UF UNIVERSITY of FLORIDA

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January 25, 2009

McKnight Brain Research Foundation Trustees
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Dear MBRF Trustees:

It has been another good year for our Evelyn F. and William L. McKnight Brain Institute, with many important discoveries made, numerous superb quality publications from our investigators appearing in high impact journals, and the recruitment of one of the most promising young brain scientists in the world, Dr. Matthew Sarkisian, who will be working in our newly launched Program in Aging Brain Plasticity. In addition, we were able to help settle in Dr. Brent Reynolds, who we brought here from Queensland Brain Institute, and who is the discoverer of stem cells in the adult brain; he has begun his work on potential applications of stem cell biology and neurogenesis toward enhancing lifelong learning and memory. The very successful first McKnight Inter-Institutional meeting also brought together new collaborators, including, e.g. Dr. Gavin Rumbaugh from the UAB MBI who is working with our Dr. Nick Muzyczka who is making viral vectors for Dr. Rumbaugh for his studies on actin-myosin interactions involved in learning in the CA1 of the hippocampus.

In addition to investing Age-Related Memory (ARML) funds in new recruits, we have also funded a small number of continuing and new competitive RFA grant proposals in the areas of: " Microglia as Therapeutic Targets in Age-Related Memory Loss" (Dr. Jake Streit, MBI Dept. of Neuroscience); "Development of Nanoencapsulated Orexin As A Potential Therapeutic for Age-Related Memory Loss" (Dr. Mike King, Dept. of Pharmacology/Therapeutics); "Effects of Resveratrol on Memory Performance in Older Adults" (Drs. Manini and Pahor, UF Aging Institute); "Implantation of Adult Human Progenitors into an Animal Model of Cortical Dysplasia" (Dr. Steve Roper, Dept. of Neurosurgery, MBI); "Do Different Neurons Age Differently? Direct Single-Cell Genomic Profiling of Neuronal Subtypes in a Memory Forming Network as a Function of Aging" (Dr. Leonid Moroz, Whitney Laboratory); "Genome-Scale Approaches to the Discovery of Novel Targets for Neuroprotection" (Dr. Hendrik Luesch, UF Dept. of Medicinal Chemistry); and "Neural Stem Cells in the Aging Brain" (Dr. Eric Laywell, Dept. of Anatomy and Cell Biology, MBI). We also completed the one-time funding of a contract-like proposal for Dr. Gerd Kempermann (Dept. of Genomics and and Regeneration, Dresden, Germany) to do gene expression profiling of at-risk memory and learning genes in a study, "Genetics of Gene Expression Associated With Neurogenesis in the Aging Mouse Hippocampus". We will continue to fund small numbers of proposals, more contract-like fundings with milestones, that have the potential to advance our understanding of ways to increase aging brain plasticity; these will be solicited RFAs that act as "flyer grants" to also help the principal investigators obtain competitive federal grants.

In addition to the numerous collaborations between the Foster lab and the other McKnight Institutes, we are also collaborating with Dr. Geoffrey Ahern from our sister Institute at the University of Arizona, who will be working with us on hopefully our first inter-institutional clinical trial for enhancing memory function in the aging that is being planned for the very near future (a new R01 also submitted by Drs. Brent Reynolds and Hubert Fernandez, Co-PIs, and along with myself, Drs. Wu, Haas, Ahern and Christiana Leonard as Co-Is and collaborators). Substantial plasticity is possible in the aging brain, with physical losses being potentially reversed, and aspects of sensory, motor, and cognitive systems can be potentially restored to more youthful levels of functioning. Two of the strongest effectors have been behavioral interventions (cognitive stimulation and exercise) suggesting that safe and cost effective programs can be implemented to produce a meaningful improvement in cognitive ability that will persist after training and transfer to non-trained domains. We intend to pilot, in this near-future trial, the implementation of cognitive training and exercise and measure its effectiveness on a number of outcomes including cognitive ability, transference to everyday tasks, maintenance beyond the training period, conversion to mild cognitive impairment, and importantly, to anatomical changes in the brain (in addition to measurements of the hippocampus before and after the training period, we will be using MRI and other tools to measure potential positive effects on area 39, or the angular gyrus of the cerebral cortex, a structure we believe to be very important in memory and learning) that will be analyzed using our powerful high resolution imaging facilities in our MBI. We are proposing in new grants, a three-center, randomized, double-blind, sham-controlled, parallel-group study of auditory/visual system cognitive training (Posit Science Brain/Visual Training Program; this program was developed, as Dr. Dockery knows, by one of my mentors from the University of California San Francisco, Dr. Michael Merzenich) combined with a computer-based exercise program (Sony Wii based) in elderly patients. It is our long term goal to develop a globally accessible cognitive/exercise training program that can be implemented at in anyone's home using computer and television- guided modules that will ultimately allow a simple, cost and clinically effective cognitive enhancement program to gain wide acceptance and deep penetration into the our rapidly expanding aging population. A trademarked MBI webpage is in process, with help from Dr. Brent Reynolds, "agingwithme.com", that will allow us to offer computer-based behavioral enhancements to improve memory function to literally everyone with access to the internet.

In recent years I have asked our McKnight Chair, Dr. Tom Foster, to help form several "memory" working groups to be associated with our Institute. He has tried to bring together investigators from around campus who have an interest in the aging brain and enhancing memory function with aging, and his progress report is below:

"...Human Memory Focus Group
This was the first group to be organized and was initiated in 2006.

The three main goals of the focus group were:

1) Facilitate interactions across UF researchers with an interest in memory function.
2) Disseminate results from research supported by McKnight Brain Research funding.
3) Generate collaborative research on age-related memory function.

The steering committee consisted of David Loring (Chairman), Bruce Crosson, Michael Marsiske, Steve Nadeau, Jeffrey Kleim and Tom Foster (ex-officio member).

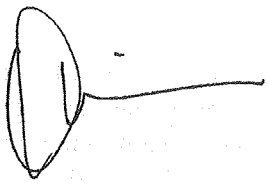
The benchmarks and mechanisms for success as identified by the committee included:

1) Have seminars that focus on aging and memory function.
    a. Generate a list of individuals interested in human memory function and changes in memory during aging.
    b. Secure the meeting time and location

regenerative medicine, high resolution imaging of brain and brain cell structure and function, and basic as well as clinical neuroscience, we will continue to tap the diversity of brain research being done in the Institute to provide unique perspectives toward the development of interventions for age-related cognitive decline. Along this line, in a new memoir from Dr. Harold Varmus, former Director of the NIH and Nobel Prize winner, he describes the difficulties of trying to predict where and how great scientific discoveries will be made. He notes that serendipity in science often leads to the biggest breakthroughs, whereby, "...Spending funds to seize a chance to understand a fundamental principle in biology is often a more effective approach to disease than mandating funds for research on a specific disease. Furthermore, efforts to understand another disease, even one that does not affect neurons [for example], might prove to be a more valuable means to understand ALS [for example] than work on ALS itself..." (The Scientist, January, 2009). With this in mind, our wonderful Institute will continue to make significant discoveries in brain science and especially Aging Brain Plasticity, by tapping our greatest resource: Our highly diverse and talented investigators.

Thank You MBRF for Your Continued Interest and Support of Our Work!

Sincerely Yours,



Dennis A. Steindler, Ph.D.
Executive Director
The Evelyn F. and William L. McKnight Brain Institute
University of Florida

programs that will be directed at elucidating how cellular and molecular mechanisms of brain plasticity change during aging. As a faculty member in neuroscience, the candidates will be expected to participate in graduate and medical educational programs in the College of Medicine. Minimum requirements include a doctorate or medical degree in a relevant discipline, and 2-5 years of post-doctoral experience. The University of Florida offers a competitive salary and start-up package. Salaries are negotiable and commensurate with experience.

Applicants should submit curriculum vitae, a brief description of research interest, and the names and contact information of three references to: Dr. Dennis Steindler, Neurobiology Search Committee Chair, Department of Neuroscience, McKnight Brain Institute, PO Box 100244, Gainesville, FL 32610. Electronic applications can be made to Sabrina McLaughlin at sabrina.mclaughlin@mbi.ufl.edu. Visit us on the web at www.mbi.ufl.edu. Review of applications will begin March 1, 2009."
Search Committee:

Dennis Steindler, Chair of the Committee
Faculty Members:
Tom Foster
Harry Nick
Maury Swanson
Brandi Ormerod
Matt Sarkisian
Jackie Hobbs

All of us in the Institute are excited about our expanding research in "Aging Brain Plasticity". As Dr. Michael Merzenich has pointed out, brain development does not stop in adolescence, but rather, even the very aged human cerebral cortex is amenable to lifelong training and reorganization. In line with and championing this philosophy, the journal *Nature* has recently participated in a conference that focused on how aging and development have much in common. This "Symposium on Biological Complexity: Processes of Aging" advertised, that the "...Aging process is a multi-faceted process, where cellular aging, metabolism, the DNA damage machinery and stem cell dynamics interact to influence aging of an entire organism..." The McKnight Brain Institute of the University of Florida has exceptional strengths in the areas of brain development and plasticity, and we will continue to mobilize a growing group of gifted and dedicated investigators in this area to provide new insights into how to teach and reshape the aging human forebrain to accommodate for deleterious anatomical, biochemical, and physiological changes that can accompany the aging process. In particular, Dr. Matthew Sarkisian has been given the charge of mobilizing UF and MBI laboratories and technologies involved in gene, molecular and cellular therapies to develop a new brain imaging and manipulation platform to reprogram aging cerebral cortical neurons to assume more immature structure-function relationships. Reprogramming higher forebrain circuitries that are involved in higher cognitive functions has the potential to reverse the negative consequences of brain aging and facilitate both neurogenesis-based and synapse/circiuitry-associated learning and memory function. This is an unprecedented and extremely ambitious endeavor that will bring together some of our best investigators skilled in conventional memory research with regenerative medicine, a clear strength in our Institute, to methodically but creatively generate new models and paradigms for enhancing Aging Brain Plasticity. Our planned behavioral/physical enhancement clinical trials for improving memory function first in retirement communities, and eventually through the internet, will nicely dovetail with this endeavor; we look forward to bringing together our colleagues in the other McKnight Institutes to offer their unique expertises in memory loss and enhancement models to be tested in this new platform of Aging Brain Cell Reprogramming.

The Evelyn F. and William L. McKnight Brain Institute of the University of Florida is unique in its incredibly wide breadth of scope in nervous system research. With everything from gene therapy to adult stem cell and

training on the Neurobiology of Aging and to enhance collaboration on research related to the neurobiology of aging. We have successfully funded a grant for Training in Neurobiology of Aging through the Center for the Neurobiology of Aging. The training grant supplies support for three graduate students and three postdoctoral fellows. In addition, this group has obtained one additional postdoctoral position from the University of Florida. Selection of trainees is determined by a steering committee consisting of Drs Meyers, Scarpace, King and Foster.

A course on the Neurobiology of Aging is sponsored by the training grant and this course is required for trainees. In addition, there are several journal club which meet to focus on neurophysiology, neuroplasticity, brain aging, and neurodegenerative diseases of aging.

A number of collaborative projects are on going. Some relevant examples of projects that I am involved in include: Studies of diet and memory function, Biomarkers of healthy cognition across the lifespan, Anorexin as a pharmacological treatment for age-related memory decline. A larger collaborative group is examining autophagy as a target pathway for modulation of cellular aging and includes studies examining the role of autophagic regulation on memory function. A program project grant related to this research was submitted in 2008 and is currently being revised. This project will probably be resubmitted in late 2009 or early 2010.

Currently: This group is active and continues to sponsor talks and seminars, there is an underlying theme of education related to the neurobiology of aging and a network of collaborations are ongoing with shared ideas and resources.

Molecular, Cellular and Neurogenesis Group

Discussions related to the start of this focus group began in 2007. Dr Eric Laywell volunteered to be the head of this group. Individuals associated with this group attended the first MBRF Inter Institutional meeting in 2008. My lab has been interacting with Dr Muzyczka's group examining synaptic function following viral vector treatments for genes associated with age-related cognitive decline.

Currently: The Molecular, Cellular, and Neurogenesis group is in the process of undergoing revisions in direction ..."(Dr. Tom Foster, 1/09).

As mentioned above, the group will become a new Program in Aging Brain Plasticity since understanding how the brain maintains the ability to respond to changes in the way it responds to and processes sensory and other stimuli will certainly have a huge impact on discovering ways to maintain and enhance cognitive prowess throughout life.

Toward the goal of meeting our recruitment of several new aging brain plasticity researchers to join our efforts, a new faculty search has just begun to fulfill our commitment to use McKnight endowment-associated funds to expand our aging brain and plasticity faculty researchers. Even though the endowment has been dramatically reduced due to the economic downturn, we still plan on trying to fill this position as soon as possible. The ad for this position appears below:

"Tenure-Track Assistant Professor Position in Neurobiology, University of Florida

A new tenure-track position has been established at the level of Assistant Professor by the McKnight Brain Institute of the University of Florida and the Department of Neuroscience at the University of Florida College Of Medicine. The Institute and the Department are seeking candidates with research interests in brain plasticity during aging and development. Successful candidates are expected to establish extramurally funded research

c. Generate a list of speakers with a focus on those that have received McKnight Brain Research funding.

2) Generate potential research groups
3) Submit research proposals to NIH

In fulfilling goals 1 and 2, the group had a series of ~17 talks/seminars from the group members which spanned a two year period. The talks included data presentation from eight individuals who had previously received MBRF support.

In addition, individuals associated with this group were involved in organizing and participating in the 1st Annual North Florida BETRR Workshop Building Effective Translational Rehabilitation Research which was held in 2007. This workshop included interactive talks and discussion on cognition, designed to address the following questions.

1. What are the best animal models available to test human impairments?
2. How could either animal models or human outcome measures be altered to improve translation of animal research into clinical research?
3. What are the most important, clinically relevant issues that clinicians want basic scientists to address?

Concerning the generation of collaborative projects (Goal 3), it should be noted that I was not involved in all the discussions of collaborative projects that have materialize as a result of the Human Memory and Aging seminar series. However, below is a sample of some of the efforts toward collaborative projects that have emerged.

The Director of the Institute on Aging, Dr. Marco Pahor, submitted a multi-site "LIFE TRIALS" pilot proposal to NIA. This proposal is designed to examine the effects of exercise on measures of successful aging. This proposal included projects to examine cognitive factors (executive function, attention, speed of processing and memory).

A translational research group has generated ideas and preliminary data specific to translational models for developing treatment strategies, and methods to assess them, in the context of the current clinical cognitive program at the Brain Rehabilitation Research Center (BRRC). This group was initiated by Leslie J. Gonzalez Rothi, Director of the BRRC and consists of individuals interested in human memory, animal models, and neurological diseases.

Currently: The group has met all the stated goals. All the members willing to give seminars have presented seminars related to their published work or preliminary data. Therefore, the time slot for the scheduled seminars has been returned to neurology. It should be noted that the leader for this group, David Loring, has taken a position at another university.

I continue to maintain an e-mail list which I use to notify people of relevant talks, grant opportunities, and meetings related to human memory. Members of this group will attend the Inter-Institutional meeting at the University of Alabama in 2009. A strong core group continues to collaborate on aging and cognition research, especially as it related to language.

In addition, I continue to act as an advisor/mentor for projects which link human cognition to brain function. I am currently an advisor for an NIH supplement award to address "The effects of cardiovascular fitness on the brain in humans".

Neurobiology of Aging Focus Group

A set of individuals focused on Neurobiology of Aging was in place prior to my arrival at the University of Florida. In 2006, the goals of this group were to obtain funding from the National Institute on Aging to support

Annual Report-McKnight Brain Research Foundation
Sponsored Institutes and Research Programs
(Include activity of all McKnight supported faculty and trainees)
Bruce Crosson, Ph.D. – M-47
Report Period: 2007-2008 (calendar years)

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1. **Summary of scientific achievements since last report (calendar years 2007 and 2008)**

In keeping with the agreement between Dr. Crosson and the MBI, the M-47 project on which we have worked during the past two years is a collaborative project between David B. FitzGerald, M.D. (Neurology) and the laboratory of Dr. Crosson that allows the former to use image processing resources for a study of memory in aging. The specific aim of the study is to map changes in brain systems activity responsible for improved memory when older adults take donepezil, an anticholinesterase. The scientific achievements are as follows:

1) Completion and publication of a randomized, double blind placebo controlled study of the effects of donepezil on immediate and delayed recall of semantically and superficially processed words in healthy elderly. (FitzGerald, et al, Cognitive and Behavioral Neurology, 2008).
2) Development and validation of in-scanner stimuli for semantic and superficial processing of words as part of further evaluation of memory processing in healthy elderly using functional MRI in a randomized, double blind placebo controlled study of the effects on immediate and long-term memory in healthy elderly (i.e., the current M-47 project). This is an extension of #1 (please see above) with fMRI added to investigate changes in superficial and semantic processing before and after being given a study drug (donepezil or placebo). This study uses both in-scanner performance and post-scan out-of-scanner performance for both block analysis and event-related analysis of the fMRI data to determine:
    a. Changes seen within medication group (pre and post medication) and as compared to the placebo group
    b. Correlation between performance on and off medication and changes in activation seen in pre-defined regions of interest
3) Development and validation of MRI sequences (both structural and functional) for investigating semantic and superficial processing and recall of the words used in these tasks (see #2 above).
4) Development and validation of similar in-scanner and out-of-scanner tasks to characterize differences and similarities in performance of these tasks. Performance in and out of the scanner has been assumed to be equivalent by virtually all investigators, but there is no validation of this approach with experimental data. (as part of #2 above).

5) Acquisition of data (out-of-scanner performance and in-scanner performance with structural and fMRI data) in 5 subjects (total subjects needed is 24). We are prepared to begin the study with another 6 subjects.
6) Establishment of database (with on-going data acquisition as part of the study in #2 above) of word frequencies in semantic and categorical fluency tasks on and off medication. This is similar to work done in Foster, Drago, FitzGerald, et. al Neuropsychologia, 2008, but instead of dopamine supplementation, looks at cholinergic supplementation using donepezil.

2. **Publications in peer reviewed journals**

Peer-Reviewed Journal Articles Related to Age-Related Memory Loss and/or McKnight Funding

Wierenga, C. E., Perlstein, W. M., Benjamin, M., Leonard, C., Rothi, L. G., Conway, T., Cato, M. A., Gopinath, K., Briggs, R., **Crosson, B.** (in press). Neural substrates of object identification: fMRI evidence that category and visual attribute contribute to semantic knowledge. *Journal of the International Neuropsychological Society.*

Meinzer, M., Wilser, L., Flaisch, T., Eulitz C., Rockstroh B., Rothi, L.J.G., **Crosson, B.** (in press). Neural signatures of semantic and phonemic fluency in young and old adults. *Journal of Cognitive Neuroscience.*

Hu, J., Lee, J.-M., Gao, J., White, K. D., **Crosson, B.** (2008). Assessing a signal model and identifying brain activity from fMRI data by a detrending-based fractal analysis. *Brain Structure and Function*, **212**, 417-426.

Lee, J.-M., Hu, J., Gao, J., **Crosson, B.**, Peck, K. K., Wierenga, C. W., McGregor, K., Zhao, Q., White, K. D. (2008). Discriminating brain activity from task-related artifacts in functional MRI: Fractal scaling analysis simulation and application. *NeuroImage*, **40**, 196-212.

Wierenga, C. E., Benjamin, M., Gopinath, K., Perlstein, W. M., Leonard, C. M., Rothi, L. J. G., Conway, T., Cato, M. A., Briggs, R. W., **Crosson, B.** (2008). Age-related changes in word retrieval: Role of bilateral frontal and subcortical networks. *Neurobiology of Aging*, **29**, 436-451.

FitzGerald DB, Crucian GP, Mielke, JB, Shenal BV, Burks D, Womack KB, Ghacibeh G, Drago V, Foster PS, Valenstein E, Heilman KM. Effects of Donepezil on Semantic Processing and Verbal Memory in Healthy Older Adults. *Cognitive and Behavioral Neurology*, 2008, 21(2), 57-64.

Other Peer-Reviewed Journal Articles

Gopinath, K., **Crosson, B.**, McGregor, K., Peck, K. K., ChangY.-L., Moore, A., Sherod, M., Cavanagh, C., Wabnitz, A., Wierenga, C., White, K., Cheshkova, S., Krishnamurthy, V., Briggs, R. W. (in press). Selective detrending method for reducing task-correlated motion artifact during speech in event-related FMRI. *Human Brain Mapping.*

Rothi, L. J. G., Fuller, R., Kendall, D., Leon, S. A., Moore, A., Nadeau, S., Wu, S. S., **Crosson, B.**, Heilman, K. M. (in press). Errorless practice as a possible adjuvant to donepezil in Alzheimer's disease. *Journal of the International Neuropsychological Society.*

Crosson, B. (2008). An intention manipulation to change lateralization of word production in nonfluent aphasia: Current status. *Seminars in Speech and Language*, **29**, 188-200.

