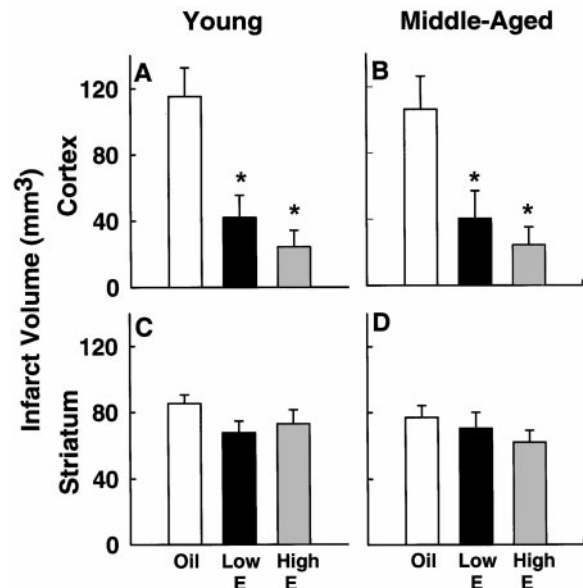


FIG. 4. Effects of estradiol in cerebral ischemia are region specific in young and middle-aged rats. Neuroprotective effects of estradiol were dramatically amplified in the cerebral cortex of (A) young (Low E:  $P < 0.01$ ; High E:  $P < 0.01$ ) and (B) middle-aged (Low E:  $P < 0.05$ ; High E:  $P < 0.03$ ) rats. No protective effect of estradiol was observed in the striatum of either (C) young ( $P = 0.41$ ) or (D) middle-aged ( $P = 0.16$ ) female rats. Values represent mean  $\pm$  SE.

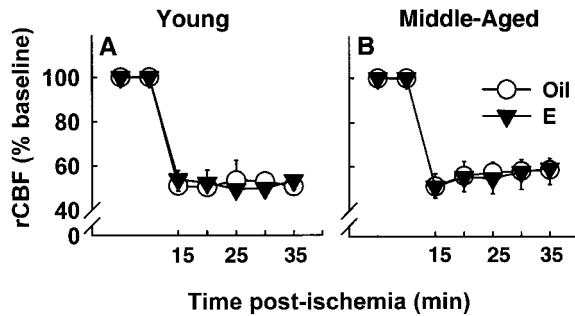
aged rats. Second, estradiol's effects are region-specific and equivalently amplified in the cerebral cortex of young and middle-aged rats. Third, in parallel to young rats, pretreatment with physiological levels of estradiol appears to protect the brains of middle-aged rats via blood flow-independent mechanisms.

Our observation that physiological levels of estradiol replacement protect the brains of young and middle-aged animals against ischemic injury highlights the vulnerability of the hypoestrogenic brain to neurodegeneration. The data suggest that hypoestrogenic, postmenopausal women may suffer greater consequences from brain injury such as stroke, compared with their counterparts who receive estradiol replacement therapy. Using a paradigm of physiological estradiol replacement in young and middle-aged rats, we

**TABLE 1.** Baseline regional cerebral blood flow (rCBF)

	Young	Middle-aged
Oil (Vehicle)	48.8 ± 3.0	45.6 ± 3.1
Estradiol	45.8 ± 2.4	49.9 ± 3.1

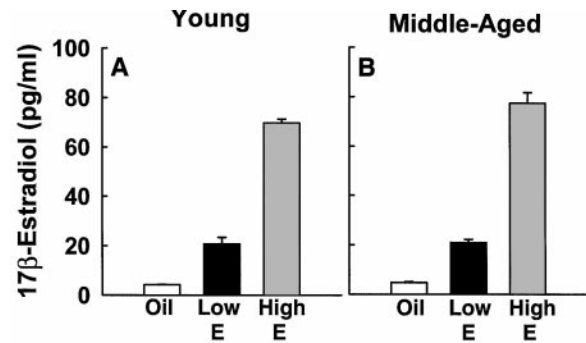
Baseline rCBF did not differ between oil- and estradiol-treated, young ( $P = 0.62$ ) or middle-aged ( $P = 0.43$ ) female rats. Values represent mean ± SE (Values for young animals were included in a previous paper [12]).



