Grant Title: *Estrogen Induced Neurogenesis*

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FINAL REPORT

Dr. Louise McCullough has completed two years of funding as a recipient of a Career Development Award from the Goddess Fund. This award has significantly contributed to Dr. McCullough's career at Johns Hopkins University. Funding from this Career Development Award has led to 9 published articles, 2 chapters in press, as well as 5 additional articles that are currently in preparation. Dr. McCullough has been invited to give several talks on the subject of gender and stroke, and has been actively involved in the "Stop Stroke" campaign in Maryland. In addition, she has obtained grant funding from the American Heart Association until 2007, and plans to submit a Culpeper grant in August and an RO1 in October 2004 that are based on findings from the period of Goddess funding. The generous support of the Fund has been integral to Dr McCullough's success as an independent researcher.

Background

Stroke is a leading cause of permanent disability and death within the United States. Our laboratory, and others, has shown that female animals sustain less tissue damage after experimental stroke. The neuroprotection seen in female animals is eliminated with the loss of female sex steroids after either surgical removal of the ovaries or with natural aging. Furthermore, the principal mammalian estrogen, 17-_ estradiol, provides robust neuroprotection from numerous types of brain injury when given to animals of either sex. What remains to be shown is whether estrogens also facilitate healing and recovery in injured brain, and if this benefit is limited to the female.

It is clear that new neurons are formed after ischemic brain injury. These new cells may play a role in improving the rate and amount of behavioral recovery after stroke. As the population ages, stroke incidence continues to rise, as does the number of patients who survive stroke. The ability to enhance

functional recovery, even to a modest degree, would have a significant impact on the lives of these patients. Strategies to increase and maintain neurogenesis after stroke and increase incorporation of neurons into damaged brain could have significant clinical potential. Estrogen is known to increase both neurogenesis and synaptogenesis in the non-injured hippocampus. No studies have examined the possibility that this hormone can increase new neuron proliferation or survival under pathological conditions such as cerebral ischemia. The overall goal of the study was to evaluate if estrogen enhances development and survival of neural progenitor cells and improves functional outcome after focal cerebral ischemia.

Experimental Design and Protocol

Mice of both genders were subjected to 90 minutes of reversible middle cerebral artery occlusion (MCAO) and allowed to survive 4 days, 14 days, or 6 weeks. The different endpoints allowed us to assess the proliferation, maintenance and survival of newly generated cells. One week prior to MCAO, female mice were subjected to ovariectomy. Under halothane anesthesia, the ovarian artery and vein were ligated and the ovary was removed. Silastic capsules (.062”ID/.0125”OD) filled either with 0.035 ml of sesame oil or 17β-estradiol (Sigma) were implanted in ovariectomized females and males.

Alzet osmotic mini-pumps containing Bromo-deoxyuridine (BrdU 50mg/kg/day for 3 days) which labels newly dividing cells were implanted in the intraperitoneal (i.p.) cavity on day 3 (2 days after stroke) or day 8 (peak neurogenesis) after stroke. Animals underwent a variety of behavioral tests on days 1, 3, 5, 7, 14, 21, 28, 35 and 42. At the conclusion of the experiment animals were sacrificed using an overdose of pentobarbital and then perfused with 4.0% cold paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The brain was removed, post-fixed for 24 hours, cryoprotected with sucrose, and sectioned (30 μm) on a freezing microtome. Free-floating serial sections were collected throughout the brain so that both the infarct and the areas involved in the highest rate of cell proliferation (the hippocampus and subventricular zone) were included. Six series of sections were obtained and analyzed. One series was used to calculate infarct volume using standard histology with cresyl violet staining. Infarction volume was determined by video microscopy and image analysis (Inquiry 3, Loats Associates). The remaining sets of serial sections were double-labeled with BrdU (a marker of cell proliferation) and a cell-type specific marker. Immature neurons were identified with TU-J1, migrating neurons with DoubleCortin, mature neurons with NeuN, endothelium with VWF, and astrocytes with GFAP. BrdU was linked to a FITC anti-rat secondary, the other antibodies were linked to a Texas red secondary (anti-mouse or anti-rabbit) ((Vector Labs 1:200). Slides were viewed with a Zeiss Axiophot fluorescent microscope equipped with rhodamine and fluorescein filters. For confirmation of the phenotype of individual BrdU cells, double-labeled sections were examined with an Axiovert confocal scanning laser microscope.