Conway, T. W., Heilman, K. M., Gopinath, K., Peck, K. K., Bauer, R. M., Briggs, R. W., Torgesen, J. K., & **Crosson, B.** (2008). Neural substrates related to auditory working memory comparisons in adult developmental dyslexics: An fMRI study. *Journal of the International Neuropsychological Society*, **14**, 629-639.

Dotson, V. M., Singletary, F., Fuller, R., Koehler, S., Bacon Moore, A., Rothi, L. J. G., **Crosson, B.** (2008). Treatment of word-finding deficits in fluent aphasia through the manipulation of spatial attention: Preliminary findings. *Aphasiology*, **22**, 103-113.

Crosson, B., McGregor, K., Gopinath, K. S., Conway, T. W., Benjamin, M., Chang, Y.-L., Bacon Moore, A., Raymer, A. M., Briggs, R. W., Sherod, M. G., Wierenga, C. E., White, K. D. (2007). Functional MRI of language in aphasia: A review of the literature and the methodological challenges. *Neuropsychology Review*, **17**, 157-177.

Crosson, B., Fabrizio, K. S., Singletary, F., Cato, M. A., Wierenga, C. E., Parkinson, R. B., Sherod, M. E., Bacon Moore, A., Ciampitti, M., Holiway, B., Leon, S., Rodriguez, A., Kendall, D. L., Levy, I. F., Gonzalez Rothi, L. J. (2007). Treatment of naming in nonfluent aphasia through manipulation of intention and attention: A phase 1 comparison of two novel treatments. *Journal of the International Neuropsychological Society*, **13**, 582-594.

Foster PS, Drago V, **FitzGerald DB**, Skoblar BM, Crucian GP, Heilman KM, Spreading Activation of lexical-semantic networks in Parkinson's Disease, Neuropsychologia, June 2008, 46(7), 1908-1914.

Drago V, Crucian GP, **FitzGerald DB**, Jeong Y, Finney GR, Heilman KM. Spatial attentional bias in normal people: object or viewer-centered. *Cortex*, 2008, 44(2), 196-199.

FitzGerald DB, Mitchell AL. Career Choices: The Fellowship Search. *Neurology*, 2008, 70(2): e5-8

FitzGerald DB, Drago V, Jeong Y, Heilman KM. Asymmetrical Alien Hands in Cortico Basal Ganglia Degeneration: Avoidance-Levitation and Tactile *Mitgehen. Movement Disorders*, 2007, 22(4): 581-584.

FitzGerald DB, Drago V, Sutherland D, Heilman, KM. Carbidopa/Levodopa responsive myoclonus. *Movement Disorders*, 2007, 22(3): 392-395

3. **Publications (other)**

Book Chapters Related to Other Topics

Leon, S. A., Nadeau, S. E., de Riesthal, M.,Crosson, B., Rosenbek, J. C., Gonzalez Rothi, L. J. (2008). Aphasia. In D. T. Stuss, G. Winocur, I. H. Robertson (eds.), *Cognitive Rehabilitation: Evidence and Application* (second edition). (pp. 435-448). New York: Cambridge University Press.

Crosson, B., Benjamin, M., Levy, I. (2007). Role of the basal ganglia in language and semantics: Supporting cast. In J. Hart, Jr. & M Kraut (eds.), *Neural Basis of Semantic Memory*, (pp. 219-243). New York: Cambridge University Press.

Crosson, B. (2007). Functional neuroimaging of impaired language: Aphasia. In J. DeLuca (Ed.) *Functional Neuroimaging in Impaired Populations*, (pp. 219-246). New York: Guilford.

4. **Presentations at scientific meetings**

Scientific Papers Related to Age-Related Memory Loss and/or McKnight Funding

Benjamin, M. L., McGregor, K. M., Chang, Y., White, K. D., Rackleman, C., Sherod, M., Levy, I., Crosson, B. (2008). Hemispheric asymmetry reductions in older adults during category exemplar generation. *International Neuropsychological Society: 36th Annual Meeting Program & Abstracts*, 156. (36th Annual International Neuropsychological Society Meeting, February 2008, Waikoloa, HI).

McGregor, K. M., Benjamin, M. L., Chang, Y., Zlatar, Z., Rackleman, C., Sherod, M., Levy, I., White, K. D., Crosson, B. (2008). Age group comparisons in category member generation tasks: Effects of task design. *International Neuropsychological Society: 36th Annual Meeting Program & Abstracts*, 172. (36th Annual International Neuropsychological Society Meeting, February 2008, Waikoloa, HI).

Other Scientific Papers

Kluger, B., McGregor, K., Drago, V., Benjamin, M., Mizuno, T., White, K., Crosson, B., Heilman, K. (2007). An fMRI study of the crossed response inhibition task in Parkinson's disease. *Journal of Cognitive Neuroscience*, **suppl.**, 101. (2007 Annual Meeting of the Cognitive Neuroscience Society, New York, NY).

Crosson, B., McGregor, K., Benjamin, M., Chang, Y.-L., Bacon Moore, A., Gopinath, K., Peck, K., Wierenga, C., Wabnitz, A., Gaiefsky, M., Soltysik, D., Cavanagh, C., Gonzalez Rothi, L., Briggs, R., White, K. (2007). Can manipulating intention change frontal lateralization for word production during aphasia treatment? *Journal of Cognitive Neuroscience*, **suppl.**, 255. (2007 Annual Meeting of the Cognitive Neuroscience Society, New York, NY).

Tyner, C. E., Deltoro, C., Gaiefsky, M. E., Klenberb, K., Levy, I., White, K. D., Crosson, B., Moore, A. B. (2007). Homographs: Bringing norms to present. *International Neuropsychological Society: 35th Annual Meeting Program & Abstracts*, 141. (35th Annual International Neuropsychological Society Meeting, February 2007, Portland, OR).

Moore, A. B., Li, Z., Tyner, C. E., Crosson, B. (2007). Frontal-subcortical circuits in verbal working memory: An fMRI study. *International Neuropsychological Society: 35th Annual Meeting Program & Abstracts*, 234. (35th Annual International Neuropsychological Society Meeting, February 2007, Portland, OR).

Altmann, L. J., Crosson, B., Mikell, E., Deltoro, C. M., Leon, S., Blonder, L. X., Gonzalez Rothi, L. J. (2007). Change in discourse quality following an attentional treatment for anomia. *International Neuropsychological Society: 35th Annual Meeting Program & Abstracts*, 255. (35th Annual International Neuropsychological Society Meeting, February 2007, Portland, OR).

Other National and International Addresses and Workshops

Crosson, B. (August, 2008). *A Model for Analyzing fMRI Data during Spoken Responses in Aphasia.* Presented at the National Institute on Deafness and Other Communication Disorders, Bethesda, Maryland, August 5, 2008.

Crosson, B. (May, 2008). *Left-Right Confusion in Aphasia Research: Which Hemisphere Supports Language in Aphasia?* Presented at the conference "Neural and Neuropsychological Correlates of Language," 29th Annual Conference of the New York Neuropsychology Group and Joint Meeting with the Psychology Section of the New York Academy of Sciences, Weill Medical College of Cornell University, New York, New York, May 17, 2008.

Crosson, B. (November, 2007). *Targeting Neuroplastic Change in Cognitive Rehabilitation: Can an Intention Manipulation in Aphasia Treatment Affect Lateralization?* Grand Rounds Presentation for the Department of Physical Medicine and Rehabilitation, Emory University, Atlanta, Georgia, November 6, 2007.

Crosson, B. (October, 2007). *An Intention Manipulation in the Treatment of Aphasia: Can Left-Hand Movement and Gesture Relateralize Language Production in Nonfluent Aphasia?* Lecture Presented at the Max Planck Institute for Psycholinguistics, Nijmegen, The Netherlands, October 10, 2007.

Crosson, B. (October, 2007). *Cognitive and Neural Substrates in Rehabilitation: Awareness and Intention.* Presented at the Conference in Cognitive Rehabilitation sponsored by the Netherlands Organization for Scientific Research (NWO), Zeist, The Netherlands, October 9, 2007.

Crosson, B. (September, 2007). *An Intention Manipulation for Treatment of Naming in Aphasia.* Presented at the 2nd Annual Eleanor M. Saffran Cognitive Neuroscience Conference, Temple University, Philadelphia, Pennsylvania, September 24, 2007.

Crosson, B. (February, 2007). *Functional Neuroimaging in Rehabilitation and Aphasia.* Workshop presented at the 35th Annual Meeting of the International Neuropsychological Society, Portland, Oregon, February 7, 2007.

5. **Presentations at public (non-scientific) meetings or events**

Crosson, B. (August, 2008). *Aging and the Brain.* Presentation to the Tuesday Rotary Club, Gainesville, Florida, August 12, 2008.

Crosson, B. (April, 2008). *Memory and Aging.* Presentation to weekly discussion at the Village Retirement Community, April 21, 2008.

FitzGerald, D. B. (February, 2008) Workshop: Dealing with Memory Disorders, Marion County (FL) Senior Services, 28 February 2008

6. **Awards (other)**

Crosson, B. Senior Research Career Scientist Award. Department of Veterans Affairs Rehabilitation Research and Development Service. January, 2009-December 2015.

Crosson, B. Honorary Professor, School of Health and Rehabilitation Sciences, University of Queensland, Brisbane, Australia (January 2005-present)

Crosson, B. Research Mentorship Award, University of Florida Department of Clinical & Health Psychology (October 2008)

7. **Faculty. Please include abbreviated CV with publications for previous 12 months**

Crosson's NIH Bio and FitzGerald's short CV are attached to end of this report.

8. **Trainees of Dr. Crosson**

* Indicates trainees whose current scientific work relates to age-related memory loss

Mentored Young Investigators

Tim W. Conway, Ph.D. Short- and Long-Term Reliability of fMRI in Aphasia. Department of Veterans Affairs, Rehabilitation Research and Development. Career Development Award (full salary and benefits + expenses). 2005-2008. Crosson Role: Primary Mentor.

*David B. FitzGerald, M.D. fMRI of Changes in Memory Systems in Neurologically Normal Older Adults Taking Aricept. Department of Veterans Affairs, Rehabilitation Research

and Development. Associate Investigator Award (full salary and benefits). 2006-2008. Crosson Role: Primary Mentor.

Post-doctoral

Stacy Harnish, Ph.D. Training in Treatment of Communication Disorders and Translational Neuroscience. Funded through the National Institute on Deafness and Other Communication Disorders. Grant 5-T32-DC008768-02, Leslie J. Gonzalez Rothi, Ph.D., PI. Harnish Fellowship 2008-2010. Crosson Role: Primary Mentor.

*Marcus Meinzer, Ph.D. The neural substrate of expressive language functions in healthy and pathological aging. Funded by the German Foundation for Science (DFG) DFG, ME 3161/2-1, (full salary and benefits). 2008-2010. Crosson Role: Primary Mentor.

Pre-doctoral

* Keith M. McGregor, M.S. Mitigation of the Loss of the Negative BOLD Response in Aerobically Fit Older Adults. Department of Veterans Affairs, Office of Educational Affairs Predoctoral Fellowship (full salary and benefits). 2008-2009. Crosson Role: VA Preceptor K. D. White, Dissertation Chair).

*Zvinka Zlatar. Normalization of BOLD Maps during Word-Retrieval in Aerobically Fit Older Adults. NIH Claude D. Pepper Older Americans Independence Center Minority Supplement Award. 2009-2011. Crosson Role: Primary Mentor, along with Marco Pahor.

Michelle Benjamin, M. S. Dissertation topic not yet selected. Crosson role: Doctoral Committee Chair.

Ilana F. Levy, M. S. Dissertation topic not yet selected. Crosson role: Doctoral Committee Chair.

Other

*Matthew Cohen, De-Differentiation of Brain Activity during Word Retrieval in Neurologically Normal Older Adults. Crosson Role: Masters Committee Chair.

9. **Clinical/translational programs**

New programs related to age-related memory loss

The neural substrate of expressive language functions in healthy and pathological aging. (Funded by a grant to Marcus Meinzer, Ph. D. from the German Foundation for Science to study in our laboratory for two years (see "Trainees" above). Crosson Role: Primary Mentor.

Mitigation of the Loss of the Negative BOLD Response in Aerobically Fit Older Adults. Supported by a VA Education Award Pre-Doctoral Award to Keith M. McGregor, M.S. (see "Trainees" above) and funds from the Department of Veterans Affairs Rehabilitation Research and Development, Brain Rehabilitation Research Center of Excellence (BRRC). Crosson Role: VA Preceptor.

Normalization of BOLD Maps during Word-Retrieval in Aerobically Fit Older Adults. Supported by a NIH Pepper Center Minority Supplement Award to Zvinka Zlatar (see "Trainees" above) and funds from the Department of Veterans Affairs Rehabilitation Research and Development, Brain Rehabilitation Research Center of Excellence (BRRC). Crosson Role: Primary Mentor, along with Marco Pahor.

Update on existing clinical studies

fMRI of Changes in Memory Systems in Neurologically Normal Older Adults Taking Aricept: Subject recruitment for this project has gone slowly; some of the reasons are described in item 20 of this report. However, an additional reason for slowed recruitment of subjects is that it has been difficult to find subjects who meet the criteria for both Aricept and MRI. We have been able to work with the IRB to relax criteria related to tinnitus, which has helped to some degree (see item 20 for details). We have enrolled or are prepared to enroll 11 of the 24 required subjects for the study.

Changes in Brain Activity during Word Retrieval for Neurologically Normal Older Compared to Younger Adults: Data analyses were ongoing during the last year. Two papers were presented at an international meeting in 2008 (see above), and an additional paper will be presented at the Annual Meeting of the International Neuropsychological Society in Atlanta in 2009. We plan to submit the manuscript on this study during the next 12 months.

10. **Technology transfer**

Patents applications

None

Revenue generated from technology

None

11. **Budget update (last year's budget and actual results - with an explanation of material variances)**

Status of matching funds

See "extramural funding" below.

Projected budget for coming year

From our original budget for the current project, we have spent the following:

For Personnel, we budgeted $20, 535, and spent $22,891, overspending this line item by $2,356.
For Scanning, we budgeted $49,600, and spent $25,000, leaving a remainder of $24,600.
For Pharmacy, we budgeted $415, and spent that amount, leaving no remainder.

Hence, subtracting the personnel overage from the scanning remainder, we should have $22,244 remaining for scanning. We request an extension of these funds through December 31, 2009 to complete the project.

Extramural funding

Dr. FitzGerald received an 2-year Associate Investigator award from the VA Rehabilitation Research and Development Service to perform the current M-47 project. Although the funding for that award ended in July 2008, he has received bridge funding from the BRRC, which currently pays his salary. He has been approved to begin a Career Development Award-2 from the VA Rehabilitation Research and Development Service in the spring of 2009. This award pays his full salary, and he will have enough time under this award to complete the current M-47 project. He has recruited a volunteer to help him with this project.

See "new clinical/translational programs" for a description of other extramural funding.

12. **Educational programs focusing on age related memory loss**

Scientific

None

Public

None

13. **Collaborative programs with other McKnight Institutes, institutions and research programs**

None

14. **Collaborative program with non McKnight Institutes, institutions and research programs**

The neural substrate of expressive language functions in healthy and pathological aging.
Supported by a grant to Marcus Meinzer, Ph. D. from the German Foundation for Science to study in our laboratory for two years (see "Trainees" above).

Mitigation of the Loss of the Negative BOLD Response in Aerobically Fit Older Adults. Supported by a VA Education Award Pre-Doctoral Award to Keith M. McGregor, M.S. (see "Trainees" above) and funds from the Department of Veterans Affairs Rehabilitation Research and Development, Brain Rehabilitation Research Center of Excellence (BRRC).

Normalization of BOLD Maps during Word-Retrieval in Aerobically Fit Older Adults. Supported by a NIH Pepper Center Minority Supplement Award to Zvinka Zlatar (see "Trainees" above) and funds from the Department of Veterans Affairs Rehabilitation Research and Development, Brain Rehabilitation Research Center of Excellence (BRRC).

15. **Briefly describe plans for future research and/or clinical initiatives**

New programs related to age-related memory loss

The neural substrate of expressive language functions in healthy and pathological aging. Supported by a grant to Marcus Meinzer, Ph. D. from the German Foundation for Science to study in our laboratory for two years (see "Trainees" above).

Mitigation of the Loss of the Negative BOLD Response in Aerobically Fit Older Adults. Supported by a VA Education Award Pre-Doctoral Award to Keith M. McGregor, M.S. (see "Trainees" above) and funds from the Department of Veterans Affairs Rehabilitation Research and Development, Brain Rehabilitation Research Center of Excellence (BRRC). Crosson Role: VA Preceptor.

Normalization of BOLD Maps during Word-Retrieval in Aerobically Fit Older Adults. Supported by a NIH Pepper Center Minority Supplement Award to Zvinka Zlatar (see "Trainees" above) and funds from the Department of Veterans Affairs Rehabilitation Research and Development, Brain Rehabilitation Research Center of Excellence (BRRC). Crosson Role: Primary Mentor, along with Marco Pahor.

16. **If applicable, please provide endowment investment results for the report period.**

N/A

17. **Were any funds used for a Prohibited Purpose during the report period?**

No

18. **Do you recommend any modification to the Purpose or mandates in the Gift Agreement?**

Yes, we recommend an extension of the second year of funding under M-47 through December 31, 2009. We have experienced many delays, described in detail under item 20 of this report,

which explain why the work on M-47 was not completed within the original year plus the extension. It will take another year to complete the project.

19. **Did all activities during the report period further the Purpose?**

Yes

20. **Please describe any negative events (loss of personnel, space, budget, etc.) that occurred during the report period and the possible impact on carrying out the Gift Agreement.**

Negative events affecting the current M-47 project have been of two kinds: events affecting the 3 T Philips Achieva MRI scanner used to scan humans and events related to safety, data security, and our IRB protocol. Both kinds of events are described below.

Events affecting the 3 T Philips Achieva MRI scanner

The purpose of the current M-47 project is to use functional MRI (fMRI) to determine what changes in brain systems activity account for improved memory in neurologically normal older adults taking a cholinergic agonist, the anticholinesterase, donepizil. Hence, we are dependent on the proper operation of this scanner and its peripheral equipment for running our experiment.

The first problem with the MRI scanner that delayed the start of the current M-47 project was delayed installation of Philips 3 T Achieva system. Originally, the scanner was due to be available in the fall of 2006, around the anticipated start date of the current project. Installation of this scanner was completed in January of 2007, and the scanner became available for general use in February of 2007. However, problems remained with fMRI usage, specifically synchronizing the timing of presentation of the stimuli to the start of the functional MRI runs. These problems were resolved by March 2007, rendering the instrument available to begin our pilot work at that time. Thus, the start time for our project was delayed about 6 months.

The scanner has experienced a few other problems. In 2008, cryogenic pumps failed twice as did gradient coil amplifiers, which slowed data acquisition somewhat. An uninterruptible power supply has been installed, which should help with electronics related reliability issues.

More importantly, however, the peripheral equipment purchased from a Philips subsidiary to present visual stimuli to subjects during fMRI has been problematic. In particular, the device used to display visual stimuli inside the scanner, a hood with a video display, has been both unreliable and unavailable during much of 2008. The hood was broken and unavailable from mid December 2007 to mid January 2008. The AMRIS facility was subsequently provided with a loaner hood, which helped with availability of in-scanner video display for a while. However, the loaner hood was returned in mid 2008, and intermittent problems continued. Starting in December of 2008, the scanner hood failed and was returned to the vendor, but has not been repaired yet. Current scanning is taking place using the vendor's loaner hood. An alternate in-house design for visual stimuli has been created by a member of Dr. Crosson's Brain Imaging, Rehabilitation and Cognition (BIRC) laboratory, Dr. Keith White, and installed in the magnet

room to reduce disruptions in scanning related to visual stimuli presentation. Other scanner-related software problems, related to licensing renewal, and hardware problems, specifically the scanner table, also kept the scanner inoperative from mid-December to the beginning of January.

