

## Review

# Estrogen Replacement Therapy for Stroke

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Stroke is the third most common cause of death and severe disability among Western populations. Overall, the incidence of stroke is uniformly higher in men than in women. Stroke is rare in women during the reproductive years and rapidly increases after menopause, strongly suggesting that estrogen (E2) plays an important role in the prevention of stroke. Ongoing studies are currently evaluating both the benefits and the risks associated with E2 replacement therapy and hormone replacement therapy in stroke. Equally important is the role of E2 receptor (ER), as studies indicate that ER populations in several tissue sites may significantly change during stress and aging. Such changes may affect the patient's susceptibility to neurological disorders including stroke and greatly affect the response to selective E2 receptor modulators (SERMs). Replacement therapies may be inefficient with low ER levels. The goal of this review paper is to discuss an animal model that will allow investigations of the potential therapeutic effects of E2 and its derivatives in stroke. We hypothesize that E2 neuroprotection is, in part, receptor mediated. This hypothesis is a proof-of-principle approach to demonstrate a role for specific ER subtypes in E2 neuroprotection. To accomplish this, we use a retroviral-mediated gene transfer strategy that expresses subtypes of the ER gene in regions of the rat brain most susceptible to neuronal damage, namely, the striatum and the cortex. The animal model is exposed to experimental stroke conditions involving middle cerebral artery occlusion (MCAo) method, and eventually the extent of neuronal damage will be evaluated. A reduction in neuronal damage is expected when E2 is administered with specific ER subtypes. From this animal model, an optimal E2 dose and treatment regimen can be determined. The animal model can help identify potential E2-like therapeutics in stroke and screen for beneficial or toxic additives present in commercial E2 preparations that are currently available. Such studies will be informative in designing drug therapies for stroke.

Key words: Estrogen; Stroke; Replacement therapy; Estrogen receptor; Neuroprotection;  
Selective estrogen receptor modulators (SERMs)

## INTRODUCTION

The availability of a relevant animal model will help evaluate and optimize estrogen (E2) treatment strategies designed to reduce infarct size and minimize neuronal damage in stroke. Clinical studies show that E2 pretreatments may benefit ischemic stroke patients. However, studies also suggest that E2 used in replacement therapy may pose a risk for stroke in postmenopausal women. These seemingly conflicting observations may be due, in part, to the various E2 derivatives and additives in the preparations, the dosage used, the testing parameters, and other genetic and environmental factors that influence the overall outcome. In addition, studies also indicate that E2 receptor (ER) populations in several tissue sites may change during stress and aging. Such changes in ER

levels may affect the overall response to changing levels of E2.

Our hypothesis is that E2 neuroprotection is, in part, receptor dependent. This hypothesis testing is a proof of principle to evaluate how E2 treatments may benefit stroke victims using an animal model expressing various ER subtypes. This will be accomplished using a retroviral gene delivery system to express various ER subtypes in rat neuronal sites most susceptible to stroke damage. We expect that effective neuroprotection can be achieved at a lower E2 dose in specific neuronal sites expressing functional ER. The objectives that need to be addressed include: (1) to develop an animal model expressing human ER gene subtypes in brain regions most susceptible to stroke and (2) to evaluate E2 neuroprotection in the animal models.

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If successful, the animal model can be used to identify similar E2 derivatives and screen for additives demonstrating neuroprotective properties during experimental stroke conditions. Using the viral strategy, various ER subtypes can be expressed in animals with a genetic susceptibility for specific disorders and evaluate their role in stroke.

## BACKGROUND AND SIGNIFICANCE

Stroke is an acute neurologic event leading to death of neural tissue of the brain, resulting in loss of motor, sensory, and cognitive function. It is the third leading cause of death in the US. About 20% of strokes are hemorrhagic, resulting in bleeding into the brain (31). Ischemic strokes accounting for the remainder usually result from vascular occlusion.

### *Available Treatment Strategies for Stroke*

Currently, there are three treatment strategies for stroke: prevention therapy, therapy immediately after stroke, and poststroke therapy (62). Prevention therapy focuses on identifying and treating a patient's risk factors associated with stroke, which includes correcting diet and high blood pressure (63). Medical treatment approved for the treatment of ischemic stroke includes tissue plasminogen activator (tPA), a thrombolytic factor, which has to be administered within 3 h after stroke. Only 1% to 2% of stroke patients meet the criteria for treatment with this thrombolytic agent. Aspirin and anticoagulants are used as preventative therapy. For most stroke patients, physical and occupational therapy is the cornerstone of the rehabilitation process. Current preventive and therapeutic research strategies for stroke involve surgical intervention (carotid endarterectomy, extracranial and intracranial bypass, intravascular procedures including carotid angioplasty) and brain transplants. For intracerebral stroke hemorrhage, research has focused on lowering blood pressure and outcome (25). Several promising drug compounds are considered in stroke clinical trials, including drugs to improve blood flow, like antithrombotic, antiplatelet, fibrinogen depleting, and thrombolytics. Neuroprotective agents include calcium channel blockers, free radical scavengers,  $\gamma$ -aminobutyric acid (GABA) agonists, glutamate antagonists, growth factors, leukocyte adhesion inhibitors, and much more (43). Stroke preventive candidates include anticoagulants, anti-hypertensive agents, E2, and vitamins (56). A list of drugs used in completed and ongoing stroke clinical trials is available at the Stroke Trials Directory (76).

### *Evidence That E2 is Important in Stroke Therapy*

The strongest evidence that E2 may have a protective and therapeutic role in stroke comes from observations on gender differences among men and women. E2, a sex hormone primarily responsible in reproductive function and sex characteristics in females, declines with age.