**FIG. 5.** Ischemic regional cerebral blood flow (rCBF) does not differ between oil- and estradiol-treated, young and middle-aged rats. Ischemia significantly reduced rCBF in oil- and estradiol-treated, (A) young and (B) middle-aged female rats ( $P < 0.0001$ ). Estradiol treatment did not alter the extent of decrease in regional cortical flow, compared with respective oil-treated controls in young ( $P = 0.92$ ) or middle-aged ( $P = 0.94$ ) female rats. Panel A of this figure was modified from Dubal *et al.* (12). Values represent mean ± SE.

specifically investigated effects of physiological levels of estradiol. Our treatment paradigms produced serum levels that are equivalent to basal and proestrous levels of estradiol that circulate in the rat estrous cycle, respectively (17). Our observations clearly demonstrate that in middle-aged female rats, the cerebral cortex remains responsive to the neuroprotective effects of physiological levels of estradiol, compared with young rats. Whether or not older animals remain responsive to estradiol is an important question. However, this question is extremely difficult to approach using the current methods of artery occlusion because arteries become less elastic and, consequently, occlusion using the suture method becomes ineffective (19).

Our finding that estradiol replacement following ovariectomy exerts equivalent neuroprotection between young and middle-aged female rats was unexpected because the ability of estradiol to stimulate a variety of hypothalamic responses required to regulate estrous cyclicity is attenuated in middle-aged rats. This includes attenuation of estradiol's ability to (1) activate GnRH neurons that leads to LH surges (20–22) (2), organize diurnal rhythmicity in the hypothalamic neurotransmitter activity (21, 23) or gene expression (24, 25), and (3) stimulate progesterone receptor binding (26). In addition, slightly, but significantly lower, levels of estrogen receptor- $\alpha$  messenger RNA (mRNA) are expressed in the cerebral cortex of middle-aged compared with young rats (Wilson, M. E., K. L. Rosewell, M. L. Kashon, P. J. Shughrue, I. Merchenthaler, and P. M. Wise, manuscript submitted). For all of these reasons, we had hypothesized that estradiol would be less able to protect the brains of older animals against ischemic brain injury. Interestingly, accumulating evidence from studies in middle-aged peri- and early postmenopausal women,



**FIG. 6.** Serum 17 $\beta$ -estradiol levels (pg/ml) are equivalent in young and middle-aged rats that were ovariectomized and estradiol-replaced. Low and high physiological estradiol replacement in (A) young and (B) middle-aged rats produced serum 17 $\beta$ -estradiol levels that are equivalent to basal, circulating levels and proestrous levels in the rat estrous cycle, respectively. The low range of sensitivity of the assay was determined to be 4 pg/ml. Values represent mean ± SE.

suggests that, in a parallel manner, the hypothalamic/pituitary axis of women becomes less responsive to estradiol (27). Because our data demonstrate that middle-aged rats remain equally responsive to the neuroprotective actions of estradiol compared with young animals, our findings imply that, despite changes in hypothalamic responsiveness, middle-aged women may continue to remain responsive to the nonreproductive, neuroprotective actions of estradiol in the cerebral cortex.

Interestingly, in this model of cerebral ischemia, estradiol's neuroprotective actions in young and aging female rats are dramatic and amplified in the cerebral cortex, compared with the striatum. The region-specific effect may partially reflect differential blood perfusion to the cortex and striatum following MCA occlusion (28). It is possible that because MCA occlusion blocks blood flow to a greater extent in the striatum compared with the cortex, more necrotic than apoptotic cell death may occur in the severely ischemic, striatal region. Data from our laboratory (29 and our unpublished observations) and others (30, 31) suggest that estradiol protects through mechanisms that attenuate apoptosis. Thus, if cell death in the striatum is predominantly through necrotic mechanisms, we would not expect protection in the striatum. Furthermore, several lines of evidence demonstrate that the cerebral cortex is an important target for estradiol-mediated neuroprotection via estrogen receptor (ER)-mediated mechanisms (29). We discovered that though ER $\alpha$  mRNA is not normally expressed in the cerebral cortex or the striatum of adult rats (32), cerebral ischemia dramatically and selectively up-regulates ER $\alpha$ , but not ER $\beta$ , gene expression in the cortex (29). Furthermore, we investigated the direct roles of estrogen receptor (ER) subtypes, ER $\alpha$  and ER $\beta$ , in mediating neuroprotection and found that ER $\alpha$  plays a pivotal role in the ability of physiological levels of estradiol to protect the cerebral cortex against ischemia (33).