Events related to safety, data security, and IRB protocol

One of the investigators on this project incurred a temporary loss of hearing in June of 2007 after an extended period of time in the MRI scanner while debugging the in-scanner presentation of stimuli for this study. This incident resulted in a one month pause in scanning, while the Institutional Review Board (IRB-01 at the University of Florida) reviewed the event. The risk of hearing damage is now much better understood and multiple procedural changes have been made to prevent a recurrence. This event and our interactions with the IRB led to exclusion of potential subjects with tinnitus from the IRB protocol, and any subjects who had experienced "ringing in their ears" were excluded. Unfortunately, a large proportion of potential normal older subjects have experienced "ringing in their ears" a few times during their life, and the protocol required that we exclude them. This exclusion criterion thus made it difficult to recruit older subjects, and we were excluding many more subjects than we were screening into the protocol. Dr. FitzGerald worked with the IRB to change the exclusion criterion to persons with persistent tinnitus, and it is easier to recruit subjects now, but dealing with this issue caused a delay of several weeks.

Finally, Dr. FitzGerald has been funded by a VA grant to perform the current M-47 study, and as a result, he is a VA employee. In the spring of 2007, data security issues arose in the Veterans Administration. Because of the volume of data we handle and our need to transfer images from the scanner to our image processing computers, we work on the UF network. The VA network is not capable of handling our volume of data or image transfer. This arrangement was questioned, and it delayed VA approval of the protocol for the current M-47 study starting in March of 2007 for approximately 3 months.

The impact of all these negative events has been to impede progress on scanning research subjects, as development of the protocols has been complete for several months. The cumulative delays are perhaps a year or more. However, we have successfully dealt with each obstacle and anticipate that we can complete the protocol in another year.

21. **Please provide any general comments or thoughts not covered elsewhere – a response is not required. Please respond only if you would like to add something not otherwise covered elsewhere.**

I am not certain what the current model for these McKnight grants is. In the past, I know the model was to provide investigators with money to perform a single study, with the expectation that the investigators would convert a portion of their research portfolio to memory and aging research. I have noticed that at least some investigators have been unable to make this conversion with a single study. In my own experience, it has taken more effort and support than a single study to make this conversion. However, the projects in memory and aging that we are on the verge of starting (see description of new projects above) have solid funding underneath them and indicate that a significant portion of our research portfolio is now dedicated to memory and aging. Further, this portion of our portfolio is well integrated with our other research

programs. If the MBI has not already done so, I would suggest that you consider longer term models of funding if you are attempting to get promising investigators to convert a portion of their research portfolios to memory and aging.

22. **Signature, date and title of person submitting the report.**

2/3/09

Bruce Crosson, Ph.D., Professor — Date

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Crosson, Bruce	Professor of Clinical and Health Psychology
eRA COMMONS USER NAME BCROSSON	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Southern Methodist University	B.A.	1968-1972	Political Science
Southern Methodist University	M.A.	1972-1974	Psychology
Texas Tech University	Ph.D.	1974-1978	Psychology

A. Positions and Honors

Positions

1978-1983 Staff Psychologist, VA Medical Center, Leavenworth, KS
1983-1985 Director, Clinical Neuropsychology, VA Medical Center, Oklahoma City, OK
1983-1985 Assistant Professor of Psychiatry and Behavioral Sciences at University of Oklahoma Health Sciences Center, Oklahoma City, OK
1985-1989 Director, Head Injury Resource Center at Irene Walter Johnson Rehabilitation Institute, St. Louis, MO
1985-1989 Research Assistant Professor (1985-1989)/Research Associate Professor (1989) of Neurology, Psychiatry, and Psychology at Washington University Medical Center, St. Louis, MO
1989-present Associate Professor (1989-1994)/Professor (1994-present), Department of Clinical and Health Psychology, University of Florida, Gainesville, FL
1995(Sep-Dec) Visiting Professor, Section of Neuropsychology, Medical College of Wisconsin
1996-2000 Associate Chair for Research Department of Clinical and Health Psychology, University of Florida,Gainesville, FL

Selected Honors

1995 Fellow American Psychological Association (Division of Clinical Neuropsychology)
1996-2001 Board of Directors, American Board of Clinical Neuropsychology
1998-2001 VA Merit Review Subcommittee for Mental Health and Behavioral Sciences
2000-2003 University of Florida Research Foundation Professor
2001-present Assoc. Editor and Dept. Editor, Journal of the International Neuropsychological Society
2002-2005 Board of Directors, International Neuropsychological Society
2005-2010 Honorary Professor, Sch. of Health and Rehab. Sci., Univ. of Queensland, Brisbane, Australia

B. Selected peer-reviewed publications (from 85 refereed journal articles)

Crosson B, Cato MA, Sadek JR,Gökçay D, Bauer RM, Fischler IS, Maron L, Gopinath K, Auerbach EJ, Browd SR, Briggs RW (2002) Semantic Monitoring of Words with Emotional Connotation during fMRI: Contribution of Anterior Left Frontal Cortex. *Journal of the International Neuropsychological Society* 8:607-622.

Lu LH, **Crosson B**, Nadeau SE, Heilman KM, Rothi LJG, Raymer A, Gilmore RL, Bauer RM, Roper SN (2002) Category-Specific Naming Deficits for Objects and Actions: Semantic Attribute and Grammatical Role Hypotheses. *Neuropsychologia* 40:1608-1621.

Richards K, Singletary F, Rothi LJG, Koehler S, **Crosson B** (2002) The Activation of Intentional Mechanisms through Utilization of Nonsymbolic Movements in Aphasia Rehabilitation. *Journal of*

Rehabilitation Research and Development 39:445-454.

Crosson B, Benefield H, Cato MA, Sadek JR, Moore AB, Wierenga CE, Gopinath K, Soltysik D, Bauer RM, Auerbach EJ, Gökçay D, Leonard CM, Briggs RW (2003) Left and Right Basal Ganglia and Frontal Activity during Language Generation: Contributions to Lexical, Semantic, and Phonological Processes. *Journal of the International Neuropsychological Society* 9:1061-1077.

Glueckauf RL, Blonder LX, Ecklund-Johnson E, Maher L, **Crosson B**, Rothi LG (2003) Functional Outcome Questionnaire for Aphasia: Overview and preliminary psychometric evaluation. *Neurorehabilitation* 18:281-290.

King TZ, Fennell E, Williams L, Algina J, Boggs S, **Crosson B**, & Leonard C. (2004) Verbal memory abilities of children with brain tumors. *Child Neuropsychology*, 10, 76-88.

Peck KK, Wierenga CE, Bacon Moore A, Maher M, Gopinath K, Gaiefsky M, Briggs RW, & **Crosson B**. (2004). Comparison of Baseline Conditions to Investigate Syntactic Production using Functional Magnetic Resonance Imaging. *NeuroImage* 23:104-110.

Soltysik DA, Peck KK, White KD, **Crosson B**, Briggs RW (2004) Comparison of Hemodynamic Response Nonlinearity across Primary Cortical Areas. *NeuroImage* 22:1117-1127.

Cato MA, **Crosson B**, Gökçay D, Soltysik D, Wierenga C, Gopinath K, Himes N, Belanger H, Bauer RM, Fischler IS, Gonzalez Rothi L, Briggs RW (2004) Processing Words with Emotional Connotation: An fMRI Study of Time Course and Laterality in Rostral Frontal and Retrosplenial Cortices. *Journal of Cognitive Neuroscience* 16:167-177.

Peck KK, Moore AB, **Crosson B**, Gaiefsky M, Gopinath KS, White K, Briggs RW (2004) Pre and Post fMRI of an Aphasia Therapy: Shifts in Hemodynamic Time to Peak during an Overt Language Task. *Stroke* 35:554-559.

Crosson B, Bacon Moore A, Gopinath K, White KD, Wierenga CE, Gaiefsky ME, Fabrizio KR, Peck KK, Soltysik D, Milstead C, Briggs RW, Conway TW, Rothi LJG. (2005). Role of the Right and Left Hemispheres in Recovery of Function during Treatment of Intention in Aphasia. *Journal of Cognitive Neuroscience* 17:392-406.

Blonder LX, Heilman KM, Ketterson T, Rosenbek J, Raymer A, **Crosson B**, Maher L, Glueckauf R, Rothi LG. (2005). Affective facial and lexical expression in aprosodic versus aphasic stroke patients. *Journal of the International Neuropsychological Society* 11:677-85.

Wierenga CE, Maher LM, Bacon Moore A, White KD, McGregor K, Soltysik DA, Peck KK, Gopinath, KS, Singletary F, Rothi LJG, Briggs RW, **Crosson B**. (2006) Neural Substrates of Syntactic Mapping Treatment: An fMRI Study of Two Cases. *Journal of the International Neuropsychological Society* 12:132-146.

Grande LJ, **Crosson B**, Bauer RM, Heilman KM, KilduffP, McGlinchey R. (2006). Visual Selective Attention in Parkinson's Disease: Dissociation of Exogenous and Endogenous Inhibition. *Neuropsychology* 20:370-382.

Cato Jackson MA, **Crosson B**. (2006). Emotional connotation of words: Role of emotion in distributed semantic systems. *Progress in Brain Research*, 156, 2006:205-216.

Crosson B, McGregor K, Gopinath KS, Conway TW, Benjamin M, Chang Y-L, Bacon Moore A, Raymer AM, Briggs RW, Sherod MG, Wierenga CE, White KD. (2007) Functional MRI of language in aphasia: A review of the literature and the methodological challenges. *Neuropsychology Review* 17:157-177.

Crosson B, Fabrizio KS, Singletary F, Cato MA, Wierenga CE, Parkinson RB, Sherod ME, Bacon Moore A, Ciampitti M, Holiway B, Leon S, Rodriguez A, Kendall DL, Levy IF, Gonzalez Rothi LJ. (2007) Treatment of naming in nonfluent aphasia through manipulation of intention and attention: A phase 1 comparison of two novel treatments. *Journal of the International Neuropsychological Society*, 13:582-594.

Dotson VM, Singletary F, Fuller R, Koehler S, Bacon Moore A, Rothi LJG, **Crosson B**. (2008). Treatment of word-finding deficits in fluent aphasia through the manipulation of spatial attention: Preliminary findings. *Aphasiology* 22:103-113.

Wierenga CE, Benjamin M, Gopinath K, Perlstein WM, Leonard CM, Rothi LJG, Conway T, Cato MA, Briggs, RW, **Crosson B**. (2008). Age-related changes in word retrieval: Role of bilateral frontal and subcortical networks. *Neurobiology of Aging*, 29:436-451.

Lee J-M, Hu J, Gao J, **Crosson B**, Peck KK, Wierenga CE, McGregor K, Zhao Q, White KD. (2008). Discriminating brain activity from task-related artifacts in functional MRI: Fractal scaling analysis simulation and application. *NeuroImage*, 40:196-212.

Hu J, Lee J-M, Gao J, White KD, **Crosson B**. (2008). Assessing a signal model and identifying brain activity from fMRI data by a detrending-based fractal analysis. *Brain Structure and Function*, 212:417-426.

Conway TW, Heilman KM, Gopinath K, Peck KK, Bauer RM, Briggs RW, Torgesen JK, **Crosson B**. (2008). Neural substrates related to auditory working memory comparisons: An fMRI study. *Journal of the*

International Neuropsychological Society, 14:629-639.

Crosson B. (2008). An intention manipulation to change lateralization of word production in nonfluent aphasia: Current status. *Seminars in Speech and Language*, 29:188-200.

Gopinath K, **Crosson B**, McGregor K, Peck KK, Chang Y-L, Moore A, Sherod M, Cavanagh C, Wabnitz A, Wierenga C, White K, Cheshkova S, Krishnamurthy V, Briggs RW. (in press). Selective detrending method for reducing task-correlated motion artifact during speech in event-related FMRI. *Human Brain Mapping*.

Rothi LJG, Fuller R, Kendall D, Leon SA, Moore A, Nadeau S, Wu SS, **Crosson B**, Heilman KM. (in press). Errorless practice as a possible adjuvant to donepezil in Alzheimer's disease. *Journal of the International Neuropsychological Society*.

Meinzer M, Wilser L, Flaisch T, Eulitz C, Rockstroh B, Rothi LJG, **Crosson B**. (in press). Neural signatures of semantic and phonemic fluency in young and old adults. *Journal of Cognitive Neuroscience*.

Wierenga, C. E., Perlstein, W. M., Benjamin, M., Leonard, C., Rothi, L. G., Conway, T., Cato, M. A., Gopinath, K., Briggs, R., **Crosson, B**. (in press). Neural substrates of object identification: fMRI evidence that category and visual attribute contribute to semantic knowledge. *Journal of the International Neuropsychological Society*.

C. Research Support

Ongoing Research Support

VA549-P-0027 (TO 4.15) Crosson, B. (PI) 7/11/08-7/10/10
VA/University of Southwestern Medical Center Subcontract
Complex Verbal Functions and the Basal Ganglia in Gulf War Illness of the Neuroimaging and Biomarkers Studies.
This contract uses fMRI, semantic priming, and neuropsychological to determine whether Haley Syndrome 2 Gulf War illness is cognitively more similar to Parkinson's disease, basal ganglia stroke, or frontal stroke.
Role: PI

R01 DC007387 Crosson (PI) 9/1/06-8/31/09
NIH/NIDCD
Treatment of Intention in Aphasia: Neural Substrates
This grant uses fMRI to determine if an intention treatment for aphasia shifts lateralization of language production in frontal structures, as it was designed to do.
Role: PI

B3470S Crosson (PI) 1/1/04-12/31/08
VA/RR&D
Research Career Scientist Award: This is a salary award to the PI to sustain and enhance the VA research career of the awardee.
Role: PI

B3149C Rothi (Center Director), Crosson (Coordinator) 6/01/99-5/31/09
VA/RR&D
Center of Excellence Grant: Brain Rehabilitation Research Center
This grant provides infrastructure to leverage other grant support in the area of brain rehabilitation.
Role: Coordinator of Functional Neuroimaging Core

Project M 47 Crosson (PI) 7/01/05-6/30/08
E. F. McKnight Brain Research Investigator-Initiated Grant Program of the MBI-UF
Functional Neuroimaging of Memory in Healthy Aging
This study uses fMRI to determine the neural substrates of memory in normal older adults and what treatments can be used to address memory loss in normal aging
Role: PI

Industry sponsored trial Fernandez (PI), Crosson (Co-PI) 7/01/07-6/30/08
Novartis Pharmaceuticals

Rivastigmine for Treatment of Visual Hallucinations in PD
This trial uses fMRI to determine if decrease in hallucinations in Parkinson's disease patients on the drug Rivastigmine is accompanied by changes in visual cortex activity.
Role: Co-Investigator

Completed Support

DAM17-98-1-8616 Heilman (PI), Crosson (Co-I) 9/01/98-8/31/06
DoD
Cognitive Changes in Presymptomatic Parkinson's Disease
The purpose of this project is to develop cognitive tests that predict the onset of motor symptoms in presymptomatic Parkinson's disease patients.
Role: Co-Investigator

P50 03888 Rothi (Program Director), Crosson (PI) 6/01/00-5/31/05
NIH/NIDCD
Treatment of Aphasia and Related Disorders: Subproject 5. Treatment of Intention and Attention in Aphasia
This subproject is a clinical trial of two new treatments for aphasia.
Role: Subproject PI

P50 03888 Rothi (Program Director), Crosson (PI) 6/01/00-5/31/05
NIH/NIDCD
Treatment of Aphasia and Related Disorders: Core B. Neuroimaging
This core uses fMRI to examine neural plasticity during the various treatments studied in the program project grant.
Role: Neuroimaging Core PI (6/01/03-5/31/05) Co-PI (6/01/00-5/31/03)

Project M28 White (PI), Crosson (Co-PI) 12/16/03-2/31/04
E. F. McKnight Brain Research Investigator-Initiated Grant Program of the MBI-UF
Techniques enabling longitudinal fMRI studies of age-related memory changes
The purpose of this project is to develop nonlinear dynamical systems analysis techniques to identify active voxels and eliminate motion artifacts from fMRI scans.
Role: Co-PI

McKnight Grant Crosson (PI) 4/01/02-7/31/03
McKnight Brain Institute of the University of Florida
Changes in Word Retrieval in Normal Aging: Executive-Frontal vs. Semantic-Temporal Substrates
This study uses fMRI to determine the neural substrates of word-finding difficulty in normal older adults
Role: PI

R01 03455 Crosson (PI) 8/01/97-7/31/01
NIH/NIDCD
Medial Frontal Cortex in Intentional Aspects of Language
The studies in this grant used fMRI to explore the role of medial frontal cortex in language initiation.
Role: PI

Brooks Grant Crosson (PI) 9/01/98-8/31/00
Brooks Health Foundation, Jacksonville, FL
Treatment of Intention and Attention in Aphasia
The purpose of this grant was to gather pilot data on two new treatments of aphasia for federal grant applications.
Role: PI

David B. FitzGerald, MD

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Home
5960 SW 35th Way
Gainesville, FL 32608
(352) 376-8199

Career Goals

Develop skills in describing and diagnosing higher order cognitive dysfunction and develop ways of characterizing location of function as well as type of dysfunction by using structural and functional imaging techniques.

Education

University of Massachusetts Medical School, Worcester, MA (MD) 1996-2000
Graduate Division of the Wharton School of Commerce and Finance 1978-1980
University of Pennsylvania, Philadelphia, PA (MBA)
Lafayette College, Easton PA (BSEE) 1972-1976

Certification and Licensure

Unrestricted medical license from the State of Florida
Diplomate American Board of Psychiatry and Neurology #54463

Professional Experience

Courtesy Assistant Clinical Professor-Neurology University of Florida 2006-present
Associate Investigator, Research Division
Malcom Randall VAMC Gainesville, FL 2006-2008
Staff Physician-Neurology Service, Associate Investigator-Research Service
Malcom Randall VAMC Gainesville, FL 2008-present

Neurology Fellow, University of Florida Medical School Gainesville, FL 2004-2006
Neurology Resident, University of Massachusetts Memorial Medical Center,
Worcester, MA 2001-2004

Medical Intern, St. Elizabeth's Medical Center Brighton, MA 2000-2001

Researcher, NMR Center, Massachusetts General Hospital
Supervised by Bruce R. Rosen MD, PhD and Randall R. Benson, MD focusing on language lateralization and localization using fMRI with clinical correlation

Charlestown, MA1995-1996

Employee, Analog Devices Inc. 1980-1994

Regional Marketing Manager, Mixed Signal ASICS

Responsible for all aspects of business generation of Mixed Signal ASICS for Eastern US and Japan. Wilmington, MA 1991-1994

National Marketing Manager, Linear CMOS Products

Responsible for all aspects of $50M business. Wilmington, MA 1989-1991

Regional Marketing Manager, Linear CMOS Products

Supported standard products designed and manufactured in Ireland.
Wilmington, MA 1983-1989

Senior Product Marketing Engineer

Supported day-to-day product marketing activities of division to sales forces in Germany and US and Japan Limerick, Ireland 1981-1983

Marketing Analyst

Supported Analog Devices Enterprises (internal corporate venture capital fund) in identifying and evaluating venture capital investments. Norwood, MA 1980-1981

Publications

Journal articles:

Parkinson, RB, Raymer A, Chang, Y-L, FitzGerald DB, Crosson B, Lesion Characteristics Related to Treatment Improvement in Object and Action Naming for Patients with Chronic Aphasia, Brain and Language, in-revision

FitzGerald DB, Crucian GP, Mielke, JB, Shenal BV, Burks D, Womack KB, Ghacibeh G, Drago V, Foster PS, Valenstein E, Heilman KM, Effects of Donepezil on Semantic Processing and Verbal Memory in Healthy Older Adults, Cognitive and Behavioral Neurology, June 2008, 21(2), 57-64 (PMID 18541979)

Foster PS, Drago V, FitzGerald DB, Skoblar BM, Crucian GP, Heilman KM, Spreading Activation of lexical-semantic networks in Parkinson's Disease, Neuropsychologia, June 2008, 46(7), 1908-1914 (PMID 18325544)

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Drago V, Crucian GP, FitzGerald DB, Finney GR, Mizuno T, Pisani F, Heilman KM, Ipsilesional attentional-approach, neglect or cross-over effect? Case-report, Neurocase, August 2006, 12(4): 207-211 (PMID 17000588)

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Posters/Abstracts:

FitzGerald, DB, Crucian, GP, Mielke, J, Valenstein, E, Heilman, KW, The effect of donepezil on memory in older normal subjects, University of Florida College of Medicine Research Day, 20 March 2007, Gainesville, FL

FitzGerald, DB, Crucian, GP, Mielke, J, Valenstein, E, Heilman, KW, The effect of donepezil on memory in older normal subjects, 2nd Annual Neuromuscular Plasticity Symposium, 1 December 2006, Gainesville, FL

FitzGerald, DB, Crucian, GP, Mielke, J, Valenstein, E, Heilman, KW, The effect of donepezil on memory in older normal subjects, 1st Florida Residents and Fellows Research Day, September 23 2006, Orlando, FL, Poster 19

FitzGerald, DB, Crucian, GP, Mielke, J, Valenstein, E, Heilman, KW, The effect of donepezil on memory in older normal subjects, 58th annual meeting of the American Academy of Neurology, April 2006, San Diego, P06.071

Drago, V, Crucian, G P., FitzGerald, DB., Mizuno, T, Pisani, F, Jeong, Y, Finney, GR., Heilman, KM., Spatial Attentional Bias in Normal People: object or viewer-centered, 58th annual meeting of the American Academy of Neurology, April 2006, San Diego,

Drago, V, Crucian, G P., FitzGerald, DB., Mizuno, T, Pisani, F, Jeong, Y, Finney, GR., Heilman, KM., Spatial Attentional Bias in Normal People: object or viewer-centered, 34th annual meeting of the International Neuropsychological Society, February 2006, Boston

Tsao JW, Efros DB, Knight AM, Jeong Y, FitzGerald DB, Heilman KM, Hydrocephalic ideomotor apraxia, 18th World Congress of Neurology, 5-11 November 2005, Sydney, Australia

Jeong Y, Efros, DB, Tsao JW, Mizuno T, Finney GR, FitzGerald DB, Heilman KM, Callosal Neglect in Hydrocephalic Patients, Poster P03.021, 57th Annual Meeting AAN, Miami Beach, FL, 9-16 April 2005

FitzGerald DB, O'Reilly C, Buchbinder BR, Paskavitz, J, Komolgorov-Smirnov (KS) vs. t tests, which is more sensitive for detection of language? Senior Scholar Research Program, University of Massachusetts Medical School, June 2000

Letter to the editor:
Neurology Overseas- Other resources, Neurology Today, December 2004 Letter to the Editor: "How will we solve our traffic problems?", Gainesville Sun, 14 March 2005
Letter to the Editor: "Trials and Tribulations of ASPJ", Aviation Week,
Letter to the Editor: "Saunders Associates", IEEE Spectrum 1981?