Overall, the incidence of stroke is uniformly higher in men than in women. While stroke is rare in women during the reproductive years, it rapidly increases after menopause. Experimental stroke studies show that female rats sustain less brain damage than males and that the gender difference in ischemic outcome can be eliminated by ovariectomy (1). Endogenous E2 improves stroke outcome during vascular occlusion by exerting both neuroprotective and flow-preserving effects. Furthermore, a growing body of evidence suggests that exogenous E2 treatments may reduce tissue damage resulting from experimental stroke for both sexes (38,69). However, early last year, the Women's Health Initiative (WHI) trial showed that hormone replacement therapy (HRT) containing E2 and progesterin significantly increased the risk of invasive breast cancer and blood clots in the legs and lungs and did not protect women from heart disease and stroke. Women taking HRT had a higher risk of heart attack and stroke [reviewed in (36,48,60)]. Researchers argue that the clinical trials may lack consistency in stroke endpoints, a definition of the HRT user, E2 preparation, and influence of combined regimen, and may account, in part, for the unclear relationship (65). Clearly, further investigation is necessary in regard to E2 and the role of its receptor, ER.

### *Role of the ER in Stroke*

Potential mechanisms for E2-mediated neuroprotection include vasodilation and improved cerebral blood flow, mediated through the ER, or by a nonreceptor event [reviewed in Hurn and McCrae (37)]. Several studies provide evidence that the ER is required. Dubai et al. show that the ER- $\alpha$ , and not the ER- $\beta$ , is critical in mediating E2's protective role during stroke (23). Using physiological doses of E2, ovariectomized ER- $\alpha$  knockout mice were more susceptible to experimental stroke damage compared with either wild-type mice or ovariectomized ER- $\beta$  knockout mice. These results suggest that E2 mediates its neuroprotective role through ER- $\alpha$ , possibly by activating E2-responsive target genes [reviewed in Wise et al. (83)]. Toran-Allerand et al. describe a novel plasma membrane-bound ER called ER-X. Normally, ER-X expression is detected in postnatal, but not adult, brains (80). Interestingly, the receptor is reexpressed in adult brains following ischemic stroke injury. The receptor, predominantly located in caveolar-like microdomains, is phosphorylated by mitogen-activated protein (MAP) kinases in response to E2 and supports an alternative mechanism of E2 action. Wang et al. report morphological abnormalities in brains of ER- $\beta$  knockout mice (81). Severe neuronal deficits were detected in the somatosensory cortex, especially layers II, III, IV, and V. As mice age, neuronal deficits become more pronounced. By 2 years of age, there is degeneration of neuronal cell bodies throughout the brain. They further speculate that this

gene could have an important influence on the development of degenerative diseases of the central nervous system (CNS), such as Alzheimer's disease and Parkinson's disease, as well as those resulting from trauma and stroke in the brain. Other evidence suggesting a role for the ER in stroke comes from the use of antagonists to block ER-mediated activity. For instance, Sawada et al. provide evidence that during experimental stroke conditions, the pure ER antagonist, ICI 182,780, was able to enhance ischemic brain injury in female, but not in male mice (73). Physiological doses of E2, in the presence of the antagonist, were unable to amplify cerebral blood flow or induce vasodilation, suggesting that E2 signaling would be mediated through its cognate receptor. However, studies also suggest that the ER may play a minor role during neuroprotection or that the actions of E2 may be receptor independent. Tissue damage from experimental stroke is not enhanced in ER- $\alpha$  knockout mice (21,71).

#### *Evidence that ER Populations Change*

Several studies report that ER populations in several tissue sites may change significantly during stress and aging. Tohgi et al. evaluated mRNA expression patterns of several receptor types, including the ER, in the postmortem brains and reported age-related reductions in the hippocampus (79). Increased ER gene expression was noted in patients with Alzheimer's disease (40). Animal studies show similar age-related changes in ER- $\alpha$  and ER- $\beta$  gene expression in rat brain (82,83) and in nonneuronal tissues for ewes (87). Tamir et al. report that oxidative damage may reduce ER- $\alpha$  and ER- $\beta$  gene expression at brain sites susceptible to stroke (42,78). Since E2 upregulates expression of its receptor in selected tissue sites (27,87), postmenopausal women with low E2 levels may have lower levels of the ER. Such changes may affect the patient's susceptibility to neurological disorders including stroke and greatly affect their response to selective ER modulators (SERMs) (50,51). Replacement therapy may be inefficient with low ER levels.

#### *Animal Models in Evaluating Stroke*

At present, there are no ideal stroke animal models to identify E2 therapeutic candidates in the context of the ER. ER knockout mice demonstrate the importance for E2 in stroke (23,81) but cannot be used to identify potential drug candidates. Current animal models use ovariectomized female animals during stroke, to exclude potential hormonal influences (29,83). Approaches using ER antagonists and SERMs as therapeutics in normal animals demonstrate a role for the ER in stroke (57,67,73). Other strategies used stroke-prone animals (54) that may have variable ER populations at sites susceptible to neuronal damage. These studies have produced mixed results, largely contributing to an ongoing debate on whether

ER promotes neuroprotection via receptor-dependent or receptor-independent mechanisms (21,71). Several mechanisms have been proposed for E2-mediated neuroprotection including vasodilation, thus improving cerebral blood flow, free radical scavenging, and promoting neurogenesis [reviewed in (4,37)].