Preliminary data analysis demonstrates that estrogen enhances both basal and stroke-induced

neurogenesis. Sham animals of either gender treated with estrogen had a small increase in the number of BrdU+ cells, primarily in the hippocampus. The majority of these cells (70%) were co-labeled with the immature neuronal markers doublecortin and TUJ1 at 2 weeks after sham surgery (3 weeks of estrogen treatment). By 6 weeks, a few NeuN/BrdU+ cells were present but no immature co-labeled cells were seen. The number of BrdU+ cells was significantly lower at 6 weeks, suggesting that some of these original cells died or lost staining. The absolute increase in hippocampal neurogenesis in sham animals was small with approximately 2-3 more cells per 20x field than oil-treated animals (at 2 weeks). Some baseline BrdU staining was seen in sham SVZ, but there were no differences between oil and estrogen treated animals. This likely represents the low baseline neurogenesis seen in the rostral migratory stream providing input to the olfactory bulb. In contrast, there was a dramatic increase in TUJ1/BrdU+ cells in both the SVZ and HC animals subjected to MCAO (>10 cells/hpf) of both genders. There was a significant increase in cell number in the ipsilateral hemisphere, although even the contralateral hemisphere had much larger pools of BrdU/DCTX+ (and later NeuN+) cells than that of sham animals. Small numbers of immature cells were throughout the stroke hemisphere, including the ischemic cortex. Treatment with estrogen increased stroke-induced neurogenesis in both male and female mice compared to oil treated animals, primarily in the dorsal SVZ (16 cells/hpf).

In addition, during the funding period, a novel behavioral battery was designed to evaluate functional recovery after stroke. Previously, correlation between post-stroke histology and behavior was challenging, especially in murine models of stroke where behavioral recovery is rapid. We utilized a separate cohort of animals to develop a long-term behavioral assessment battery that can be used for months after stroke (see Li et al., Experimental Neurology, 2004).

The role of estradiol aromatized from testosterone to neuroprotection was also examined. Animals deficient in cytochrome P450 aromatase (ARKO mice), with minimal endogenous estradiol production, were found to have exaggerated tissue injury after focal stroke. This effect was reversed by the administration of exogenous estrogen. Similar results were found in female mice administered a pharmacological inhibitor of aromatase, Fadrozole. Aromatase inhibitors are now clinically utilized as first-line treatment for breast cancer due to their anti-estrogenic effects. The effect of these compounds on stroke may have clinical implications (see McCullough et al., Journal of Neuroscience, 2003).

In addition to our studies above, The Goddess Fund has played a substantial role in the description and analysis of an exceptionally novel and exciting finding; that cell death pathways are sexually dimorphic. Our emerging data suggests that neuronal cell death may follow differing mechanistic paths depending on gender as well as sex steroid exposure. One well-studied mechanism of neuronal cell death in cerebral ischemia and trauma occurs by a serially linked set of events: overstimulation of neuronal nitric oxide synthase (nNOS) leading to toxic local elaboration of nitric oxide (NO), subsequent peroxynitrite formation and nitrosative DNA damage, activation of the DNA repair enzyme poly-ADP ribose polymerase-1 (PARP-1), and PARP 1-induced mobilization of pro-apoptotic molecules such as apoptosis inducing

factor (AIF). Key evidence in establishing NO toxicity/PARP-1 activation as a major cytotoxic mechanism has accumulated from exclusively male animals with targeted deletions of nNOS (nNOS-/-) or PARP (PARP-/-). In each case, these knockouts are resistant to brain injury from focal and global cerebral ischemic insults, as are primary neuronal cell cultures derived from fetal brain obtained from mixed sexes. Similar findings of neuroprotection using pharmacological inhibitors of nNOS and PARP-1 in male animals and mixed sex neuronal cell cultures have been widely reported. In contrast, we have observed that female nNOS-/- and PARP -/- mice subjected to focal stroke do not benefit from the genetic mutation as do their male counterparts. In addition, pharmacological treatment with PARP and nNOS inhibitors exacerbated damage in females. Therefore, we believe that the nNOS-PARP pathway of ischemic cell death is sexually dimorphic in the brain; mediating cell death in males and cell survival in females (manuscript submitted PNAS).

In addition we have recently taken this in vivo data and extended it to the cellular level. Utilizing sex-specific hippocampal slices taken from day 8 rat pups, we have found that female cells are protected compared to males after oxygen-glucose deprivation (OGD). This female neuroprotection is not mediated by estrogen but appears to be a fundamental property of the cells themselves. At this point the role of nNOS, PARP and AIF in this gender dependent cell death is unknown.