***ad hoc* reviewer**

European Journal of Neurology	November 2007
Neuroimage	October 2001
Cerebral Cortex	May 2007

Awards and Honors

Career Development Award-2 "Diffusion Tensor Imaging of White Matter After Traumatic Brain Injury" B6698W Veterans Health Administration (4 year award) (full salary & research support)	13 Nov 2008
Career Development Award-1 "Effects of Cholinergic State on Semantic Memory in the Elderly: An fMRI Investigation into Anatomic-Functional Relationships of Semantic Memory" E4733-H Veterans Health Administration (2 year award) (full salary support)	1 July 2006
Systems-Based Practice Competency Award (University of Massachusetts Medical School Neurology Department)	June 2003 and June 2004
The Nickols Award "For exemplary scholarship, teaching and compassionate care in the Neurology Residency, in the tradition of Jess Nickols, MD"	June 2004
Resident Travel Award- American Academy of Neurology	2003 and 2004
Resident Travel Award- National Epifellows Foundation	2004

Nominated for Intern of the Year at St. Elizabeth's Medical Center	2000
Volunteer- Massachusetts General Hospital 2000+ hours	1996

Professional Society Memberships

Society for Behavioral and Cognitive Neurology	2005-present
Massachusetts Medical Society	2000-present
American Academy of Neurology	2002-present
Institute of Electrical and Electronic Engineers	1973-present

Presentations

Psychiatry Grand Rounds- fMRI for pre-surgical planning 1999
University of Virginia-Department of Neurology
"Semantic Memory in the Elderly" 2007

Invited Talks

Workshop: Dealing with Memory Disorders, Marion County (FL) Senior Services 28 February 2008

Committees

3T users group- chair- University of Florida/Malcom Randall VAMC	2007-present
Graduate Medical Education Committee- UMMHC, Worcester	2002-2004
Clinical Information Systems Committee- UMMHC, Worcester	2003-2004

Quoted in

Conversations on Networking, The Newsletter. May 2007 and June 2007, Smolinsky S. and Keenan K.

Photography/Imaging

- Photo used in Tikkun, "Neuroscience and Fundamentalism", Kenneth M. Heilman and Russell S. Donda, September 2007
  htttp://www.tikkun.org/magazine/tik0709/frontpage/neuroscience/base_view
- Figure 4 from FitzGerald, et. al AJNR, 1997 used in Neuroimaging, William W. Orrison, editor, 2000, W.B. Saunders, Philadelphia, Figure 4-20, page 76

Miscellaneous

Read, write, speak Japanese at intermediate level.

Annual Report-McKnight Brain Research Foundation
Sponsored Institutes and Research Programs
(Include activity of all McKnight supported faculty and trainees)
Report Period: January 1 – December 31, 2008

Some gift agreements require both Institute reports and Chair reports. If applicable, please clearly state whether a particular response relates to a Chair or Institute.

This report relates to the Evelyn F. McKnight Chair, Thomas Foster.

Any capitalized terms used on the template are intended to have the same meaning as the term is defined in the Gift Agreement.

1. Summary of scientific achievements since last report

Viral vector gene therapy: Two manuscripts were published using gene delivery as a treatment for neural dysfunction. The results from the first study show that delivery of the alpha estrogen receptor (ER□) to ER□ knockout mice (Foster et al., 2008). The results indicate that ER□ is required to obtain the full spectrum of rapid estrogenic effects in the hippocampus of young adults. In addition, we show that constitutive expression of ERα in the adult can support hippocampus-dependent behavior, an observation that suggests that cognitive deficits associated with impaired ERα function are not entirely due to the absence of ERα activity during development. Our findings, together with recent reports on estrogen's mechanisms of action during aging, suggest that ERα is important for sustaining hippocampus-dependent memory, possibly through indirect mechanisms involving the maintenance of cell health. Finally, the improvement in behavior was observed in the absence of estrogen treatments indicating that, at least for young animals, the beneficial effects can result from ligand-independent activity or through locally generated estrogen. The results suggest that treatments to enhance the signaling cascades may provide a possible alternative to current hormone treatments for age-related memory decline. Current studies are focusing on gene regulation by ER□ and ER□ across the lifespan. The second study was performed as part of collaborations with individuals in the ARB and examined the ability of gene therapy to delay retinal degeneration (Pang et al., 2008).
Regulation of neuronal health during aging: A study has been published which examined a novel mechanism for regulating the vulnerability of hippocampal neurons with aging (Jackson et al., 2008). Our data suggest that regional differences in the activity of the PI3K/AKT cell survival pathway may contribute to regional differences in hippocampal vulnerability and implicate a novel molecule, PHLPP1, as a potential target for therapeutic intervention to improve hippocampal health. Currents studies are examining the signaling pathways regulating PHLPP1.

2. Publications in peer reviewed journals

Foster, T.C., Rani, A., Kumar, A., Cui, L. and Semple-Rowland, S.L. (2008) Viral vector mediated delivery of estrogen receptor-alpha to the hippocampus improves spatial learning in adult estrogen receptor-alpha knockout mice. Molecular Therapy, 16: 1587-93.

Pang, J., Sanford, L.B., Kumar, A., Dinculescu, A., Deng, W., Li, J., Li, Q., Rani, A., Foster, T.C., Chang, B., Hawes, N.L. Boatright, J.H., Hauswirth, W.W. (2008) AAV-mediated gene therapy delays retinal degeneration in the *rd10* mouse containing a

recessive PDEß mutation. Investigative Ophthalmology & Vision Science, 49: 4278-4283.

Jackson, T.C., Rani, A., Kumar, A., and Foster, T.C. (2008) Nuclear Hippocampal PHLPP1 Correlates with Nuclear pAKTSer473 Across Lifespan: Implications for CA3-CA1 Vulnerability with Aging. Cell Death and Differentiation, in press.

3. Publications (other)

4. Presentations at scientific meetings

Forum for European Neuroscience, Geneva Switzerland. Threshold changes in plasticity: Relation to memory decline.

Barshop Institute for Longevity and Aging Studies, UTHSCSA, San Antonio, TX. From molecules to senescent physiology: Relating brain aging to cognitive decline.

5. Presentations at public (non-scientific) meetings or events

6. Awards (other)

7. Faculty. Please include abbreviated CV with publications for previous 12 months

8. Trainees
Post doctoral
Ashok Kumar
Zane Zaire

Pre-doctoral
Christina Aenlle
Travis Jackson
Karthik Bodhinathan
Wei-Hua Lee

Other
Asha Rani

9. Clinical/translational programs
New programs
Update on existing clinical studies

10. Technology transfer
Patents applications
UF disclosure # 12975 "Methods for Treatment of Memory Dysfunction"
The Office of Technological License is currently conducting "patentability and marketability study of our technology"; based on which they will decide whether to patent or waive rights to our technology.

Revenue generated from technology NA

11. Budget update (last year's budget and actual results - with an explanation of material variances)
Status of matching funds, if applicable
Projected budget for coming year

Extramural funding

12. Educational programs focusing on age related memory loss
Scientific
Public

13. Collaborative programs with other McKnight Institutes, institutions and research programs

We have started a study with Caterina Maria Hernandez, Ph.D., who was in Dr Sweatt's lab and has since moved to take a position as a Postdoctoral Fellow at the University of Texas Medical Branch, Department of Neurology.

14. Collaborative program with non McKnight Institutes, institutions and research programs

We have started a study with Caterina Maria Hernandez, Ph.D., who was in Dr Sweatt's lab and has since moved to take a position as a Postdoctoral Fellow at the University of Texas Medical Branch, Department of Neurology.

15. Briefly describe plans for future research and/or clinical initiatives

1) Studies are designed to test the hypothesis that general lifestyle factors which delay or reverse cognitive decline (mental or physical activity, caloric restriction) will modify biological markers of aging. Other studies are designed to test the hypothesis that oxidative stress underlies Ca2+ dysregulation, senescent physiology, and memory decline during aging. In some cases the research examines gene therapy as a means for rescue of age-related cognitive decline.

2) Studies are designed to test the hypotheses that estrogen influences transcription of functionally associated genes involved in brain aging. In addition the studies will determine which hippocampal genes are linked to estrogen receptor activity.

3) Studies are designed to identify behavioral, biochemical and molecular biomarkers of healthy cognitive aging. This study is in collaboration with Dr Omerod at the University of Florida and a grant has been submitted to NIH.

4) On going collaborative studies with in the University of Florida include:
a) Studies of Anorexin as a treatment of age-related memory decline (Dr King).
b) Studies of diet on age-related memory decline and neurodegenerative diseases (Drs Carter, Notterpek, Leuwenburgh).
c) Development of behavioral tests for longitudinal investigation of age-related memory decline (Dr Carter).

16. If applicable, please provide endowment investment results for the report period.

17. Where any funds used for a Prohibited Purpose during the report period?

No

18. Do you recommend any modification to the Purpose or mandates in the Gift Agreement?

No

19. Did all activities during the report period further the Purpose?

Yes

20. Please describe any negative events (loss of personnel, space, budget, etc.) that occurred during the report period and the possible impact on carrying out the Gift Agreement.

21. Please provide any general comments or thoughts not covered elsewhere – a response is not required. Please respond only if you would like to add something not otherwise covered elsewhere.

22. Signature, date and title of person submitting the report.

CURRICULUM VITAE

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NATIONAL AND INTERNATIONAL PROFESSIONAL ACTIVITY

- Reviewer ZAG1-ZIJ-05 11/14/2008
- Reviewer ZRG1-IFCN-B-02M 1/28/2008

NATIONAL AND INTERNATIONAL SPEAKING ENGAGEMENTS

International

2008 Forum for European Neuroscience, Geneva Switzerland. Threshold changes in plasticity: Relation to memory decline.

National

2008 Barshop Institute for Longevity and Aging Studies, UTHSCSA, San Antonio, TX. From molecules to senescent physiology: Relating brain aging to cognitive decline.

CURRENT EXTRAMURAL GRANT SUPPORT (2008)

As Principal Investigator

NIA R01 AG14979 Mechanisms of altered synaptic function during aging 6/01/07 to 5/31/2012, $184,500

NIMH R01 MH59891 Estrogen and cognition over the lifespan, 3/16/05 to 3/15/10, $1,250,000.

As Co-Investigator

NINDS RO1 NS041021 (Notterpek PI) Cellular events in heritable peripheral neuropathies 07/01/05 to 06/30/2010, $225,000.

As Preceptor

NIA T32 (Meyer PI, Scarpace Co-PI) Training in Neurobiology of Aging. 5/1/06 to 4/30/11, $1,183,067 direct costs.

Submitted grants currently under review
Apoptosis and Life-Long Caloric Restriction (Leeuwenburgh PI) 4/01/2009 to 3/31/2014
$133,901 direct costs over the 5 yr period.

Biomarkers of healthy cognition across the lifespan. (Foster PI) 7/01/2009 to 6/30/2013

PUBLICATIONS (60 peer reviewed manuscripts, 10 chapters/reviews)

ARTICLES

Foster, T.C., Rani, A., Kumar, A., Cui, L. and Semple-Rowland, S.L. (2008) Viral vector mediated delivery of estrogen receptor-alpha to the hippocampus improves spatial learning in adult estrogen receptor-alpha knockout mice. Molecular Therapy, 16: 1587-93.

Pang, J., Sanford, L.B., Kumar, A., Dinculescu, A., Deng, W., Li, J., Li, Q., Rani, A., **Foster,** T.C., Chang, B., Hawes, N.L. Boatright, J.H., Hauswirth, W.W. (2008) AAV-mediated gene therapy delays retinal degeneration in the *rd10* mouse containing a recessive PDEβ mutation. Investigative Ophthalmology & Vision Science, 49: 4278-4283.

Jackson, T.C., Rani, A., Kumar, A., and **Foster**, T.C. (2008) Nuclear Hippocampal PHLPP1 Correlates with Nuclear pAKTSer473 Across Lifespan: Implications for CA3-CA1 Vulnerability with Aging. Cell Death and Differentiation, in press.

Bodhinathan, K., Kumar, A., Jackson, T.C. and **Foster**, T.C. Decreased NMDA receptor function during aging is mediated by oxidative stress effects on CaMKII activity. Submitted.

Aenlle, K., and **Foster**, T.C. Age-related alteration of hippocampal responsiveness to estradiol is linked to transcriptional context. Submitted.

Carter, C.S., Leeuwenburgh, C., Daniells, M., and **Foster**, T.C. Contribution of increased physical activity to learning and memory performance after calorie restriction in a rodent model of aging. Submitted.

RECENT ABSTRACTS

KUMAR, T. JACKSON, A. RANI, K. BODHINATHAN, T. **FOSTER**; (2008) 17β-estradiol induces rapid increase in hippocampal synaptic transmission in estrogen receptor beta WT and KO mice. Soc for Neurosci

W.-H. LEE, T. C. **FOSTER**; (2008) The role of SOD1 in brain aging. Soc for Neurosci

K. BODHINATHAN1, A. KUMAR2, T. C. **FOSTER** (2008) Impaired CaMKII function in the hippocampus contributes to age-related deficits in NMDA receptor function. Soc for Neurosci

Viral Vector–mediated Delivery of Estrogen Receptor-α to the Hippocampus Improves Spatial Learning in Estrogen Receptor-α Knockout Mice

Thomas C Foster[1], Asha Rani[1], Ashok Kumar[1], Li Cui[1] and Susan L Semple-Rowland[1]

[1]Department of Neuroscience, McKnight Brain Institute, University of Florida, Gainesville, Florida, USA

Estrogen, which influences both classical genomic and rapid membrane-associated signaling cascades, has been implicated in the regulation of hippocampal function, including spatial learning. Gene mutation studies suggest that estrogen effects are mediated by estrogen receptor-α (ER-α); however, because gonadal steroids influence the organization of the hippocampus during development, it has been difficult to distinguish developmental effects from those specific to adults. In this study we show that lentiviral delivery of the gene encoding ER-α to the hippocampus of adult ER-α-knockout (ER-αKO) mice restores hippocampal responsiveness to estrogen and rescues spatial learning. We propose that constitutive estrogen receptor activity is important for maintaining hippocampus-dependent memory function in adults.

Received 29 November 2007; accepted 6 March 2008; advance online publication 1 July 2008. doi:10.1038/mt.2008.140

INTRODUCTION

Estrogen treatments can have a positive impact on hippocampus-dependent memory in mammals and delay cognitive decline associated with aging; however, the molecular mechanisms underlying these beneficial effects remain unknown. Studies aimed at understanding estrogen effects on hippocampal processes have been problematic because estrogen can act through both classic genomic nuclear events and rapid membrane-mediated mechanisms to modify the structure, biochemistry, and physiology of the hippocampus.[1] Moreover, examination of estrogenic mechanisms is complicated because hippocampus expresses two estrogen receptor subtypes: estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β). Pharmacological studies suggest different roles for these receptors in the hippocampal and cortical memory systems,[2,3] each influencing the structure, physiology, and biochemistry of hippocampal synapses.[4–6]

The functional importance of receptor-mediated signaling pathways can be examined by disrupting the function or synthesis of specific receptors. Studies in humans and genetic mutants reveal the importance of hippocampal ER-α receptors in memory function. ER-α polymorphisms have been associated with age-related memory deficits and an increased incidence of Alzheimer's disease among women.[7–10] In the same manner, knockout of ER-α (ER-αKO) in female mice has been shown to produce deficits in learning and memory assessed using hippocampus-dependent tasks.[11,12] Studies of mutant mice have also provided evidence that ER-α has a role in mediating estrogen's rapid influences. Application of estrogen to normal hippocampal slices produces a rapid increase in synaptic transmission that is muted in hippocampal slices prepared from male and female ER-αKO mice.[13] These observations suggest that ER-α is required to obtain the complete spectrum of hippocampal synaptic responses that are induced by estrogen-triggered activation of rapid signaling pathways.

Unfortunately, the interpretation of findings from ER-αKO mice is clouded by the fact that estrogen receptor activity influences the organization of the developing brain. Thus, it is unclear whether the differences observed in the ER-αKO mice are due to the absence of ER-α activity during a critical developmental period or whether they are due to a lack of constitutive expression/function of ER-α in the adult. To distinguish between these possibilities, we examined hippocampus-dependent memory and hippocampal synaptic transmission in adult ER-αKO mice that received hippocampal injections of a lentiviral vector carrying ER-α. We hypothesized that if the ER-α is critical for hippocampal function in adults, expression of this receptor in the hippocampi of ER-αKO mice should restore hippocampus-dependent memory and the rapid excitatory modulation of synaptic transmission induced by estrogen. We found that expression of ER-α in the hippocampi of ER-αKO adult mice significantly improved spatial discrimination learning and restored estrogen's ability to induce rapid increases in hippocampal synaptic transmission. These results suggest that adult ER-α expression is important for maintaining hippocampus-dependent memory function.

RESULTS

Functional characterization of ER-α

COS-7 cells, which do not normally express ER-α, were transiently transfected with a bicistronic lentiviral ER-α–expression vector. The open-reading frame encoding murine ER-α was flanked upstream by an EF1-α promoter and downstream by complementary DNA (cDNA) encoding green florescent protein (GFP) that was separated from ER-α by a polio internal ribosomal entry site

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(EF1α-ERα-GFP). Control COS-7 cultures were transfected with a lentivirus GFP-expression vector in which the cDNA-encoding GFP was flanked upstream by an EF1-α promoter (EF1α-GFP). The results of this experiment showed that cells transfected with EF1α-ERα-GFP expressed ER-α and GFP (**Figure 1a**). ER-α staining was not observed in cultures transfected with EF1α-GFP. To establish that the encoded ER-α was functional, COS-7 cultures were co-transfected with either EF1α-ERα-GFP or EF1α-GFP and a reporter vector encoding secreted alkaline phosphatase (SEAP) under the control of an estrogen response element (ERE-SEAP). Cultures expressing ER-α exhibited approximately a fourfold increase in SEAP levels above baseline after incubating 24 hours with 1 nmol/l β-estradiol 3-benzoate (EB) (**Figure 1b**). A small increase in SEAP (less than twofold) was observed in ER-α-transfected cells in the absence of EB application. An analysis of variance indicated a significant difference across groups [$F(3,8) = 4.35$, $P < 0.05$] and *post hoc* tests indicated increased SEAP expression in cultures transfected with ER-α and treated with estradiol relative to the other three groups. Similar results were obtained using TE671 cell cultures. Co-transfection of TE671 cultures with EF1α-ERα-GFP and ERE-SEAP produced a tenfold greater effect than that observed in COS-7 cultures: addition of 1 nmol/l EB to these cultures produced a 401 ± 148% increase in SEAP relative to SEAP levels observed in TE671 cultures co-transfected with ERE-SEAP and EF1αGFP (data not shown).