While some of these postulated mechanisms have been shown to be nonreceptor-mediated events (21,49), equally compelling evidence demonstrates that ER is required for neuroprotection (23,55,71,83). Evidence suggesting a role for the ER in stroke comes from the use of receptor antagonists to block ER-mediated activity (73). Physiological doses of E2 in the presence of the antagonist were unable to amplify cerebral blood flow or induce vasodilation, suggesting that E2 signaling would be mediated through its cognate receptor. Using physiological doses of E2, ovariectomized ER- $\alpha$  knockout mice were more susceptible to experimental stroke damage compared with either wild-type mice or ovariectomized ER- $\alpha$  knockout mice. These results suggest that E2 mediates its neuroprotective role through ER- $\alpha$ , possibly by activating E2-responsive target genes (4,83); the observation that E2 may be preferentially acting on ER- $\alpha$  over ER- $\beta$  using knockout mice does not unequivocally discount the possibility of redundancy in ER- $\alpha$  and ER- $\beta$  gene expression in that, in the absence of ER- $\beta$ , ER- $\alpha$  may substitute for the functional role of ER- $\beta$ . One could further postulate, based on the receptor knockout paradigm, that ER- $\beta$  is not able to reciprocate the loss of ER- $\alpha$ . Accordingly, a direct gene transfer "overexpression" paradigm, rather than knocking out the gene, will be able to provide equally important insights into the role of ER- $\alpha$ , ER- $\beta$ , or the combination of both. Additive facilitative effects of both ER- $\alpha$  and ER- $\beta$  on the neuroprotection produced by E2, SERMs, and other E2-like drugs can be examined using this paradigm. Moreover, the use of human ER- $\alpha$  and human ER- $\beta$  genes will allow in-depth examinations into the contribution of exogenous and endogenous expression of these receptors to neuroprotection. The lineage of ER-transfected cells (e.g., neurons and glia) can also be monitored with such lentiviral vector strategy, further characterizing the phenotypic target sites of E2 neuroprotection. In view of accumulating evidence demonstrating the active role of glial cells and astrocytes in neuroprotection, being able to track the phenotype of ER-transfected cells will offer additional insights into the neuron-glia interaction during cell survival and cell death. Furthermore, the recent finding of E2-mediated neurogenesis (58,72) will also be an exciting research theme in which the utility of lentiviral vector strategy of ER overexpression can be further exploited. The most important contribution of this work would be to confirm which combination of one or both of the two ERs activated by what dose of E2 achieves maximal

neuroprotection in a well-established in vivo model of stroke. If successful, this approach would provide a valuable new tool for preclinical testing of E2 derivatives that could be useful in designing prospective trials in human subjects. A transgenically altered rat would be superior to a transgenic mouse that cannot be used to test candidate therapeutic drugs.

### OUR HYPOTHESIS

Stroke, as mentioned previously, is the third most common cause of death and severe disability in the US (75). Effective preventive and therapeutic treatment strategies are necessary to minimize neuronal damage in stroke victims (19). We envision generating an animal model specifically designed to evaluate E2, E2 derivatives, and additives in the preparation, for use as potential therapeutics in stroke. The retroviral vectors used here to generate the animal model are currently used in gene therapy applications to deliver genes as a therapeutic strategy for several neurodegenerative disorders (33,34,45,46). Although these studies have focused on animal models, an interesting possibility is using drug therapy data from this animal model with retroviral vectors in gene therapy. With the recommended E2 dose, a gene therapy approach to deliver the ER may be a feasible preventive and therapeutic measure for both male and female patients susceptible to stroke and other neurodegenerative disorders.

The mechanisms involved in E2 neuroprotection are, in part, receptor dependent. To evaluate E2 activity in a receptor context, an animal model expressing the human ER at brain sites most vulnerable to neuronal damage during experimental stroke procedures will be developed. This will be accomplished by a retroviral-mediated gene transfer strategy. Three rat models are planned, each expressing the transgene regulated by a constitutive cytomegalovirus (CMV) promoter: (1) ER- $\alpha$  gene, expressed as a green fluorescent protein (GFP) fusion protein; (2) ER- $\beta$  gene, expressed as *Discosoma* sp. red fluorescent protein (ds-red) shift fusion protein; and (3) coexpression of ER- $\alpha$  and ER- $\beta$  genes by simultaneous delivery of the two vectors above. There are three critical steps in generating the animal model: (1) design and preparation of high-titer retrovirus carrying the ER gene, (2) stereotaxic injection into rat brains, and (3) evaluating transgene expression in the animal model.

#### Retroviral Design and Strategy

The retroviral vector used to develop the animal model is a kind gift from Dr. Didier Trono, University of Geneva, Switzerland. The vector is derived from the human immunodeficiency virus. It is a third-generation retroviral vector with safety modifications (24). The replication-defective virions are limited to a single round of infection in target cells and do not spread to surrounding

tissue. The viral vector is used by several researchers to investigate a role for specific genes in context of a specific disorder (34,46). The retroviral strategy uses a four-vector plasmid system, whereby accessory viral proteins required for viral packaging and assembly in producer cells are encoded and expressed separate from the transfer construct [described in detail in (24,88)]. In this way, only the minimal gene sequences are expressed in host target cells, greatly reducing the possibility of viral replication and recombination, in vivo. Only three of the nine viral genes are used: group-specific antigen (*gag*), polymerase (*pol*), and regulator of expression of virion proteins (*rev*). The transactivator of transcription (*tat*) gene is replaced by a strong CMV promoter to regulate transgene expression. Accessory genes responsible for pathogenesis, viral protein R (*vpr*), negative regulatory factor (*nef*), viral infectivity factor (*vif*), and virus protein U (*vpu*), have been deleted. The viral envelope (*env*) gene is replaced by a vesicular stomatitis virus glycoprotein (VSV-G) gene from an unrelated virus, essentially generating VSV-G-pseudotyped virus.