Other studies have examined ischemic brain injury in older animals using similar methods (19, 34). However, because the vasculature becomes less elastic as animals age, it becomes increasingly difficult to occlude the MCA. Indeed, we found that a larger diameter suture was required to occlude the MCA to the same extent in middle-aged rats

compared with young rats. Thus, other models of experimental brain injury have been used to assess the effects of neurotoxic stimuli in aging animals (35, 36). For example, older male animals exhibited greater cerebral infarction in response to focal ischemia (37–39). However, global ischemia has resulted in inconsistent effects in older animals, and this may be related to several variables including strain (15, 40), sex (35, 39, 41), experimental conditions (41) and location of the infarct (39).

A previous study reports effects of stroke in gonadally intact middle-aged female and male rats and found no differences in the extent of ischemic injury (34), concluding that older female rats were less responsive to the protective effects of endogenous estradiol. Several differences between our experimental paradigms may account for the differences in our conclusions. First, intact, reproductively senescent rats, display differing levels of ovarian estradiol, progesterone, and inhibin compared with young rats that depend on the state of reproductive senescence (42, 43). Because progesterone has been shown to exacerbate (Hoffman, Murphy, Le, and Koski, unpublished) and protect (44) against neuronal injury, it is unclear whether the aging animals used in the previous study were as vulnerable to injury because of decreased levels of estradiol or increased levels of progesterone. Second, in the previous study, some gonadally intact middle-aged rats were exposed to exogenous estradiol treatment, in addition to endogenous circulating levels, and this exposure of high levels of estradiol afforded neuroprotection. In contrast to the previous study, all rats in our study were ovariectomized to eliminate endogenous ovarian steroids including estradiol, and then replaced with vehicle or physiological doses of estradiol. Our use of a controlled endocrine paradigm allowed us to specifically test whether physiological levels of estradiol protect the brain against stroke injury.

Estradiol may protect through a variety of cellular and molecular mechanisms. Previous studies have used pharmacological levels of estradiol and demonstrated that these high levels can protect the brain against ischemic injury in transient models of ischemia (10, 45–48). In some of these studies, pretreatment was not necessary (10, 47, 48). Whereas, in our previous work that uses physiological levels of estradiol replacement in a permanent occlusion model, a pretreatment period was essential to afford protection (12). From these complementary approaches, we hypothesize that when the doses are high and/or when the blood flow is decreased for short periods of time, estradiol may act via nonreceptor mediated mechanisms, such as decreasing free radical generation (49–52), increasing blood flow (11, 45, 47, 53), decreasing intracellular calcium accumulation (46), inhibiting excitatory amino acid-induced excitotoxicity (51, 52, 54–56) or rapidly activating second messenger signaling pathways (57–60). On the other hand, more physiological levels of estradiol replacement are likely to use estrogen receptors that involve changing gene transcription. We have previously found that injury and estradiol alter the expression of several genes, including ER $\alpha$ , ER $\beta$ , and members of the bcl-2 (29) and immediate early gene family (61). Our most recent findings demonstrate a functional role for ER $\alpha$  as the critical estrogen

receptor subtype that mediates neuroprotective effects of physiological levels of estradiol in stroke injury (33).

In summary, our results clearly establish that estradiol plays a neuroprotective role in the injured brain in both young and middle-aged rats. The data strongly imply that older women may also benefit from the protective effects of estrogen replacement therapy that uses relatively low concentrations of hormone.

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