Lentiviral transduction of hippocampus

Before examining the effects of EF1α-ERα on spatial learning, we examined the medial–lateral and anterior–posterior extent of cellular transduction produced by delivery of our lentiviral vectors to the dorsal hippocampus. Lentiviral vector carrying cDNA-encoding placental alkaline phosphatase (PLAP) driven by an EF1-α promoter (1×10^6 transducing units of virus in 0.5 µl) was injected into the dorsal hippocampi of male heterozygous ER-αKO ($Erα^{+/-}$) mice. Histochemical staining of the injected brains revealed strong expression of PLAP in CA1 pyramidal cells (**Figure 2a**) that extended ~1,000 µm along the anterior–posterior axis of the hippocampus. For behaviorally characterized mice, the transduction patterns observed in dorsal hippocampi of ovariectomized female ER-αKO mice injected with either lentivirus carrying EF1α-ER-α or EF1α-GFP were similar to that observed for EF1α-PLAP. Immunohistochemical staining showed that expression of ERα was most pronounced in CA1 with numerous cells in the pyramidal cell layer staining positively for ER-α (**Figure 2b**). In some cases, ER-α–positive cells were observed outside region CA1 within the dentate gyrus, stratum radiatum, and along the injection track in

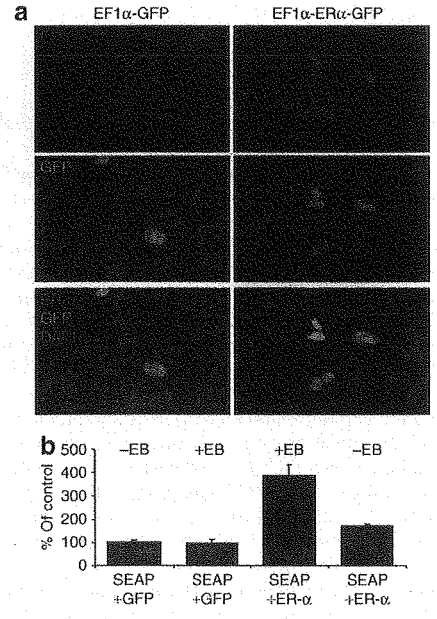

Figure 1 Analyses of estrogen receptor-α (ER-α) expression and function *in vitro*. COS-7 cultures were transiently transfected with ERE-SEAP reporter vector and (**a**) either EF1α-GFP (left) or EF1α-ERα-GFP plasmid DNA (right) to assess production and function of the ER-α protein. Immunofluorescent detection of ER-α (top, red), auto fluorescence of GFP (center, green), and colocalization of ER-α and GFP signals (bottom). Cell nuclei were stained with 4′,6-diamidio-2-phenylindole (DAPI) (blue). (**b**) Quantification of secreted alkaline phosphatase (SEAP) secreted from COS-7 cells transfected with ERE-SEAP and either EF1α-ERα-GFP or EF1α-GFP. After transfection, cultures were incubated with (+EB) or without (–EB) 1 nmol/l estradiol. The amount of SEAP detected in each culture was normalized to the average amount of SEAP detected in control cultures transfected with ERE-SEAP and EF1α-GFP. Each bar represents the mean value of four replicates. Error bars = SEM. EB, β-estradiol 3-benzoate; GFP, green fluorescent protein.

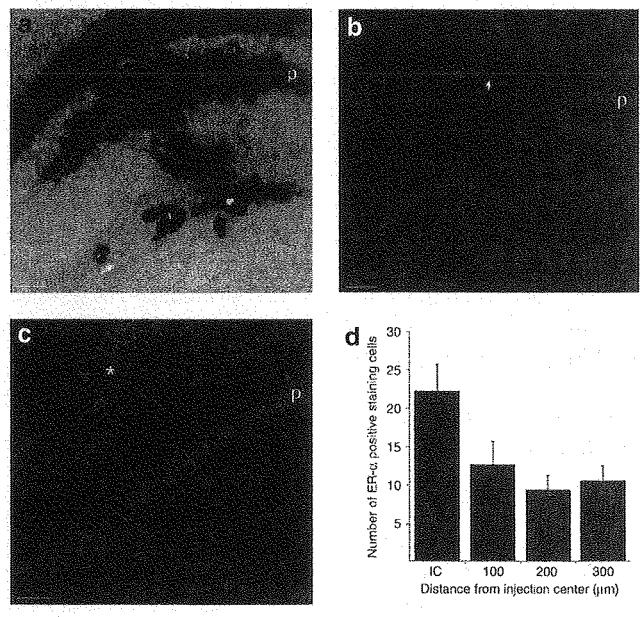

Figure 2 Expression of lentiviral vectors in the hippocampus. (**a**) Expression of placental alkaline phosphatase (PLAP) in region CA1 of a male mouse that received a hippocampal injection of EF1α-PLAP lentivirus. Eight days after injection, the animal was perfused and its brain was stained for PLAP. Hippocampal expression of PLAP (dark purple stain) was largely limited to the CA1 region of the hippocampus and included the pyramidal cell layer. (**b**,**c**) Expression of estrogen receptor-α (ER-α) in the hippocampi of two female ER-α knockout (ER-αKO) mice 4 weeks after the mice received hippocampal injections of EF1α-ERα lentivirus. ER-α was visualized using an ER-α antibody (red). Staining was observed in the CA1 pyramidal cell region and in cortical cells along the pipette track (asterisk in **c**). (**d**) Quantification of ER-α–positive cells in CA1 of female ER-αKO mice injected with EF1α-ERα. Cell counts were measured in 100-µm segments starting with the 100 µm surrounding the injection center. p, pyramidal cell layer. Calibration bars represent 100 µm The arrows in the pyramidal cell layer in **b** indicate the area surrounding the injection tract.

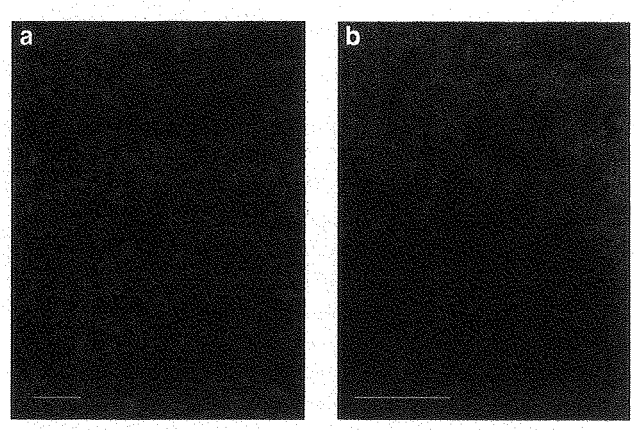

Figure 3 Estrogen receptor-α (ER-α) immunostaining in the dendrites of CA1 pyramidal cells from female ER-α knockout (ER-αKO) mice injected with EF1α-ERα. (**a**) Example of dendritic expression of ER-α in a single cell in the CA1 pyramidal cell layer. Note that the somas of several cells exhibit lower levels of ER-α immunostaining. (**b**) Example of dendritic expression in multiple CA1 pyramidal cells. Calibration bars represent 50 μm.

the overlying cortex (**Figure 2c**). An examination of brightly fluorescing cells in consecutive sections indicated that expression was observed in CA1 throughout the dorsal hippocampus with an average anterior–posterior distance of 1,066 ± 211 μm. The medial–lateral extent of expression in CA1 was estimated by counting the brightly fluorescing cells near the injection site and averaging the number of cells medial and lateral to the injection site in consecutive 100-μm segments. As expected, the number of brightly fluorescing cells was greatest near the injection site; however, considerable expression was also observed for at least 300 μm on either side of the injection (**Figure 2d**). The cell counts likely underestimate the total number of cells expressing ER-α, because numerous dimly fluorescing cells were detected, but were not counted in our analyses. Examination of transduced CA1 pyramidal cells revealed that proteins encoded by the viral vector could be detected in the dendritic processes of many of these cells (**Figure 3**).

Effects of expression of ER-α on spatial learning in ER-αKO mice

In this experiment, we limited our analyses to female mice because, unlike male ER-αKO mice, female ER-αKO mice exhibit learning and memory deficits relative to wild-type (WT) littermates.[11,12] All behavioral experiments were conducted using female mice that had been ovariectomized at 3 months of age. The experimental groups included WT littermates (n = 12), ER-αKO mice injected bilaterally with EF1α-ERα lentivirus (n = 13), uninjected ER-αKO controls (n = 8), and a second control group consisting of ER-αKO mice injected bilaterally with EF1α-GFP lentivirus (n = 10). All groups exhibited a decrease in escape latency as a function of training on the cue discrimination task. Repeated analysis of variance indicated an effect of training on escape latency [$F(3,117) = 17.35$, $P < 0.0001$] and escape distance [$F(3,117) = 11.33$, $P < 0.0001$] in the absence of group effects (**Figure 4**). No difference was observed in swim speed between groups. Spatial discrimination training was initiated 3 days after cue training and was continued for 3 consecutive days. No effect of training or groups was observed for swim speed. The latency [$F(3,78) = 37.44$, $P < 0.0001$] and distance [$F(3,78) = 49.19$, $P < 0.0001$] to find the hidden platform decreased over training days (**Figure 5**). In addition, there was a tendency for a group effect for latency ($P = 0.08$) and distance ($P = 0.09$), suggesting differences between some groups. *Post hoc* comparisons indicated no difference in latency or path length between ER-αKO mice and ER-αKO mice treated with EF1α-GFP. Similarly, no difference in latency or path length was observed between WT mice and ER-αKO mice treated with EF1α-ERα. In contrast, *post hoc* comparisons ($P < 0.05$) indicated

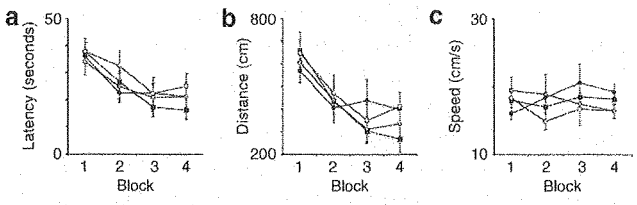

Figure 4 Cue discrimination learning in the water maze. The (**a**) latencies, (**b**) path lengths, and (**c**) swim speeds to escape to a visible platform on the water maze task were not different for wild type (filled boxes), knockout of ER-α (ER-αKO) treated with EF1α-ERα (filled circles), untreated ER-αKO (open circles), and ER-αKO treated with EF1α-GFP (gray circles). ER-α, estrogen receptor-α.

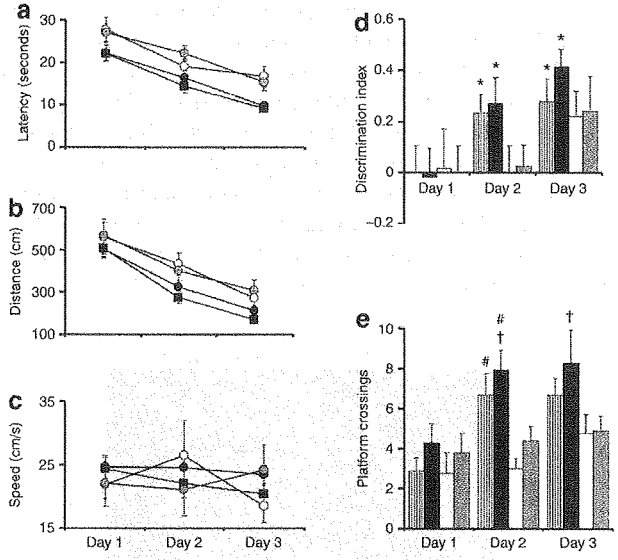

Figure 5 Expression of EF1α-ERα in the hippocampus improves spatial learning in female ER-α knockout (ER-αKO) mice over 3 days of training. Mean (**a**) latencies, (**b**) distances, and (**c**) swim speeds to reach the hidden escape platform across 3 days of training for wild-type (WT) (filled boxes), ER-αKO treated with EF1α-ERα (filled circles), untreated ER-αKO (open circles), and ER-αKO treated with EF1α-GFP (gray circles). WT and ER-αKO mice treated with EF1α-ERα showed reduced latencies to escape compared with untreated ER-αKO mice and ER-αKO mice treated with EF1α-GFP. A similar pattern was observed for escape distances. (**d**) Discrimination index for WT (striped bar), ER-αKO treated with EF1α-ERα (filled bar), untreated ER-αKO (open bar), and ER-αKO treated with EF1α-GFP (gray bar) calculated from probe trials delivered at the end of training each day during 3 days of spatial discrimination training. (**e**) Number of platform crossings during each probe trial. Asterisks in **d** indicate that the discrimination index was significantly ($P < 0.05$) different from that expected by chance (discrimination index score = 0). Pound signs indicate significant differences relative to the untreated ER-αKO mice. Dagger indicates a significant difference relative to the ER-αKO mice treated with EF1α-GFP. ERα, estrogen receptor-α.

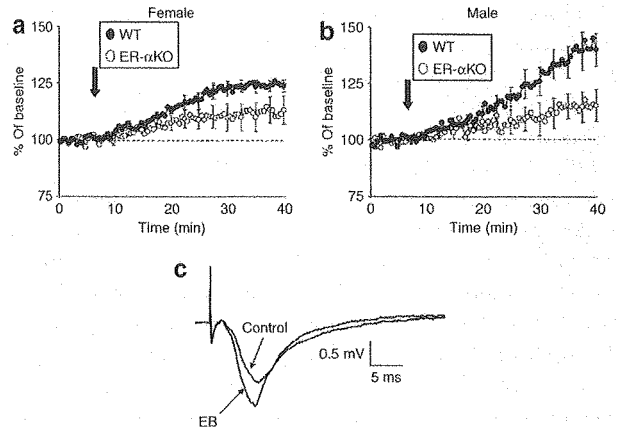

Figure 6 Estrogen receptor-α (ER-α) contributes to the rapid increase in synaptic transmission after β-estradiol 3-benzoate (EB) application to hippocampal slices. Time courses for changes in hippocampal synaptic responses after EB application (arrow) in (**a**) female and (**b**) male wild-type (WT) (filled circles) and ER-α knockout (ER-αKO) (open circles) mice. The increase in the synaptic response from baseline (dashed line) was reduced in ER-αKO slices relative to WT slices. (**c**) Examples of synaptic responses obtained during baseline recordings (control) and 30 minutes after application of EB to a hippocampal slice obtained from a WT male mouse. The averages were generated from recordings of 8–13 slices and error bars = SEM. The SEMs are presented for every fifth record to preserve clarity.

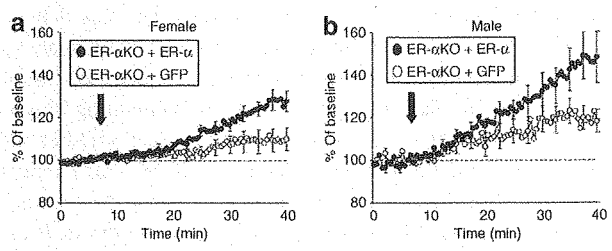

Figure 7 Expression of ERα in ER-αKO hippocampal slices restores synaptic responses to β-estradiol 3-benzoate (EB). EF1α-ERα lentivirus was injected unilaterally into the hippocampus and EF1α-GFP lentivirus was injected into the contralateral hippocampus of (**a**) female and (**b**) male ER-α knockout (ER-αKO) mice. Expression of estrogen receptor-α (ER-α) (filled circles) was associated with enhanced EB responsiveness relative to slices expressing green fluorescent protein (GFP) (open circles).

that the escape latency was reduced for WT mice and ER-αKO mice treated with EF1α-ERα compared with ER-αKO mice and ER-αKO mice treated with EF1α-GFP. *Post hoc* comparisons also indicated that the escape distance was reduced for WT mice compared with ER-αKO mice treated with EF1α-GFP.

A repeated analysis of variance on the discrimination indices for the three probe trials that were conducted at the end of training each day indicated an effect of training [$F(2,78) = 9.92$, $P < 0.0001$]. WT and ER-αKO mice treated with EF1α-ERα exhibited discrimination index scores above chance on days 2 and 3 (**Figure 5d**), indicating that these groups had acquired a spatial search strategy. In contrast, the control groups (uninjected ER-αKO and ER-αKO mice treated with EF1α-GFP) did not exhibit a differential search strategy even after 3 days of training. As with the discrimination index, the number of platform crossings increased over the period of training [$F(2,78) = 11.07$, $P < 0.0001$], and a significant main effect of group was observed [$F(3,78) = 3.49$, $P < 0.05$]. For day 2, the total number of platform crossings made by WT mice and ER-αKO mice treated with EF1α-ERα was greater than the number of crossings made by uninjected ER-αKO controls. Furthermore, ER-αKO mice treated with EF1α-ERα exhibited more crossings than ER-αKO mice treated with EF1α-GFP and the difference between WT mice and ER-αKO mice treated with EF1α-GFP approached significance ($P = 0.09$). Similarly, ER-αKO mice treated with EF1α-ERα exhibited more crossings than ER-αKO mice treated with EF1α-GFP and the difference between ER-αKO mice treated with EF1α-ERα and uninjected controls approached significance ($P = 0.05$) during day 3.

Effects of lentiviral ERα treatment on estrogen-induced hippocampal synaptic responses

Using ER-αKO and WT littermates, we confirmed that the responsiveness of the hippocampus to estrogen is diminished in male and female ER-αKO mice.[13] No genotype or sex differences were observed in the half maximal synaptic response. Consistent with previous reports, the increase in the slope of the synaptic response was enhanced in WT hippocampi relative to ER-αKO hippocampi 30 minutes after EB application [$F(1,37) = 7.75$, $P < 0.01$] in the absence of a difference between males and females (**Figure 6**). The effects of EB application on the synaptic field potential of hippocampal slices obtained from mice treated with EF1α-ERα are shown in **Figure 7**. No treatment or sex differences were observed for the half maximal synaptic response employed as a baseline response. Analyses of the changes observed in the synaptic responses of the slices 30 minutes after addition of EB showed a treatment effect [$F(1,29) = 16.43$, $P < 0.0005$)], indicating that EB application produced a larger increase in the synaptic response in slices transduced with EF1α-ERα compared to those transduced with EF1α-GFP. In addition, there was a significant effect of gender [$F(1,32) = 7.0$, $P < 0.05$], the estrogen-induced responses obtained from male hippocampi being larger than those obtained from female hippocampi.

DISCUSSION

Role for ER-α in learning and memory in adults: The site of estrogen influence on memory is likely to reside in the frontal cortex or hippocampus.[3,14–16] A distinctive role for ER-α in maintaining memory function is suggested by human studies involving polymorphisms in the *ER-α* gene[7–10] and the observation that spatial learning is impaired in female ER-αKO mice.[11] Although studies of *ER-α* gene mutations point to the importance of ER-α in preserving memory function, it is unclear from previous work whether the deficits reflect receptor influences on brain organization during development or whether ER-α contributes to memory in the adult.

Expression of ER-α and aromatase is developmentally regulated in the hippocampus and plays an important role in the masculinization of hippocampal structure and function.[17,18] Thus, one might expect that knockout of ER-α would affect the behavior of male mice more than that of female mice. However, studies of the relationship between ER-α polymorphisms and memory in humans suggest that the deficits are greatest in females and studies of ER-αKO mice indicate that spatial learning impairments are limited to females.[11] This study demonstrates that lentivirus-mediated expression of ER-α in the hippocampus rescues spatial learning deficits in female ER-αKO mice providing evidence for the idea that ER-α plays a significant role in the functioning of the hippocampus in

adult females. These results also indicate that the learning deficits observed in female ER-αKO mice cannot be attributed solely to developmental changes in brain organization. Rather, expression of ER-α appears to play an important role in maintaining hippocampal function in the adult. However, it is unclear whether the benefits of expression of ERα in adult female ER-αKO mice reflect renewed signaling through normal ER-α pathways or whether activation of the expressed ER-α is acting on a compensatory pathway to alleviate a developmental defect in hippocampal organization.