The transfer vector contains a chimeric 5'-long terminal repeat (LTR) sequence, packaging signals, the gene of interest regulated by a strong promoter, and the 3'-LTR/self-inactivating (SIN)-18 sequences. These are the only viral sequences transferred to host target tissue sites. Viral proteins required for packaging and assembly of the viral genome into infectious virions are encoded and expressed from three separate plasmids: (1) The packaging construct contains the *gag* and *pol* genes. (2) The regulatory construct contains the *rev* gene responsible for nuclear export of unspliced viral transcripts. (3) The envelope construct contains the VSV-G gene from vesicular stomatitis virus. Viral entry with VSV-G occurs through the endocytic pathway. This change in the viral entry route drastically changes the viral vector properties, compared with the pathogenic wild-type HIV. In summary, the retroviral design generates replication-defective virions in producer cells, which are limited to a single round of infection in target cells, without spreading.

#### Viral Vector Modifications

We considered developing a single internal ribosome entry site (IRES) construct with both genes coexpressed. However, after much careful deliberation about viral vectors, we decided to pursue the development of two IRES constructs, namely, ER- $\alpha$ -IRES-GFP and ER- $\beta$ -IRES-Red. Cell transduction achieved with coexpression of ER- $\alpha$ -IRES-ER- $\beta$  is believed to be similar to ER- $\alpha$ -IRES-GFP combined with ER- $\beta$ -IRES-Red. However, the advantages of transducing cells with double IRES constructs over the single IRES construct include being able to follow the lineage of the transduced cells (using both GFP and Red markers), as well as more stable continuous



expression of the transgenes. Indeed, a recent study has shown the feasibility, transduction efficiency, and functional efficacy of this double transgene expression using two lentiviral vector constructs (47).

#### *Choice of CMV Promoter*

Studies show that the viral LTR is nonfunctional in host cells, after it has integrated into host DNA (24). A strong promoter included in the viral vector will regulate the expression of the ER gene. In this strategy, the constitutive CMV promoter from the immediate early region of CMV is selected. Previous studies have shown that the CMV promoter is responsive in most cells, including neuronal cells, in vivo (59). Preliminary data show that transgene expression with the CMV promoter can be detected, in vivo, as early as 3 to 5 days postinjection, and stable for up to 6 months. Other studies show no evidence of morphological abnormalities or cell toxicity, in vivo (59).

#### *Possible Drawbacks With the CMV Promoter*

Transgene expression from the constitutive CMV promoter may result in toxic accumulation of the ER transgene after an extended period of weeks or months. For this reason, the animals will be evaluated within 7 days to 2 weeks postinjection. Analysis and verification include morphological and apoptotic assays, discussed in "Evaluating the animal model." Alternative strategies include development of the hypoxia-responsive element (HRE) sequences with the Simian virus 40 (SV-40) promoter to regulate transgene expression. In this way, ER expression will occur only during hypoxic conditions. The HRE has been evaluated in vitro, using the adeno-associated virus (AAV) delivery system (74,77). Because of the large number of treatment permutations with the present design, we have decided at this time to pursue the MV promoter for proof-of-concept studies, but we will use the HRE promoter in future studies.

#### *Preparation of High-Titer Retrovirus Carrying the ER Gene*

Two viral preparations, each encoding the human ER- $\alpha$  and ER- $\beta$  genes, are required to generate two animal models. The third animal model will use both viral preparations. Generation of high-titer retrovirus is described in detail (24). Briefly, the ER- $\alpha$  and ER- $\beta$  genes, isolated from a Clontech PCR library, are inserted into the transfer vector using standard molecular biology techniques. All four plasmids are transiently transfected into 293/human embryonic kidney (HEK) producer cell lines. Infectious virions are secreted into the medium and harvested after 24 to 72 h. The medium is pooled, filtered through a 0.23- $\mu$ m cellulose acetate filter, and concentrated by ultracentrifugation at 28,000 $\times$ g, 4°C, 90 min. The pellet

is resuspended in suitable media; 20- $\mu$ l aliquots are stored at -80°C until needed.

#### *Evaluation of Viral Titer*

Assays to evaluate viral titer are described in detail (88). Briefly, five serial 1:2 dilutions of filtered vector stock are used to transduce HeLa cells in six-well plates at 2–3 $\times$ 10<sup>5</sup> cells/well. The highest and lowest inocula correspond to 100 and 6.25 ml of undiluted supernatant, respectively. Vector particles were added to 2 ml of culture medium. After 48- to 60-h incubation, the percentage of GFP-positive cells was determined with a fluorescence-activated cell sorter on a Beckton Dickinson FACScan. To calculate titers [transducing (TU) per milliliter], 2–3 $\times$ 10<sup>5</sup> cells/well were multiplied by the percentage of GFP-positive cells, and this product was divided by the number of microliters in the inoculum. Typical titer ranges from 10<sup>8</sup> to 10<sup>9</sup> TU/ml.