The knowledge gained from these studies will be far-reaching. We anticipate that gender differences will be present in many, if not all, cells in the body making these studies directly relevant to all investigators examining cell death. These findings may help explain why translation of neuroprotective agents from the lab to the clinic has been largely unsuccessful for the treatment of stroke.

Scholarly Progress

Publications since grant inception:

1) McCullough LD, Zeng Z, Blizzard K, Debchoudhury I, Hurn PD. Ischemic nitric oxide and poly (ADP-Ribose) polymerase-1 activation in brain: male toxicity, female protection. PNAS (submitted).
2) McCullough LD, Zeng Z, Landree L, Ronnett G. The role of AMPK in stroke (In preparation for submission; Nature).
3) Li X, Blizzard K, Zeng Z, Derives AC, Hurn PD, McCullough LD. (2004). Behavioral assessment after stroke: Role of gender. Experimental Neurology 187:94-104.
4) McCullough LD & Hurn PD (2003). Estrogen and ischemic neuroprotection: An integrated view. Trends in Endo and Metabolism 14: 228-35.
5) McCullough LD, Blizzard KK, Oz O, Simpson E & Hurn PD (2003). Aromatase Cytochrome P450 and extragonadal estrogen play a role in ischemic neuroprotection. J of Neuroscience 23:8701-8705.
6) Murphy SJ, McCullough LD, Littleton-Kearney M & Hurn PD (2003). Estrogen and selective estrogen receptor modulators: Neuroprotection in the Women's Health Initiative Era. Endocrine 21: 17-26.

7) Murphy SJ, McCullough LD, Smith JM. Stroke in the female: Role of Biological Sex and Estrogen. ILAR 45 (In press).
8) Murphy SH, Littelton-Kearney MT, McCullough LD & Hurn PD (2003). Sex, hormones, and the endot helium. In: Principles of sex based physiology: 34: 71-84. Miller V. and Hay MH, eds. Elsevier. (In press).
9) Hurn PD, Ardelt AA, Alkayed NJ, Crain BJ, Hu W, Kearney ML, McCullough LD, Murphy SJ, Toung TJK, Traystman RJ, Wang MM. (2002). Estrogen and Testosterone as Neuroprotectants in Stroke. In: Pharmacology of Cerebral Ischemia: Krieglstein J, editor. Medpharm Scientific Publishers, Stuttgart, Germany.

In Press:

1) Blakely J., McCullough LD. Thrombolytic therapy in acute stroke. In: Acute Stroke Management: Johns Hopkins University Press.
2) Cortese I., McCullough LD. Hormones and stroke: In: Acute Stroke Management: Johns Hopkins University Press.

In Preparation:

1) Li H, Zeng Z, Li X, Hurn PD, McCullough LD. Estrogen-induced neurogenesis after stroke.

2) Li H, Zeng Z, Andreasson K, Wang M, Hurn P, Pin S, McCullough, LD. Gender differences in ischemic cell death in vitro; results from sex-specific hippocampal slices.

3) Li H, Savitt J, Zeng Z, Pin S, Gearhardt J, McCullough LD. Effects of estrogen and exogenous stem cell implantation on stroke-induced neurogenesis.

4) Savitt J, Li H, Zeng Z, Pin S, Gearhardt J, McCullough LD. Enhanced neurogenesis and behavioral recovery following exogenous stem cell transplantation.

5) Li H, McCullough LD. Gene expression analysis of sex-specific hippocampal slices.

Data has also been presented at:

1. The Society for Neuroscience (oral platform), 11/2003.
2. The Society for Neuroscience (poster presentation), 11/2002.
3. American Heart Association stroke meeting (poster presentation), 2/2003.
4. American Heart Association stroke meeting (oral presentation), 2/2002.

Ad Hoc Reviewer:

Stroke, Annals of Neurology, Experimental Neurology, Journal of Neurochemistry.

Invited Talks, Panels:
Neuroscience Lecture Series, Department of Neuroscience, Johns Hopkins University May, 2003.
American Association for Orthopedic Surgery, Hunt Valley MD, April 2004.
American Heart Association Woman's Board, McLean VA, May 2004.

Grants awarded as a result of the Goddess Fund Career Development Award
American Heart Association Fellow to Faculty Award: 2002-2007.
Invited submission for The Charles Culpeper Award (due 8/04).