The fact that hippocampal ER-α expression was able to improve memory in ovariectomized ER-αKO mice indicates that the memory improvements were not dependent on steroids released from the gonads. In this regard, it is important to note that considerable estrogen receptor–mediated transcriptional activity occurs in the brain of ovariectomized animals.[19] In the absence of gonadal steroids, activation of ER-α may result from ligand-independent mechanisms involving estrogen receptor phosphorylation.[20] There is also evidence that hippocampal ER-α can be activated by locally synthesized estrogen, and that activation of ER-α exerts neuroprotective[21,22] and trophic effects on synaptic structure and function.[23–26] Taken together, these results are consistent with the idea that constitutive ER-α activity through ligand-independent or -dependent mechanisms can influence memory indirectly by regulating transcription of genes that function to maintain hippocampal health.[27]

The temporal constraints associated with estrogen treatments are important such that hormone treatment during training can impair learning/memory, and beneficial effects are generally observed when estrogen is delivered several days before or shortly after learning.[1] The effects of treatment several days before behavioral testing likely involve transcriptional/translational changes and, in the case of age-related cognitive decline, ER-α–dependent neuroprotection.[27–29] In contrast, activation of either ER-α or ER-β immediately after training is sufficient to enhance recognition on some tasks.[2,3] Mounting evidence indicates that ER-β activation facilitates memory for aversive task such as the water escape task and inhibitory avoidance,[12,30,31] possibly through anxiety and memory-modulating systems such as the amygdala.[32,33] Indeed, interactions with systems for behavioral stress may mediate the dose-dependent relationship between estrogen and learning/memory.[1,34,35]

The temporal constraints on memory facilitation combined with biochemical studies suggest that agonists can act on rapid membrane-associated signaling cascades that are involved in laying down memories. ER-α-mediated rapid membrane effects, which include increases in synaptic transmission and altered synaptic plasticity, involve activation of G-protein and Ca^{2+} signaling cascades that lead to altered protein kinase and phosphatase activity. Research from other systems indicates that membrane ER-α induces kinase activation.[36,37] Similar mechanisms are present in hippocampal cells including activation of extracellular signal mitogen-activated protein kinase,[38–40] CaM kinase II,[41] and cAMP-activated kinase.[42,43] We confirmed that the EB-mediated increases in synaptic transmission are blunted in male and female ER-αKO mice.[13] Lentivirus-mediated expression of ER-α enhanced EB effects on synaptic transmission, consistent with the idea that ER-α contributes to the rapid membrane effects of EB.

The link between activation of rapid signaling cascades and memory is likely to include phosphorylation of CREB (pCREB) and subsequent regulation of transcription. Both ERα and ERβ can increase pCREB in neurons.[30,44–46] Work in hippocampal cell cultures suggests that estrogen is acting on ER-α to enhance pCREB,[44] although the same pathways may be activated following overexpression of ER-β.[39]

ER-α and ER-β interactions: Application of EB produced a modest increase in synaptic transmission in hippocampi of control ER-αKO mice suggesting that other estrogenic mechanisms can contribute to synaptic function. ER-α and ER-β receptor subtypes have been localized near synaptic sites in the hippocampus[47,48] and research suggests similarities in their signaling processes.[45] However, other studies indicate ER-α and ER-β may activate different signaling cascades.[6,44] There are conflicting reports that assign one or the other receptor to mediating estrogen-induced changes in spine growth and synaptic components in the hippocampus.[4,30] ER-α activation has been shown to increase neurite length and number, whereas ER-β activation increases only the neurite length.[49] Similarly, while it has been suggested that ER-α and ER-β contribute to neuroprotection, others have argued that the level of ER-α expression or activation is critical for determining the extent of neuroprotection.[29,50,51] One possible explanation for these discrepancies is that the effects of receptor activity may differ when ER-α and ER-β are expressed alone or in combination. In cells that express multiple receptor subtypes, the subunit composition of the receptor complex (*i.e.*, homodimer or heterodimer) can have different or even opposite effects on transcription.[52,53] In the absence of estrogen treatment, ER-βKO mice exhibit normal learning.[12,30,54] ER-βKO mice do not exhibit memory facilitation after post-training estrogen treatment[30] and exhibit impaired spatial learning when estrogen is delivered chronically before learning.[54] These results support the idea that post-training ER-β activation can facilitate memory; however, ER-β is not required for spatial learning. Further, over activation of ER-α due to estrogen treatment of ER-βKO mice may impair spatial learning. Alternatively, because estrogen treatment reduced ER-α immunoreactivity in hippocampal cells of ER-βKO mice,[54] it is plausible that the impairments in spatial learning may have resulted from loss of ER-α function associated with estrogen treatment. The results of this study confirm that ER-αKO mice exhibit impairments on the water maze. Together with studies on the role of ER-α and ER-β in neuroprotection, these results suggest that, under some conditions, ER-β cannot compensate for a loss of ER-α.[21,22,51]

In summary, our results demonstrate that ER-α is required to obtain the full spectrum of rapid estrogenic effects in the hippocampus of young adults. In addition, we show that constitutive expression of ER-α in the adult can support hippocampus-dependent behavior, an observation that suggests that cognitive deficits associated with impaired ER-α function are not entirely due to the absence of ER-α activity during development. Our findings, together with recent reports on estrogen's mechanisms of action during aging, suggest that ER-α is important for sustaining hippocampus-dependent memory, possibly through indirect mechanisms involving the maintenance of cell health.[27] However, it will be important for future research to determine how ER-α is interacting with other estrogen-mediated signaling pathways. The fact that cognitive benefits were observed in ovariectomized animals suggests that treatments to enhance the signaling cascades may provide a possible alternative to current hormone treatments for age-related memory decline.

MATERIALS AND METHODS

Construction of lentiviral vectors and vector packaging.

pTYF-EF1α-ERα-IRES-GFP. The open-reading frame encoding the murine ER-α was amplified from cDNA clone pSG5.MOR (kind gift from Dr. M. Parker) using primers that introduced *Nhe*I and *Cla*I sites on the 5′- and 3′-ends of the coding region (5′-TTG CTA GCC GGC TGC CAC TTA CCA-3′ and 5′-TTA TCG ATT GTT GCA GGG ATT CTC AG-3′) and plaque-forming units Turbo polymerase (Stratagene, Cedar Creek, TX). The resulting 1,874-base pair product was subcloned into the ZeroBlunt shuttle vector (Invitrogen, Carlsbad, CA). The integrity of ER-α was confirmed by sequencing. The murine ER-α cDNA was removed from ZeroBlunt and ligated into demethylated pTYF-EF1α-IRES-GFP linker vector (http://www.mbi.ufl.edu/~rowland/vector.htm) by *Nhe*I and *Cla*I to create pTYF-EF1α-ERα-IRES-GFP (EF1α-ERα-GFP).

pTYF-EF1α-ERα. Demethylated pTYF-EFα-IRES-GFP was digested with *Kpn*I removing IRES-GFP, religated, and subsequently digested with *Nhe*I and *Cla*I. The murine ER-α cDNA was removed from the ZeroBlunt clone by *Nhe*I and *Cla*I and ligated into the digested vector backbone producing pTYF-EF1α-ERα.

pTYF-EF1α-PLAP and pTYF-EF1α-GFP. The maps and sequences of these vectors are available online (http://www.mbi.ufl.edu/~rowland/vector.htm). Both vectors have been previously described.[55]

Vector packaging. The ER-α and reporter gene vectors were packaged into lentivirus using previously described methods.[55] The titers of the viral preparations were estimated by quantifying the amounts of p24 core protein in the preparations using a Coulter HIV-1 p24 antigen enzyme-linked immunosorbent assay and converting these values into transducing units (defined as an infectious particles) using a constant value determined in our laboratory that relates p24 values to infectious particle numbers. This constant value was determined by measuring p24 levels in several pTYF-EF1αPLAP viral preparations and relating these values to the number of PLAP-positive cells in TE671 cell cultures transduced with these viruses.[55]

Cell culture analyses of ER-α function. COS-7 monkey kidney cells or TE671 human rhabdomyosarcoma cells (American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium containing 10% charcoal-filtered serum (Cocalico Biologicals, Reamstown, PA), 100 U of penicillin/ml, 100 μg of streptomycin/ml, 25 μg of gentamycin/ml, and 2 mmol/l L-glutamine. The day before transfection, cells were plated onto 12-well plates and grown overnight in Dulbecco's modified Eagle's medium containing 10% charcoal-filtered serum. On the day of transfection, the medium was removed and replaced with 0.4 ml of fresh medium. Transfection mixture for each well was prepared by adding 3.5 μg of ERE-TA-SEAP vector (Clontech, Palo Alto, CA) and 3.5 μg of either pTYF-EF1α-ERα-IRES-GFP or pTYF-EF1α-eGFP to 55 μl of serum-free Dulbecco's modified Eagle's medium, mixing, and then adding 10 μl SuperFect (Qiagen, Valencia, CA). This mixture was incubated at room temperature for 10 minutes and then 26 μl of the mixture was added dropwise into each well. Following a 5-hour incubation at 37 °C, the cultures were rinsed and allowed to grow overnight at 37 °C in fresh medium containing 10% charcoal-filtered serum. In some cases 1 nmol/l EB was added to the medium.

After overnight incubation, 30 μl of culture media was collected from each culture well, centrifuged at 12,000 rpm for 10 seconds, and the cleared supernatant was transferred into a fresh microcentrifuge tube. Levels of SEAP in the media were measured using a Tropix Phospha-Light chemiluminescent assay kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Samples were run in triplicate and light emission from the processed samples and SEAP standards was measured using a Turner 20/20 luminometer with a delay of 2 seconds and integration time of 5 seconds. The amount of SEAP in the culture media samples was calculated from the standard curve. For some cultures, triple fluorescence labeling for ERα (MC-20, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), GFP, and nuclei was carried out sequentially.

Animals and surgical procedure. All animal experiments were performed in accordance with institutional guidelines and with Institutional Animal Use and Care Committee approval. Mice were generated and screened by PCR amplification as previously described.[11–13,56] All female mice were ovariectomized.[11–13] For behavioral studies, female ER-αKO and WT littermates (2–3 months) were ovariectomized and EF1α-ERα or EF1α-GFP lentivirus (1×10^6–10^9 transducing units/μl) in a total volume of 0.25–0.5 μl was injected into the hippocampus. For *in vitro* electrophysiological studies, ovariectomized female ($n = 5$) or intact male ER-αKO mice ($n = 7$) were injected unilaterally with EF1α-ERα, and the contralateral hippocampus received an equal injection of EF1α-GFP. Animals were allowed to recover for at least 8 days (12 ± 0.8 days, mean ± SEM) before killing for *in vitro* electrophysiological recording.

Electrophysiological recordings from hippocampal brain slices. Three-month-old WT and ER-αKO mice were used in all electrophysiological experiments as previously described.[13] EB was initially dissolved in a small amount of ethanol and diluted by recording medium to a final concentration 100 pmol/l of EB and 0.001% of ethanol. Consistent with previous reports, application of 0.001% ethanol alone or cholesterol had no effect on synaptic responses.[13] At the end of each experiment, the slices were fixed in 4% paraformaldehyde for 2 hours, subsequently removed and placed into 30% sucrose in 0.01 mol/l phosphate-buffered saline (PBS) at 4 °C overnight, and then prepared for sectioning.

Behavioral characterization. Three-month-old female WT and ER-αKO mice were used for behavioral studies. Starting 3 weeks after ovariectomy and virus injection, female mice were trained on the cue discrimination version of the Morris swim task followed 3 days later by the spatial version of the task using methods which have been previously described.[11,27] The penultimate trial on each day consisted of a probe trial which served as an index of learning. The probe trial consisted of placing the mouse in the tank for 1 minute without the platform and recording both the time the animal spent in each quadrant of the tank and the number of times the animal crossed the region in which the platform had been located. A spatial discrimination index was computed according to the formula $(G_i O)/(G + O)$, where G and O represent the percent of time spent in the goal quadrant and quadrant opposite the goal, respectively.

Immunohistochemistry. After behavioral characterization, mice were perfused with 4% paraformaldehyde in PBS. Brains were harvested and postfixed in 4% paraformaldehyde for 2 hours, subsequently removed and placed into 30% sucrose in 0.01 mol/l PBS at 4 °C until permeated then used for sectioning. Brain sections for electrophysiologically characterized hippocampal slices were placed into 30% sucrose in 0.01 mol/l PBS at 4 °C until permeated then embedded in Tissue-Tek O.C.T. Compound (Ted Pella, Redding, CA) and sectioned. Sections (20 μm) were incubated with primary antibody (C-311, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C, washed, and then incubated in Alexa 594 secondary antibody (1:500 dilution; Molecular Probes, Eugene, OR) for 1 hour at room temperature. Normal mouse immunoglobulin G (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) was used as negative control. Sections were washed, counterstained with 4′,6-diamidio-2-phenylindole solution (0.1 μg/ml in PBS).

Statistical analyses. For electrophysiological studies, *t*-test comparisons were used to identify significant main effects. For behavioral studies, analysis of variance for repeated measures was used to determine significant main effects and interactions across days of training. Fisher's PLSD *post hoc* comparisons were used to localize specific differences. Finally, the discrimination index was analyzed using a one-group student *t*-test predicting that scores would be greater than chance (*i.e.*, a discrimination index of 0).

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Regional hippocampal differences in AKT survival signaling across the lifespan: implications for CA1 vulnerability with aging

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Distinct neuronal populations differ by the degree of damage caused from cellular stress. Hippocampal neurons of area CA1 are especially vulnerable to several stressors that increase as age advances. We show here that survival signaling, as measured by activated protein kinase B (AKT), was significantly reduced in the nuclear CA1 region across the lifespan compared with CA3. In agreement with these findings, the pro-apoptotic protein and AKT nuclear substrate, forkhead box O3a transcription factor (FOXO3a), were significantly higher in CA1. Further, regional differences in PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP1), a recently discovered inhibitor of AKT, inversely correlated with nuclear phosphorylated AKT at Ser473. Altogether, our data suggest that regional differences in nuclear levels of activated AKT may contribute to regional differences in hippocampal vulnerability and implicate PHLPP1 as a potential target for therapeutic intervention to improve hippocampal health.
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Aging is associated with increased vulnerability to hippocampal cell death.[1] Furthermore, aging-dependent diseases can target vulnerable neuron populations. Under these conditions, loss of select hippocampal neuron populations can compromise hippocampal function, contributing to age associated memory impairments.

Despite the similarity in cell type between the CA1 and CA3 regions, evidence suggests that these regions show different vulnerabilities to cell death depending on the stressor.[2] For example aging-dependent complications, such as cardiovascular disease,[3] stroke,[4] and decreased cerebral blood flow[5] can increase neuronal ischemic damage in the hippocampus. However, area CA1 is the most affected hippocampal region to ischemic insult.[6] Numerous *in vivo* studies using rodent models of hippocampal acute/severe ischemia,[7] or chronic/mild hypoperfusion,[8] showed targeted damage to the area CA1, while sparing area CA3.

In addition to ischemic insult, differences in hippocampal vulnerability to stressor-induced damage are observed in debilitating age-dependent diseases, such as Alzheimer's disease. Studies on the human brain show that area CA1 of the hippocampus is one of the earliest brain regions to develop the pathological markers associated with Alzheimer's disease,[9] and the rodent models have correlated disease pathology to CA1 neuronal loss.[10] Understanding what mediates regional differences in hippocampal vulnerability may provide novel solutions for treating aging-dependent decline in hippocampal function caused by decreased neuronal health and survival.

Regional differences in hippocampal vulnerability might result from intrinsic CA1/CA3 differences in the regulation of cell-survival pathways. The phosphoinositide kinase-3 pathway, through the activation of protein kinase B (AKT), is particularly important for neuron survival and has been shown to protect neurons against a vast variety of stressors, including ischemia,[11] β-amyloid,[12] and tau pathology.[13] As AKT activation can protect neurons against stressors known to increase with aging, we sought to determine whether measures of AKT activity differed between areas CA1 and CA3 of the hippocampus across the lifespan.

Results

Nuclear active AKT differs between CA3 and CA1 regions. Accumulation of nuclear phosphorylated AKT (pAKT) is critical to AKT's antiapoptotic effects. To determine regional hippocampal differences in both nuclear pAKT levels and regulators of pAKT, nuclear and cytoplasmic-enriched fractions were prepared from CA1 and CA3 hippocampal homogenates and used for western blot analysis. Cytoplasmic and nuclear fractions were probed for the nuclear protein, TATA box binding protein (TBP), to verify separation. As expected, TBP was observed primarily in the nuclear protein enriched fractions (Figure 1a) and served as a nuclear loading control in subsequent analyses. Nuclear CA1 and CA3 samples were then probed for AKT phosphorylated at Ser473 (pAKT473, ~60 kDa; Figure 1b),

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Abbreviations: AKT, Protein Kinase B; FOXO3a, Forkhead Box O3a Transcription Factor; CA3, Ammon's horn (area CA3); CA1, Ammon's horn (area CA1); TBP, Tata Box Binding Protein; PTEN, Phosphatase and Tensin Homolog; IGF-1, Insulin-like Growth Factor 1; PP2A-A, Protein phosphatase 2A, subunit A; PHLPP1, PH Domain and Leucine-Rich Repeat Protein Phosphatase 1; PKC, Protein Kinase C; PI3K, Phosphoinositide Kinase-3
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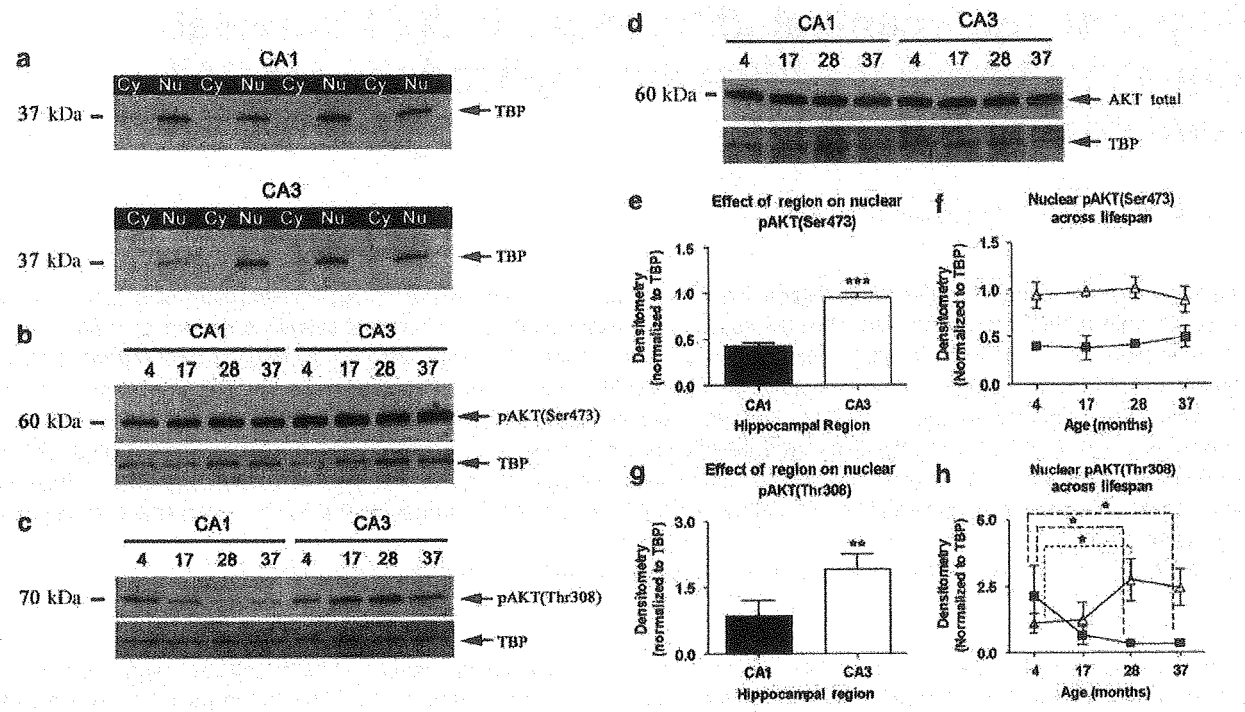

Figure 1 Activated AKT is increased in nuclear fractions from region CA3 compared with region CA1. (**a**) Control blot showing TBP is localized to nuclear enriched fractions (Nu) relative to cytoplasmic enriched fractions (Cy). (**b–d**) Representative blots of nuclear CA1/CA3 pAKT473, pAKT308, and total AKT, from animals aged 4, 17, 28, and 37 months (top blots) and TBP nuclear loading control (bottom blots). (**e** and **g**) Mean nuclear pAKT averaged across ages ($n=15$) for CA1 (filled bars) and CA3 (open bars). (**f** and **h**) Effect of age on nuclear pAKT in area CA1 (filled squares) and CA3 (open triangles) for animals aged 4 ($n=4$), 17 ($n=3$), 28 ($n=4$), and 37 ($n=4$) months. Dashed lines indicate significant aging effects in CA1 (- - -) and CA3 (·····). In this figure and subsequent figures, asterisks indicate significant differences $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$

AKT phosphorylated at Thr308 (pAKT308, ~75 kDa; Figure 1c), and total AKT (Figure 1d). No regional or aging differences were observed in nuclear total AKT levels. In marked contrast, both pAKT473 and pAKT308 were significantly higher in nuclear samples from CA3 when compared with CA1 (Figure 1e and g). Further, although age had no effect on nuclear pAKT473 in either of the regions (Figure 1f), pAKT308 levels were reduced in area CA1 and increased in area CA3 of older animals (Figure 1h). Altogether, the data show regional differences in pAKT with higher levels in area CA3.