#### *Stereotaxic Injection Into Rat Brains*

The animal model will be generated by stereotaxic delivery of viral vectors carrying the human ER- $\alpha$  and/or ER- $\beta$  gene into neuronal sites. Under strict biological containment, 4- to 6-week-old male rats (Sprague-Dawley) will be anesthetized by equithesin (300 mg/kg, IP) and positioned in a stereotaxic head frame. Following a midline incision of the skin, holes will be drilled in appropriate locations in the skull using a dental bur. A 10- $\mu$ l Hamilton syringe with needle will be used to slowly inject 5–10  $\mu$ l of viral suspension (10<sup>9</sup> TU/ml) in sterile saline into selected areas of the brain. Ischemic core coordinates: AP+1.2 mm; ML 3.4 mm; DV -5.0 mm. Ischemic penumbra coordinates: AP: +1.2 mm; ML 1.8 mm; DV -5.0 mm (41,66). During injection, the needle will be withdrawn slowly. The viral suspension will be expelled slowly at smaller increments to allow for viral diffusion. This will be repeated at different levels. Our preliminary studies show that this strategy generates several clusters of ER-positive areas at different levels from anterior to posterior and creates minienvironments or localized areas for E2 neuroprotection.

Our preliminary studies show that this strategy generates clusters of fluorescent cells (expressing GFP fusion proteins) clearly visible along the needle tract at different levels from anterior to posterior. Transgene expression is stable and can persist, in vivo, for as long as 6 months. This observation indicates that transgene expression at target sites is limited by viral diffusion close to the injection site and confirms the safety properties of a replication-defective virus. However, large areas of the ischemic penumbra are not appropriately targeted for transgene expression. We reason that the E2 neuroprotective effect would depend on the expression of ER target genes in these clusters, to create larger microenvironments or localized areas that

encompass portions of the penumbra. Of note, reports indicate that potential ER target genes from neuronal cells may include secreted trophic factors (28,39,52).

#### *Choice of Cortex and Striatum to Express the ER*

The striatum and cortex are selected sites to express the ER gene, as evidence shows that these areas are vulnerable sites after transient middle cerebral artery occlusion (MCAo) stroke procedures (41,44). Indeed, such sites become parts of the ischemic core and ischemic penumbra after transient MCAo procedure. Our previous studies (5,6,8,11,13) show that the ischemic core is localized within the lateral aspect of the striatum and cortex, while the immediate area next to this core, the penumbra, identified by increased glial activation (14) includes the medial aspect of the striatum and cortex (5). Thus, site-specific injection of the lentiviral vector to express ER at these sites would be optimal to evaluate E2's role in neuroprotection. A possible E2 neuroprotective mechanism mediated by the ER involves the formation of active transcriptional complexes between the receptor and its ligand, E2. Functional transcriptional complexes (TF) activate a subset of target genes responsible for neuroprotection (4). With this in mind, E2 treatments are planned 24 h before and during MCAo stroke procedures. In this way, formation of active TF complexes around this time would be optimal to regulate the expression of E2-responsive genes critical in neuroprotection. Assays to evaluate transgene expression in the animal model are discussed in the following section.

#### *Evaluating the Animal Model*

**Choice of Animals.** Adult male Sprague–Dawley rats are selected to develop the animal model. Previous studies in our laboratory have been successful in reproducing stroke conditions in this rat strain (5,11). Adult males are selected over females to eliminate potential hormonal influences associated with E2. Future studies will focus on gender differences and investigate other rat strains more susceptible to stroke, namely, the spontaneous hypertensive rats, stroke-prone (SHR-SP) and their normotensive reference strain, Wistar Kyoto rats (WKY). Such studies will determine how a genetic predisposition may affect stroke (54).

**Behavioral Testing.** Animals will be evaluated for motor and neurological functions using elevated body swing test (EBST) and Bederson neurological exam, respectively. Both these tests have been demonstrated as sensitive assays for detecting behavioral deficits in MCAo stroke animals. Reductions in motor and neurological impairments will indicate behavioral neuroprotection. Based on our preliminary data, E2 pretreatment reduces motor and neurological impairments in stroke

animals. These tests are routinely conducted in the PI's laboratory (5–8,11–13). EBST involves handling the animal by its tail and recording the direction of the swings. The test apparatus consisted of a clear Plexiglas box (40×40×35.5 cm). The animal is gently picked up at the base of the tail and elevated by the tail until the animal's nose is at a height of 2 inches (5 cm) above the surface. The direction of the swing, either left or right, is counted once the animal's head moves sideways approximately 10° from the midline position of the body. After a single swing, the animal is placed back in the Plexiglas box and allowed to move freely for 30 s prior to retesting. These steps are repeated 20 times for each animal. Normally, intact rats display a 50% swing bias, that is, the same number of swings to the left and to the right. A 75% swing bias would indicate 15 swings in one direction and 5 in the other during 20 trials. We have previously used the EBST and noted that lesioned animals display >75% biased swing activity at 1 month after a nigrostriatal lesion; asymmetry is stable for up to 6 months (5,11). About 1 h after the EBST, a neurological exam is conducted following the procedures previously described (2). The neurologic score for each rat is obtained using four tests that include (1) observation of spontaneous ipsilateral circling, graded from 0 (no circling) to 3 (continuous circling); (2) contralateral hindlimb retraction, which measures the ability of the animal to replace the hindlimb after it is displaced laterally by 2 to 3 cm, graded from 0 (immediate replacement) to 3 (replacement after minutes or no replacement); (3) beam walking ability, graded 0 for a rat that readily traverses a 2.4-cm-wide, 80-cm-long beam to 3 for a rat unable to stay on the beam for 10 s; and (4) bilateral forepaw grasp, which measures the ability to hold onto a 2-mm-diameter steel rod, graded 0 for a rat with normal forepaw grasping behavior to 3 for a rat unable to grasp with the forepaws. The scores from all four tests, which are done over a period of about 15 min on each assessment day, are added to give a neurological deficit score (maximum possible score is 12).

**Triphenyl Tetrazolium Chloride (TTC) Staining.** We routinely conduct TTC staining in our laboratory for measuring cerebral infarct size (7,12). Based on our experience, TTC staining does not interfere with epifluorescence microscopy and also allows quite good immunostaining with neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP), terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) or immunoglobulin G (IgG), and hematoxylin and eosin (H&E). Thus, the initial step following sacrifice of the animals is to capture the non-paraformaldehyde perfused or fixed TTC images and thereafter put the 2-mm TTC-stained brain slices in 4% paraformaldehyde overnight then store in 25% sucrose until subsequent cryostat sectioning for epifluorescence

and immunostaining analyses. Calculations for cerebral infarct volume will include corrections for edema as we have done in the past (84).

*ER Transgene Expression.* Several criteria will be used to verify ER transgene expression at stereotaxic injection sites. Since the ER- $\alpha$  gene is expressed as a GFP fusion protein and the ER- $\beta$  as a ds-red shift fusion protein, it is readily detected in thin section slices using epifluorescence microscopy. This strategy will also be used to monitor viral expression and possible spread in host tissue. Based on this modification, the epifluorescence strategy can be used to (a) estimate viral efficiency or “take rate” or estimate the number of virally infected cells or percentage of cells expressing the transgene, (b) statistically determine the percentage of animals infected with virus or animals expressing the transgene, (c) estimate the area of potential viral spread from injection site, and (d) estimate the amount of time the virus or ER transgene persists in weeks or months.

In addition, morphological analysis will allow identification of specific neuronal cell types infected and a crude evaluation of potential cytotoxic side effects associated with long-term viral and transgene expression. This will be confirmed with general apoptotic/inflammation assays, described below. To adjust for spontaneous GFP or red shift epifluorescence from ischemia-induced necrotic tissue, we will compare counts of transfected cells in nonischemic brain versus ischemic brain. We considered bilateral lentiviral delivery in stroke animals, but because the endpoint is neuroprotection (reduced cerebral infarcts and attenuated behavioral deficits), lentiviral transfection of the contralateral noninjured brain may present as a confounding variable. Thus, we have decided to generate a separate control group to serve as denominator. This additional treatment group will not significantly increase the sample size since the animals will be subjected only to sham surgery and therefore can receive bilateral lentiviral infusion.

*Epifluorescence and Immunohistochemical Phenotypic Characterization of Lentiviral Transfected Cells.* For epifluorescence microscopy, we refer to our published papers using such an approach (7,9,10,12,13,15). Briefly, based on our recent experience with visualizing the extent of the lentiviral transfection within the striatal and cortical ischemic penumbra, we found a maximal transfection extent of about 300  $\mu\text{m}$  (150  $\mu\text{m}$  anterior and 150  $\mu\text{m}$  posterior from the original bregma anterior–posterior stereotaxic infusion site). Accordingly, if we cut 30  $\mu\text{m}$  sections, we will end up with 10 consecutive sections that will capture the whole transfected area. An allowance of five sections anterior and five sections posterior to the infusion site will be performed. Initially, all 20 sections will be used for GFP/red epifluorescence microscopy. Since no immunostaining is required for such GFP/red

epifluorescence microscopy, we will be able to determine the 10 sections that capture the transfected area. These 10 selected sections will be subsequently stained with NeuN and GFAP antibodies to reveal the phenotype (via double labeling) of the transfected cells. Adjacent sections are not used for NeuN and GFAP antibodies to reveal phenotype (via double labeling) of the transfected cells. Adjacent sections not used for NeuN and GFAP immunostaining will be used for TUNEL and IgG immunostaining counterstained with H&E (see detailed description of cytotoxic and inflammation assays below). These assays are routinely performed in the PI's laboratory (14,84). Stereological cell counts will follow the procedures described elsewhere (35). In addition, the stroke control group that will receive empty lentiviruses (i.e., only GFP or red) can serve as the denominator for adjusting the artifact of spontaneous GFP/red fluorescence associated with ischemia-induced necrotic tissue.

*Limitations With Epifluorescence.* Transgene expression from the CMV promoter usually produces strong fluorescence. Early expression is sometimes weak and may be subject to background artifacts. Thus, to confirm ER transgene expression at the protein level, highly specific antibodies against human ER- $\alpha$  and ER- $\beta$ , but not rat ER, will be used in immunohistochemistry and Western (immunoblot) analysis. In both assays, comparisons obtained from brain tissue with and without viral infection will be performed. Transgene expression at the transcriptional level will be confirmed by reverse transcription polymerase chain reaction (RT-PCR). Primers used will flank the internal hER region and 5' end of the GFP gene. The RT kit from Ambion will be used. A functional assay to demonstrate ER activity involves gel shift binding assays (or electrophoretic mobility shift assay, EMSA) using a radiolabeled E2-responsive element (ERE). Cell extracts will be derived from cortex and striatum tissue expressing the ER transgene and compared with extracts from a mock infection or infection with an empty vector. Strong ER binding is expected in the presence of the E2 ligand. Binding of transcription factor complexes to DNA-responsive sites is a strong indication of transcriptional activation (30). The PI is familiar with such assays and has published several papers on the transcriptional properties of the c-jun protein, compared to the viral, v-jun protein (32). The gel shift assay can also be used to optimize experimental conditions to maximize the formation of active TF complexes. For instance, the E2 drug bioavailability, dose, frequency, and route of administration can be evaluated. The experimental conditions used in “Preliminary studies” will be evaluated initially and adjusted accordingly.

*Safety Assays.* The safety modifications generate replication-defective retroviral particles in producer cells.