Regional differences in hippocampal nuclear FOXO3a. Activated AKT selectively phosphorylates the pro-apoptotic transcription factor forkhead box O3a (FOXO3a) at Ser253,[14] leading to nuclear exclusion and enhanced cell survival of hippocampal neurons.[15] Consistent with the decrease in activated AKT in the nuclei of area CA1, immunofluorescent staining of hippocampal sections from a 28-month-old animal indicated lower pFOXO3a253 (red), in nuclei (blue), in area CA1 (Figure 2a–c) relative to CA3 region (Figure 2d–f). Moreover, cells that were negative for the neuronal marker tubulin III (green) exhibited little or no nuclear pFOXO3a253. To determine whether the increase in pFOXO3a253 was associated with lower nuclear FOXO3a levels, hippocampal sections were stained for FOXO3a (red). Again, total FOXO3a was primarily localized to neurons (green), however; more staining was observed in the nuclei (blue) and perinuclear areas from CA1 (Figure 3a–c) relative to CA3 (Figure 3d–f). To examine total nuclear FOXO3a levels across the lifespan, western blots of nuclear CA1 and CA3 samples were probed with a polyclonal antibody against FOXO3a and a single band was detected at ~70 kDa (Figure 3g). The results confirmed the FOXO3a immunofluorescence, exhibiting significantly higher levels of total FOXO3a in CA1 compared with CA3, consistent with the ability of pAKT to exclude FOXO3a from the nucleus (Figure 3h). No significant aging effects on nuclear FOXO3a were observed (Figure 3i).

Cytoplasmic factors contributing to regional differences in nuclear pAKT. Activation of AKT in the cytoplasm promotes nuclear pAKT translocation. To determine whether cytoplasmic pAKT showed similar regional differences compared with nuclear pAKT, cytoplasmic samples were probed for pAKT473 (Figure 4a), pAKT308 (Figure 4b), and total AKT (Figure 4c). No significant regional differences were observed for cytoplasmic AKT total or pAKT308. Only cytoplasmic pAKT473 showed significant regional differences (Figure 4d and e), and was higher in the CA3 compared with CA1. No age-dependent changes were observed for either pAKT473 or pAKT308 (Figure 4f and g). Next we sought to determine if known regulators of cytoplasmic pAKT might show regional hippocampal differences; suggesting a mechanism for increased cytoplasmic CA3 pAKT levels.

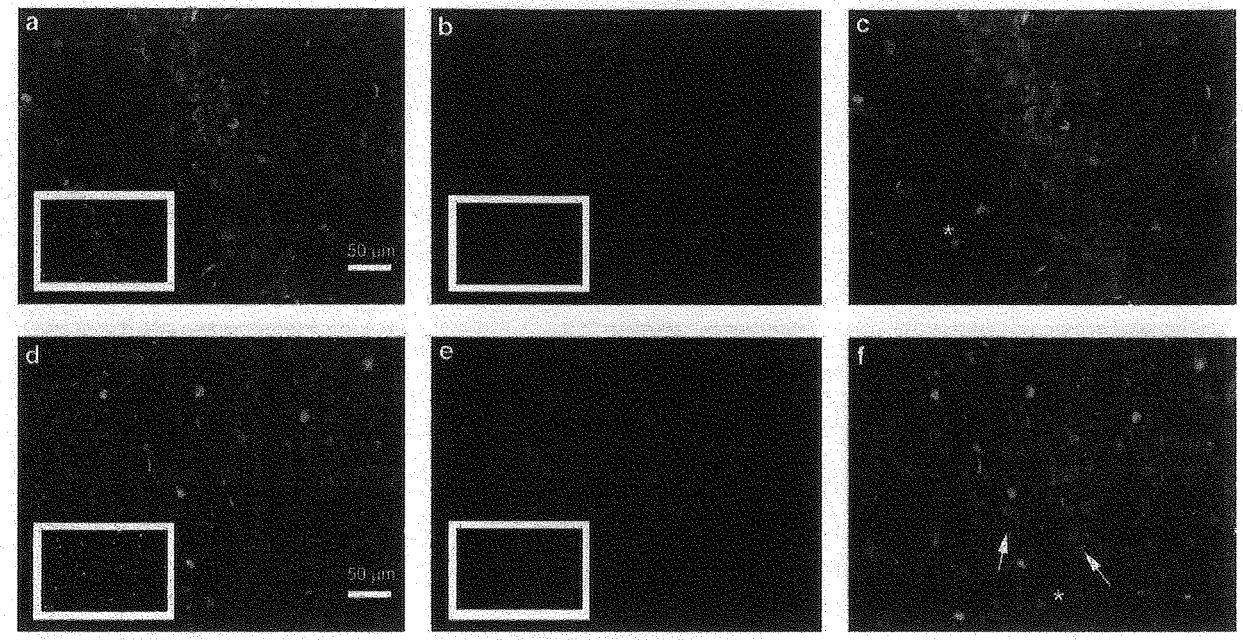

Figure 2 Nuclear pFOXO3a253 is higher in region CA3 and lower in region CA1. (**a** and **d**) Immunofluorescence showing CA1 and CA3 neuronal marker tubulin III (green), and nuclei (blue). White boxes in the lower left indicate secondary antibody-only controls. No green staining was observed. (**b** and **e**) Immunofluorescence showing CA1 and CA3 pFOXO3a253 (red). More staining is observed in region CA3 compared with CA1. No staining was observed in the secondary antibody-only controls. (**c** and **f**) Merged images of panels **a/b** and **d/e** showing pFOXO3a. pFOXO3a is localized to the nuclei of CA1 and CA3 pyramidal neurons. White arrows indicate intense nuclear pFOXO3a253 CA3 staining. White asterisks indicate non-neuronal nuclei

The insulin-like growth factor 1 (IGF-1) receptor tyrosine kinase is a robust activator of AKT signaling in hippocampal neurons. Cytoplasmic activation of PI3 kinase by IGF-1 promotes AKT phosphorylation and enhances its nuclear translocation.[16] To investigate whether expression of IGF-1 receptors contributes to the differential activation of AKT, cytoplasmic CA1 and CA3 samples were probed for IGF-1Rα, and a band was detected at ~150 kDa (Figure 5a). No region or age differences in IGF-1Rα were observed suggesting that the total receptor expression does not underlie regional differences in cytoplasmic pAKT (Figure 5c).

Next, we investigated whether the activity of IGF-1 receptors might reflect regional differences in pAKT. Activation of IGF-1 receptors requires ligand-dependent autophosphorylation of the IGF-1Rβ (95 kDa) subunit. CA1 and CA3 cytoplasmic samples were probed for phosphorylated IGF-1Rβ (Tyr1135/1136) and a dominant band was detected at ~95 kDa (Figure 5b). The existence of multiple bands above 95 kDa may be because of additional post-transcriptional modification of IGF-1Rβ. Although no significant regional differences in pIGF-1Rβ were observed, there was a significant interaction ($P=0.016$) between region and aging (Figure 5d). Activated IGF-1 receptors remained stable across the lifespan in CA1. However in CA3, activated IGF-1 receptors were much higher compared with CA1 in young animals and decreased significantly across the lifespan, falling well below CA1 levels at 37 months of age.

Phosphatase and tensin homolog (PTEN) can act as a negative regulator of AKT at the cell membrane.[17] Total cytoplasmic PTEN was detected as a single band at ~50 kDa and levels were not different across regions (Figure 6a). In contrast, PTEN levels varied as a function of age ($P<0.001$) with the highest levels observed between 17 and 28 months (Figure 6c). PTEN is regulated by multiple phosphorylation sites, including Ser370 such that the phosphorylated form (pPTEN370) is less active than the dephosphorylated form. Therefore, cytoplasmic samples were probed with an antibody specific for pPTEN370, and a band was detected at ~55 kDa (Figure 6b). The level of pPTEN370 was not influenced by age, (Figure 6d) however, pPTEN370 was significantly higher in cytoplasmic CA1 samples relative to region CA3 (Figure 6e). As pPTEN370 should enhance the level of pAKT, the activity of PTEN does not seem to explain the reduced level of nuclear pAKT in region CA1.

Phosphatases directly acting on AKT contribute to regional differences in nuclear pAKT. The serine/threonine phosphatase protein phosphatase 2A (PP2A) turns off AKT activity by dephosphorylation of AKT at Thr308.[18] CA1 and CA3 nuclear fractions were probed with an antibody against the PP2A-A subunit, and a single band was detected at ~60 kDa (Figure 7a). Comparing CA1 and CA3 hippocampi across age groups showed no significant regional or age differences in total PP2A-A, suggesting that nuclear PP2A-A levels do not underlie the robust regional differences in active AKT (Figure 7c).

The PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP1) dephosphorylates AKT at Ser473 in a highly specific fashion.[19] Examination of nuclear fractions for total PHLPP1 (Figure 7b) indicated that PHLPP1 tended ($P=0.096$) to increase with age from 17 months to 38 months (Figure 7d). Furthermore, significantly higher levels of

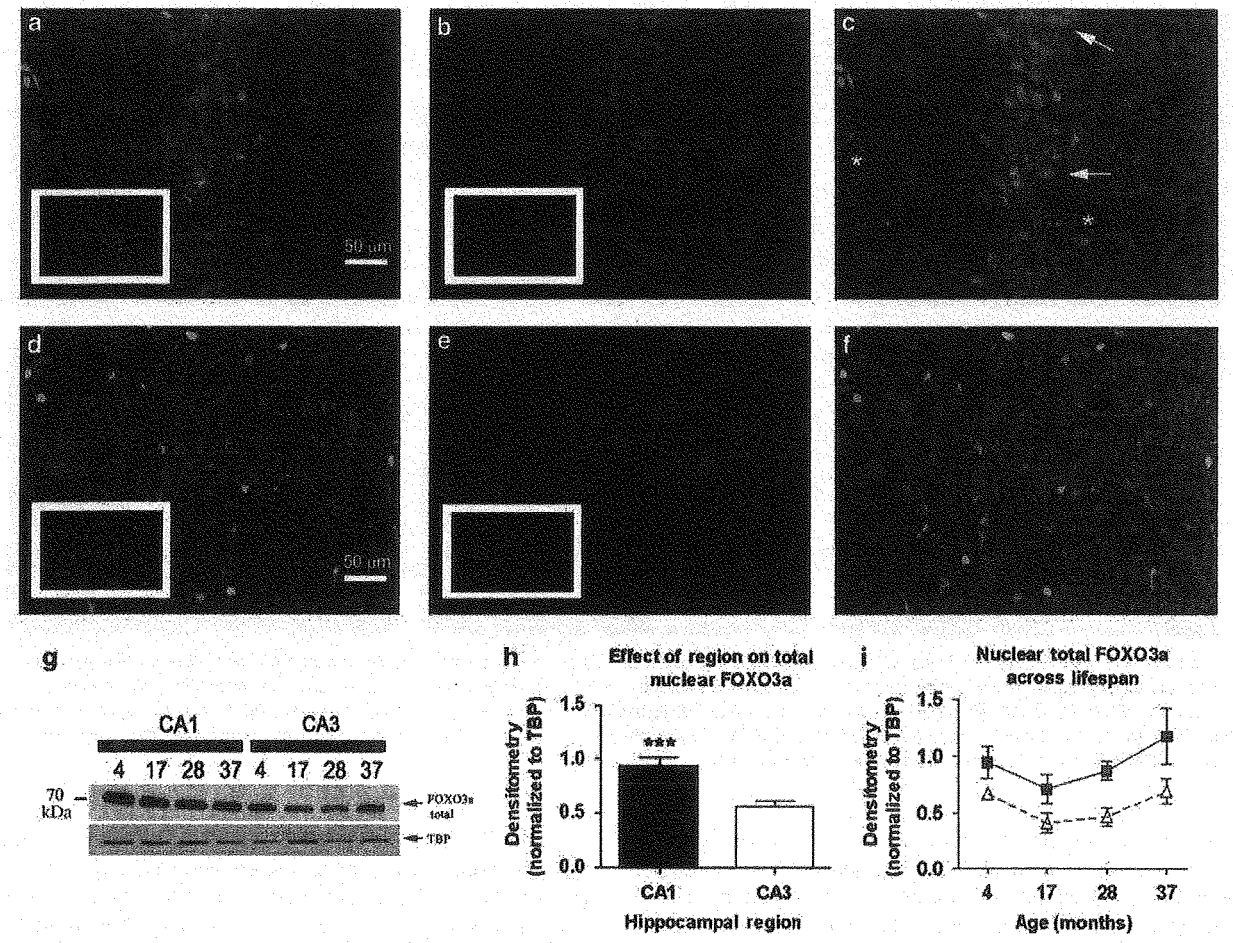

Figure 3 Nuclear total FOXO3a is lower in region CA3 and higher in region CA1. (**a** and **d**) Immunofluorescence showing CA1 and CA3 neuronal marker tubulin III (green), and nuclei (blue). White boxes in the lower left indicate secondary antibody-only controls. No green staining was observed. (**b** and **e**) Immunofluorescence showing CA1 and CA3 total FOXO3a (red). More staining is observed in region CA1 compared with CA3. No staining was observed in the secondary antibody-only controls. (**c** and **f**) Merged images of panels **a/b** and **d/e** showing total FOXO3a. FOXO3a is localized to the nuclei and perinuclear areas of CA1 and CA3 pyramidal neurons. White arrows indicate intense nuclear total FOXO3a CA1 staining. White asterisks indicate non-neuronal nuclei. (**g**) Representative blot showing nuclear CA1/CA3 total FOXO3a (top blot) and TBP (bottom blot) from animals aged 4, 17, 28, and 37 months. (**h**) Mean normalized density averaged across ages ($n=15$) in CA1 (filled bars) and CA3 (open bars). (**i**) Nuclear FOXO3a plotted as a function of age for CA1 (filled squares) and CA3 (open triangles), for animals aged 4 ($n=4$), 17 ($n=3$), 28 ($n=4$), and 37 ($n=4$) months

PHLPP1 were observed in CA1 nuclear samples compared with CA3 (Figure 7e). Consistent with recent reports that PHLPP1 dephosphorylates pAKT473, the variability in the level of nuclear pAKT473 in CA1 and CA3 regions across the lifespan was negatively correlated with levels of nuclear PHLPP1 ($P<0.001$) (Figure 7f). In agreement with western blot analysis, immunofluorescent staining from a 28-month-old animal indicated more CA1 PHLPP1 (red) compared with area CA3 (Figure 7g and h). To verify antibody specificity, nuclear enriched fractions were probed with the same PHLPP1 antibody used for immunofluorescence, and a single dominant band was observed at approximately 145 kDa (Supplementary Figure 1).

Cytoplasmic pAKT473 was reduced in CA1 across the lifespan, and immunofluorescence showed higher cytoplasmic CA1 PHLPP1 (in addition to nuclear CA1 PHLPP1). Therefore, we examined whether cytoplasmic PHLPP1 levels were higher in area CA1 across lifespan (Figure 8a). Collapsed across ages, cytoplasmic CA1 PHLPP1 was significantly higher compared with CA3 (Figure 8b). However, there was a significant interaction ($P=0.036$) between aging and region. Though CA3 cytoplasmic PHLPP1 remained low across the lifespan, CA1 PHLPP1 showed more variation; significantly dropping from 4 to 17 months of age, and significantly rising from 17 to 28 months of age (Figure 8c).

To support our conclusion that PHLPP1 contributes to the regional differences in hippocampal nuclear pAKT across lifespan, we were interested in probing an additional signaling protein known to be dephosphorylated by PHLPP1, but independent from the AKT pathway. Recently, Gao *et al.* (2008)[20] showed that knockdown of PHLPP increased phosphorylation of protein kinase Cα (PKCα) *in vitro*. Therefore, we probed for regional nuclear differences in total PKCα (Figure 9a) and PKCα phosphorylated at Ser 657 (pPKCα657; Figure 9b). Both showed a prominent band at ~75 kDa. No change in nuclear total PKCα was observed for region or age.

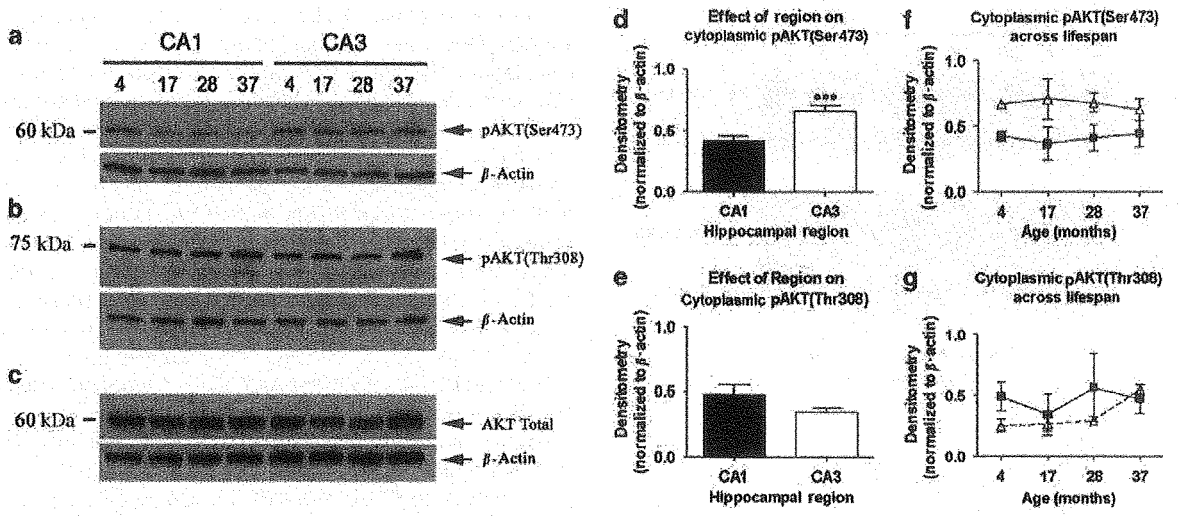

Figure 4 Only AKT phosphorylated at Ser473 is increased in cytoplasmic fractions from region CA3 compared with region CA1. (**a**–**c**) Representative blots of cytoplasmic CA1/CA3 pAKT473, pAKT308, and total AKT, from animals aged 4, 17, 28, and 37 months (top blots) and β-actin cytoplasmic loading control (bottom blots). (**d** and **e**) Mean cytoplasmic pAKT averaged across ages for CA1 ($n=14$; filled bars) and CA3 ($n=15$; open bars). (**f** and **g**) No effect of age on cytoplasmic pAKT in area CA1 (filled squares) and CA3 (open triangles) for animals aged 4 ($n=4$), 17 ($n=3$), 28 ($n=4$), and 37 (CA1 $n=3$/CA3 $n=4$) months

However, pPKCα657 was significantly higher in area CA3 consistent with our observations that PHLPP1 was lower in this region (Figure 9c). No effect of age was observed for pPKCα657 (Figure 9d).