The resulting pseudotyped virions are limited to infection at target cells at injection sites. Because of a mutation in the viral 3'-LTR, it is silenced in host target cells. A reliable safety test to verify this includes Northern blot analysis of RNA transcripts obtained from target cells after a successful viral infection. In target cells, transgene expression is regulated by the CMV promoter and should reveal a shorter transcript size. Transcripts from producer cells where the LTR is active generate longer unspliced transcripts (24). The transcriptional test mentioned above will be complemented with a p24-gag ELISA assay. The gag gene sequences, provided in *trans*, are not packaged into infectious virions and, therefore, absent in host target tissue. A sensitive anti-p24 gag antibody (Santa Cruz Biotech, CA, USA) should detect p24 gag in secreted media of producer cells, but not in lysates obtained from target tissue. The absence of p24 gag antigen in host tissue is a strong indication that no infectious virions are produced from the viral vectors. It also suggests a lack of DNA recombination between the viral vector and endogenous host retrovirus (24).

Since the pseudotyped virions can only infect host target sites once, viral infection will be limited by diffusion at stereotaxic injection sites. To verify this and monitor potential spread due to production of replication-competent virus, colocalization of epifluorescence and immunohistochemistry analysis will be performed in thin brain sections. As mentioned earlier, the transgene is expressed as a GFP fusion protein and can be detected easily. The p24 gag antibody recognizes viral gag antigen and is used to detect for productive viral infection. It will be used in immunocolocalization studies. Fluorescent sites are expected to stain negative for p24 antigen and localize close to the needle tract or stereotaxic injection site. A distant location indicates that cells expressing the transgene have migrated from the injection site. Fluorescent areas that test positive for p24 antigen indicate that a replication-competent virus was generated. Since weak epifluorescence may be difficult to detect, additional colocalization experiments will be performed using anti-ER and anti-p24 primary antibodies with different secondary antibodies, that is, Texas red for ER- $\alpha$  detection and fluorescent isothiocyanate (FITC) for the ER- $\beta$  transgene.

**Cytotoxic and Inflammation Assays.** Wild-type HIV induces premature destruction of infected cells mediated by a continuous production of viral proteins (tat, env, and nef) that modulate apoptotic factors (26). This is highly unlikely in the viral strategy described here, since the *tat*, *env*, and *nef* genes have been deleted. Excluding these genes eliminates potential DNA recombination with endogenous retrovirus. Nonetheless, tests to evaluate potential destruction of host tissues will be conducted. The TUNEL assay, as described previously (84,85), will

serve as the apoptotic assay, while IgG will be used to reveal local inflammation (14). TUNEL- and IgG-stained sections will be counterstained with H&E. Primary neuronal cells and thin brain sections, each infected with the retroviral constructs, will be compared with noninfected samples. No apoptosis is expected. Assays will be confirmed with a p24 gag ELISA assay (24) that detects gag protein expression. We expect a negative response from *in vivo* samples (thin tissue sections from brains infected with the retroviral vector) and a positive response during viral production in 293 cell lines, *in vitro*.

**Rationale for Lentiviral System.** The lentiviral strategy is an excellent strategy to express a gene of interest in specific tissue sites, *in vivo*, and has been used successfully and reproducibly by several researchers in gene therapy (45,46,53). The system is safe (88). Virulent retroviral genes have been deleted in this construct. The integrated provirus is replication incompetent; consequently, no progeny viruses are produced after an initial infection. Previous studies also indicate no evidence of genetic recombination with potential endogenous retrovirus. In addition, the virus can easily accommodate a large insert size of up to 5 kb or more and infect a wide range of tissue types, including nondividing and postmitotic neuronal cells, *in vivo* [(59), reviewed by Amado and Chen (3)]. These characteristics are ideal for investigating the target gene's potential therapeutic and preventive role in disease model systems in animals.

## LIMITATIONS

The lentiviral strategy has some disadvantages. One possibility is random integration of the viral genome into host DNA that may disrupt normal gene function. The probability is low, since the number of functional genes expressed in differentiated cells is small compared with the total number of available genes in the genome. Nonetheless, a nonreproducible phenotype could occur, if the disrupted gene is required in stroke. To overcome this limitation, increasing the number of animals per group will likely be required.

### *Evaluating the Animal Models in E2 Neuroprotection*

The next goal is to determine the effective E2 dose with specific ER subtypes that can best reduce neuronal damage during stroke injury. This is also a proof-of-principle approach to evaluate mechanisms in E2 neuroprotection. Experiments will test the hypothesis that the ER plays a role in E2-mediated neuroprotection. The genomic mechanism of action predicts that E2, acting through the ER, generates activated transcription factor complexes that in turn regulate a set of E2-responsive genes responsible for neuroprotection [reviewed in Behl (4)]. Three animal models that express either ER- $\alpha$ , ER- $\beta$ , or ER- $\alpha$ +ER- $\beta$  are



evaluated. Different ER subtypes are investigated since ER homodimer or heterodimer pairs can selectively form distinct complexes with coactivators or corepressors to regulate alternative programs of gene expression (22,64).

Animal models expressing one of the following ER transgenes—(1) ER- $\alpha$ , (2) ER- $\beta$ , or (3) ER- $\alpha$ +ER- $\beta$ —will be used in an MCAo stroke procedure. E2 (E2, 17- $\beta$ -estradiol) treatments are timed and optimized to generate transcriptional factor complexes and ER-responsive genes before stroke (18,68). To demonstrate specificity through the ER, additional groups include E2 treatments with an antagonist (ICI 182,780). Data analysis includes estimating tissue infarct size by TTC staining and viable cell counts. Statistical comparisons between animal models and among groups will be performed. Experiments will begin with animals expressing ER- $\alpha$  and described in detail below. This will be followed with animals expressing ER- $\beta$  and animals expressing both ER subtypes.

As noted above, we will use E2 dose from 10 pg to 100  $\mu$ g/kg body weight. E2 will be administered subcutaneously at 2 days before and during stroke. Previous studies show the E2 neuroprotection dose in rats to be between 25 and 100  $\mu$ g/kg body weight, depending on sex, strain, and route of administration (55). In addition, a low E2 dose of 100 pg/kg body weight is selected to reflect physiological plasma E2 levels in rats, estimated at 10 and 30 pg/ml, with peak levels during proestrus at 80 to 140 pg/ml (17,61). To demonstrate the specificity of E2 activity through the ER, groups 5–8 will use the same E2 dose range in combination with a pure antagonist, ICI 182,780. A fixed low dose of 1 pM ICI 182,780 at 1 pM/kg body weight is sufficient to block a low physiological E2 dose (100 pg/kg body weight), but not a high E2 dose (100  $\mu$ g/kg body weight). A cocktail of E2 at the specified dose will be mixed with 1 pM ICI 182,780 and injected once (SC) at 2 days before and during stroke.

The E2 antagonist, ICI 182,780, belongs to a class of SERMs that bind both ER- $\alpha$  and ER- $\beta$  subtypes, thereby blocking E2 activity (3,38,50). The antagonist dose at 1 pM/250 nl can effectively block 0.5  $\mu$ M/250 nl E2 activity at the receptor level, in vivo (69,70). Previous studies have used this compound in a stroke model to demonstrate the involvement for ER in E2 neuroprotection (83,86) and in an ischemic mouse model (73). Although the antagonist does not readily cross the blood–brain barrier (BBB) by systemic administration (20), studies by others (16) and our lab (9,10) demonstrate that stereotaxic injections compromise the BBB for up to 12 days. We maintain that systemically administered ER antagonists, in general, do not easily cross the BBB. However, since these drugs will be delivered after stereotaxic lentiviral delivery, which renders the BBB to be compromised up to 12 days post-surgery (16), such mechanical disruption would likely

facilitate the entry of these peripherally administered drugs. Indeed, our previous studies (6) demonstrate that stereotaxic infusion of saline facilitates the CNS entry of cyclosporine-A, which under normal conditions does not easily cross the BBB. Treatment schedules proposed here are within this short BBB breakdown window.

To establish a basis for comparison, control groups without ER transgene will be added and will receive E2 at the dose specified above. An additional group will receive an empty vector expressing a GFP marker protein only. This group will be used to demonstrate that the viral vector alone does not interfere with E2 neuroprotection. All animals in these control groups will receive MCAo stroke procedures.

### *Expected Outcomes*

We expect a progressive decrease in neuronal damage with increasing E2 dose when administered to animals with either normal or elevated ER levels. However, a lower effective E2 neuroprotective dose is expected in animals with elevated ER levels. We also expect that the lower E2 dose is abolished in the presence of an E2 antagonist.

### *Potential Problems With the Strategy*

Our envisioned studies are designed to express the ER transgene at appropriate levels in the brain such that active transcriptional factor complexes are generated with E2 administration. However, if significant neuroprotection is not observed, the levels of ER transcriptional activation complexes will be increased by adjusting the E2 drug bioavailability, frequency, and route of administration. The time interval for ER transgene expression will be extended from 7 to 12 days postviral injection. This approach will increase ER levels at target sites. Alternatively, the bioavailability of E2 at neuronal sites can be increased by modifying the frequency of drug administration. E2 treatments will be doubled to twice a day, before and during and after stroke. The dose will not be increased. IM injections or use of time-released E2 pellets are considered. Overall, the measures increase the probability that activation of ER target genes responsible for neuroprotection will occur.

ER viral transfection is quite limited to the original striatal infusion site. Such focal transfection somehow mimics the pattern of migration (or lack thereof) of stereotaxic transplanted fetal cells, postmitotic teratocarcinoma-derived Ntera2/D1 neuron-like (NT2N) cells, or neuronal stem/progenitor cells in similar animal models of stroke (7,8,13). Despite the limited area of transfection and cell migration in both neuroprotective/neural repair paradigms, robust functional recovery has been documented. We recently showed that trophic factor release is an equally

important mechanism underlying such functional recovery in the absence of grafted cell survival and/or migration (9–12), which could very well be the same mechanism involved in our preliminary study demonstrating reduction of cerebral infarcts by localized transfection of ER. It is possible that elevated E2 levels resulted in increased neurotrophic factors, which could have diffused some distance away from the lentiviral infusion site. Accordingly, for future experiments, we plan to assay trophic factors of tissues from within and around the stroke area to reveal such ER overexpression and neurotrophic factor release mechanism.

### CONCLUSIONS

Several lines of scientific and clinical investigations point to the key role of E2 in stroke. The benefits and risks associated with E2 replacement therapy in stroke will be critical in advancing E2 treatment in the clinic. In tandem, understanding the role of the ER will be important in improving clinical outcomes of E2 therapy for stroke. The need for a validated and standardized animal model is urgently needed to assess the potential therapeutic effects of E2 and ER in affording neuroprotection against stroke. The advent of retroviral-mediated gene transfer strategy may allow the manipulation of ER subtypes in discreet regions of the brain that will facilitate optimization of E2 dosing regimen that is safe and effective for stroke patients.

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