Discussion

Regional differences in AKT survival signaling. The main finding of this study was an increase in the activity of the AKT pathway in CA3 relative to CA1. AKT is activated by phosphorylation at Thr308 and Ser473 and we observed increased levels of pAKT in region CA3 in the absence of regional differences in total AKT expression. Nuclear pAKT473 was approximately 50% higher in area CA3 compared with CA1, and cytoplasmic pAKT473 was approximately 25% higher in area CA3 compared with cytoplasmic CA1. Further, nuclear pAKT308 was increased in area CA3. In addition, regional differences in the AKT nuclear target substrate, FOXO3a, were consistent with differences in the level of pAKT. FOXO proteins have been shown to play a pro-apoptotic role in the hippocampus, and are activated under ischemic conditions.[21] AKT negatively regulates the pro-apoptotic protein FOXO3a through the shuttling and degradation of FOXO3a.[22] This regulation involves AKT-mediated phosphorylation of FOXO3a at Ser253,[23] and the level of pFOXO3a253 provides a measure of AKT activity. Examinations of pFOXO3a253 with immunofluorescence indicated increased phosphorylation in region CA3, confirming that AKT signaling is particularly high within the nuclei of CA3 pyramidal neurons. The phosphorylation of FOXO3a is the primary step in translocation of FOXO3a out of the nucleus for degradation in the cytoplasm.[24] Thus, despite the increase in pFOXO3a253, total FOXO3a was significantly reduced in nuclear fractions from hippocampal CA3 compared with CA1. The results on pAKT and FOXO3a are consistent with the increase in the level of AKT activity in region CA3 and suggest that the differential regional vulnerability is related to decreased AKT signaling in region CA1.

In addition to AKT/FOXO3a protein levels, robust regional differences were observed for phosphorylated PKCα. Studies suggest that activated PKCα can also play an important role in cell survival and growth.[25] Moreover, marked down regulation of PKCα and other PKC isoforms in the vulnerable CA1 hippocampal region has been observed after hippocampal ischemic injury.[26] Down regulation of PKCα after ischemia was not seen in damage-resistant hippocampal regions. Therefore, the increase of phosphorylated nuclear PKCα in region CA3 may also help to protect against ischemic damage across the lifespan.

Regulation of AKT survival signaling. We were interested in determining whether differences in AKT regulators might help to show the mechanism responsible for regional differences in hippocampal nuclear pAKT. The IGF-1/PI3 kinase pathway is a classic activator of AKT. IGF-1 binding to the IGF-1α receptor subunit leads to autophosphorylation/activation of IGF-1Rβ receptor subunit, and downstream phosphorylation of AKT. It has been reported that IGF-1 receptor levels are higher in CA3 relative to CA1 in young and old animals.[27] Therefore, we expected to see higher levels of CA3 IGF-1Rα expression across the lifespan. However, we found no regional variation in IGF-1Rα total, suggesting IGF-1 receptor levels are not responsible for robust increase in CA3 pAKT. Differences in our results may be explained by the aforementioned study using radioligand-binding assays, in which IGF-1 ligand was used as the probe to evaluate receptor totals. As the brain and hippocampus express IGF-1 binding proteins and receptors, studies using IGF-1 binding do not differentiate the IGF-1 receptor from IGF-1 binding proteins.

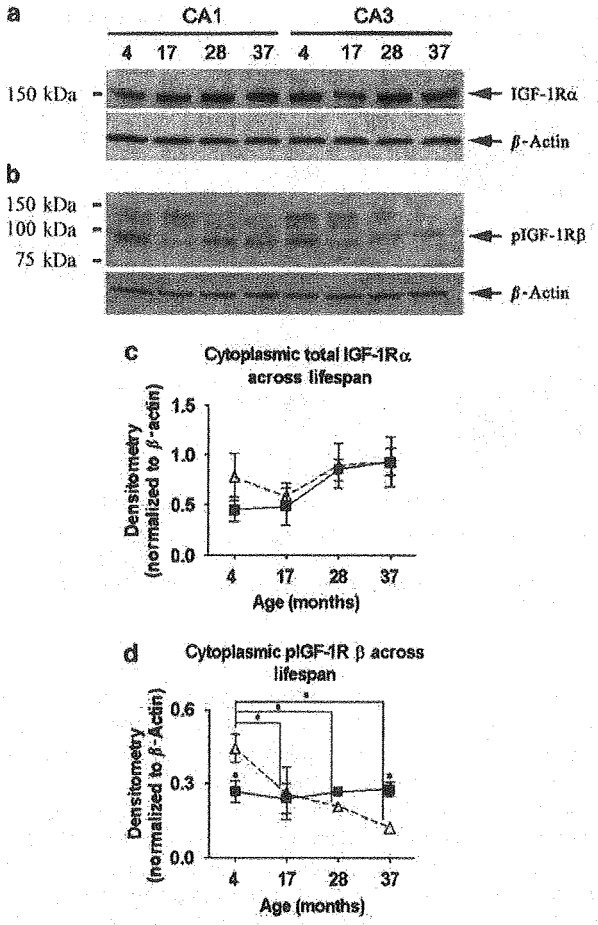

Figure 5 IGF-1 receptors do not predict regional differences in pAKT. (**a** and **b**) Representative blots showing total cytoplasmic IGF-1Rα and cytoplasmic pIGF-1Rβ from animals aged 4, 17, 28, and 37 months (top blots) and cytoplasmic loading control β-actin (bottom blots). (**c**) No effect of age or region for cytoplasmic IGF-1Rα in area CA1 (filled squares) and CA3 (open triangles) in animals aged 4 ($n=4$), 17 ($n=3$), 28 ($n=4$), and 37 ($n=4$) months. (**d**) No effect of age for cytoplasmic pIGF-1Rβ in area CA1 (filled squares). However, there is a significant age-dependent decline in area CA3 (open triangles) for animals aged 4 ($n=4$), 17 ($n=3$), 28 ($n=4$), and 37 ($n=4$) months. Solid lines indicate aging differences in CA3 pIGF-1Rβ

We next determined whether regional differences in IGF-1 receptor activity, as measured by IGF-1Rβ phosphorylation, might explain regional differences in pAKT. Intriguingly, though no change in CA1 IGF-1Rβ phosphorylation was observed, there was an age-dependent decline in CA3 IGF-1Rβ phosphorylation. However, decreased CA3 IGF-1Rβ phosphorylation did not reflect differences in either CA3 pAKT473 or pAKT308 levels. The data suggest that (1) IGF-1 does not mediate the observed increase in CA3 pAKT levels, and (2) aging differentially impacts CA1 and CA3 IGF-1 receptor activity. In support for this conclusion, earlier studies in old rats showed that intraventricular perfusion of IGF-1 failed to correct an age-dependent reduction in glucose metabolism in area CA3, however, it did improve glucose metabolism in area CA1.[28]

The phosphatases PTEN and PP2A also regulate pAKT levels. PTEN works upstream to prevent AKT activation in the cytoplasm, whereas PP2A works downstream to dephosphorylate nuclear pAKT. However, no regional differences in cytoplasmic PTEN or nuclear PP2A were observed. Unexpectedly, phosphorylated (i.e. inhibited) PTEN was increased in cytoplasmic samples from region CA1. Initial findings suggest that PTEN and PP2A do not mediate the observed regional differences in nuclear pAKT across the lifespan. Nevertheless, it should be noted that many additional post-transcriptional modifications can alter phosphatase activity of PTEN[29] and PP2A.[30] As we did not measure phosphatase activity directly, the potential contribution of PTEN and PP2A activity to pAKT levels cannot be addressed by these experiments. The role of phosphatase activity deserves future investigation; particularly because PTEN and PP2A play a direct role in the regulation of pAKT308.

In contrast to PTEN and PP2A, regional differences were observed for the expression of the phosphatase PHLPP1. Current knowledge of how PHLPP phosphatase activity is regulated remains limited. PHLPP exists as two isoforms, PHLPP1 and PHLPP2, of which the first isoform has two splicoforms, PHLPP1α (~135 kDa) and PHLPP1β (~190 kDa).[31] Studies by Gao *et al.* (2005)[19] showed that PHLPP1α directly binds and dephosphorylates pAKT at Ser473. These researchers found that knockdown of PHLPP1 in 293T cells caused an upregulation of endogenous phosphorylated PKCα. To date, no phosphorylation sites or other post-transcriptional modifications of PHLPP1 have been discovered, though they likely exist. However, Shimizu *et al.* (2007)[32]showed calpain-mediated cleavage of PHLPP could downregulate phosphatase activity on target substrates in area CA1 of the hippocampus, and prevent nuclear activation of MAPK/CREB. These results suggest that proteolytic degradation of PHLPP1 is an important regulatory mechanism of phosphatase activity.

Our results are consistent with the idea that changes in total hippocampal PHLPP1 levels are important in regulating the degree of pAKT473 phosphorylation. Thus, hippocampal nuclear PHLPP1 levels matched regional differences in nuclear pAKT473 such that PHLPP1 was elevated in region CA1, and CA1 exhibited the lowest level of nuclear pAKT473. Furthermore, nuclear PHLPP1 levels were inversely correlated with pAKT473 levels across the lifespan. Finally, the observed decrease in CA1 nuclear pPKCα657, another substrate for PHLPP1, supported our conclusion that the level of nuclear PHLPP1 is important in the regulation of target nuclear substrates.

In addition to nuclear PHLPP1, cytoplasmic levels of PHLPP1 were reduced across the lifespan in area CA3 and cytoplasmic pAKT473 levels were higher across the lifespan in area CA3. Alternatively, cytoplasmic PHLPP1 collapsed across age, was significantly higher in region CA1. Altogether, the nuclear and cytoplasmic data suggest that cellular levels of PHLPP1 are higher in CA1 region and correspond to reduced cellular levels of pAKT473. It is noted that, regional differences in cytoplasmic pAKT308 were not observed. These findings support our conclusion that PHLPP1 is important in selectively regulating pAKT473 levels across the lifespan, and suggest that regional differences in nuclear

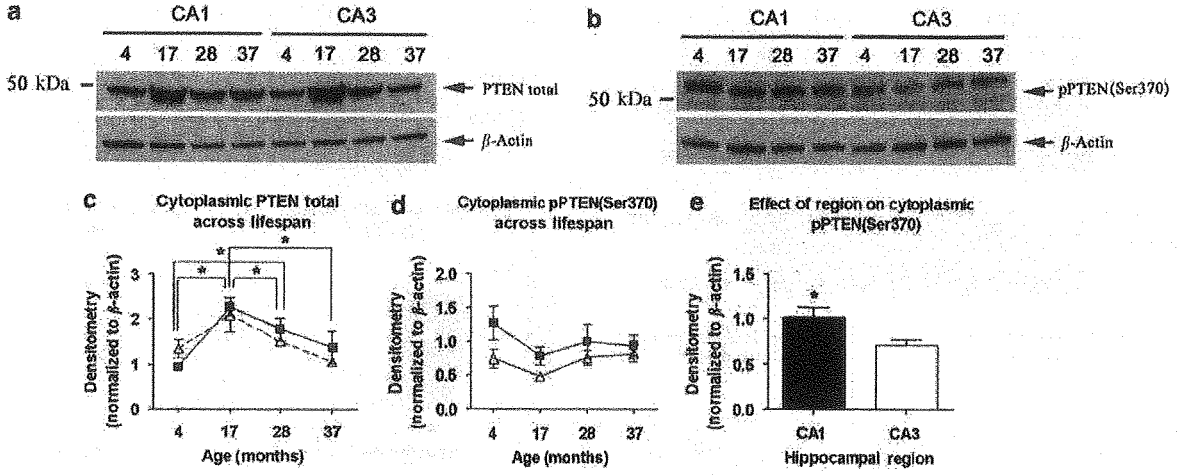

Figure 6 PTEN does not predict regional differences in pAKT. (**a** and **b**) Representative blots showing total PTEN, and pPTEN370 in animals aged 4, 17, 28, and 37 months (top blots) and cytoplasmic loading control β-actin (bottom blots). (**c**) CA1 (filled squares) and CA3 (open triangles) cytoplasmic total PTEN shows no significant regional effects but significant aging effects in animals aged 4 ($n=4$), 17 ($n=3$), 28 ($n=4$), and 37 ($n=4$) months. Solid lines indicate aging differences in CA1/CA3 total PTEN . (**d**) CA1 (filled squares) and CA3 (open triangles) cytoplasmic pPTENSer370 was not different across the lifespan in animals aged 4 ($n=4$), 17 ($n=3$), 28 ($n=4$), and 37 ($n=4$) months, however, (**e**) elevated levels of pPTEN370 were observed ($n=15$) in region CA1 (filled bars) relative to CA3 (open bars)

CA1/CA3 pAKT308 depend upon regulation (or dysregulation) by mechanisms distinct from PHLPP1.

Despite our data supporting PHLPP1's role in mediating regional differences in hippocampal pAKT473, additional regulation seems very likely; primarily because CA1 pAKT473 levels were remarkably stable across the lifespan in both the nucleus and the cytoplasm whereas CA1 PHLPP1 showed much more variability. One possibility is that regional differences in growth factor-mediated AKT activation, other than IGF-1, help to regulate AKT across the lifespan. Immunohistochemical studies in rat brain showed that BDNF levels are extremely high in area CA3 and extremely low in area CA1.[33] Further, these differences are maintained across the lifespan.[34] As BDNF can activate AKT, and has been shown to rapidly inhibit FOXO3a nuclear localization,[35] BDNF signaling may also contribute to the observed maintenance of pAKT across the lifespan. However, comparative studies of growth factor-mediated survival signaling in hippocampal cell culture show that IGF-1 strongly induces AKT although it weakly induces ERK, whereas BDNF strongly induces ERK and only weakly induces AKT.[36] Therefore, whether BDNF contributes to sustained AKT activity *in vivo* and whether its contribution is large or small remains unknown and deserves further investigation.

AKT activity across the lifespan. Aging-dependent dysregulation of proteins that mediate survival signaling may contribute to increased hippocampal vulnerability during aging. The results of this study indicate that the level of nuclear pAKT308 declines in CA1 region as age advances. Alternatively, CA1 pAKT473 was stable across the lifespan. This raises the question of whether the loss in dual pAKT exacerbates vulnerability of CA1 region as age advances. Recent *in vitro* studies examining the regulation of AKT phosphorylation and AKT activity provided evidence that dual phosphorylation of pAKT308 and pAKT473 are temporally associated/coordinated, and together predicted AKT activity.[37] In addition, studies in NIH3T3 cells showed that a pAKT308 mutant failed to activate when treated with growth factor, whereas a pAKT473 mutant showed reduced activity; thus dual phosphorylation seems to be required for full AKT kinase activity.[38] In aged animals, decreased CA1 pAKT308 levels would predict reduced total AKT activity even though pAKT473 remained stable.

It is unclear what mechanism(s) underlie the age-related decline in nuclear CA1 pAKT308. One possible explanation may be that aging differentially affects nuclear CA1/CA3 membrane phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) levels. AKT can be activated in the nucleus by identical signaling mechanisms observed at the cell membrane.[39] However, before AKT can be phosphorylated at Thr308, PI3 kinase must be activated upstream. When activated, PI3 kinase will phosphorylate membrane bound PIP_2 to PIP_3. PIP_3 will then allow both AKT and PIP_3-dependent kinase 1 (PDK-1) to bind the inner membrane through their PH domains. PDK-1 is responsible for phosphorylating AKT at Thr308, and binding of both enzymes to PIP_3 is critical to allowing PDK-1 access to AKT's active site at Thr308. Therefore, changes in the nuclear CA1 PIP_3 levels across the lifespan could alter the nuclear CA1 AKT308 levels. However, little is known about the regulation and control of nuclear PIP_3, let alone how aging might influence nuclear PIP_3 levels.

Alternatively, Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-KK) can selectively and directly phosphorylate AKT at Thr308 but not on Ser473.[40] Intriguingly, CaM-KKα is found only in the nucleus, where it acts to regulate its better known substrate Ca^{2+}/calmodulin-dependent protein kinase IV. As Ca^{2+} imbalance plays an important role in hippocampal CA1 loss of function with aging, altered hippocampal CaM-KK activity with age may also play a role in selectively altering nuclear pAKT308 phosphorylation across the lifespan in area CA1. This idea remains to be tested.

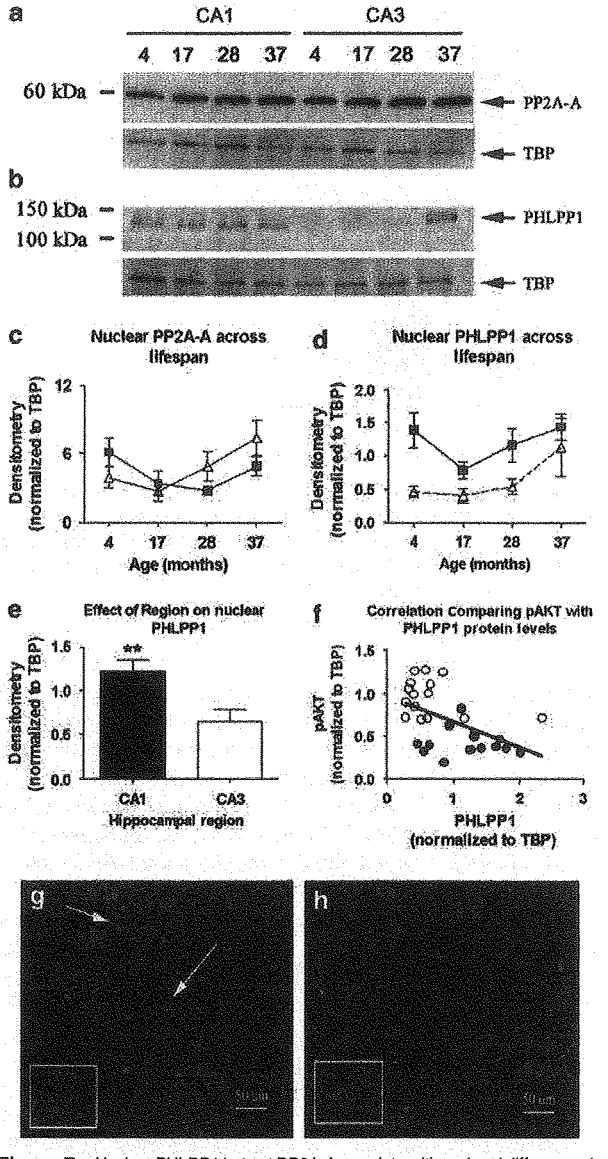

Figure 7 Nuclear PHLPP1 but not PP2A-A correlate with regional differences in nuclear pAKTSer473. (**a** and **b**) Representative blots showing nuclear total PP2A-A and PHLPP1 in animals aged 4, 17, 28, and 37 months (top blots) and TBP (bottom blots) (**c**) CA1 (filled squares) and CA3 (open triangles) nuclear PP2A-A across the lifespan. (**d**) CA1 (filled squares) and CA3 (open triangles) nuclear PHLPP1 had a tendency to increase with age, and (**e**) significant regional differences ($n = 15$) were observed in CA1 (filled bars) relative to CA3 (open bars). (**f**) Linear regression of combined CA1 (dark circles)/CA3 (open circles) samples across the lifespan showing PHLPP1 levels correlate with pAKTSer473 ($r^2 = 0.27$). (**g** and **h**) Immunofluorescence showing CA1 and CA3 PHLPP1 (red) and nuclei stained with DAPI (blue). White border squares in the bottom left indicate secondary antibody-only controls. White arrow heads indicate nuclear and perinuclear regions of intense PHLPP1 staining in area CA1. Animals aged 4 ($n = 4$), 17 ($n = 3$), 28 ($n = 4$), and 37 ($n = 4$) months were used for lifespan graphs

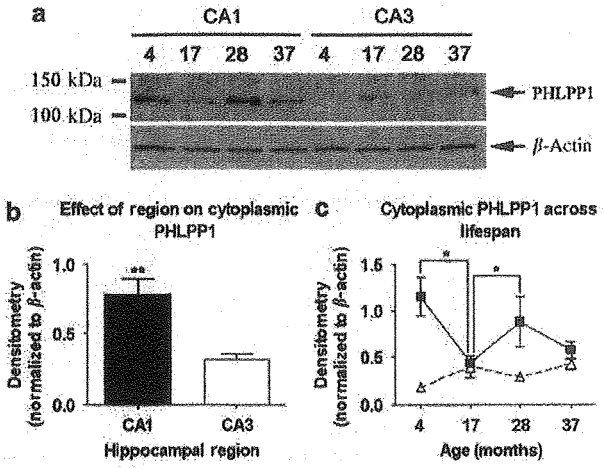

Figure 8 Cytoplasmic PHLPP1 is increased in area CA1. (**a**) Representative blot showing cytoplasmic total PHLPP1 in animals aged 4, 17, 28, and 37 months (top blot) and β-actin (bottom blot) (**b**) Mean normalized densitometry averaged across ages ($n = 15$) in CA1 (filled bars) and CA3 (open bars). Significant regional differences in cytoplasmic PHLPP1 ($n = 15$) were observed in area CA1 (filled bars) relative to area CA3 (open bars). (**c**) CA1 PHLPP1 show significant variation across the lifespan (filled squares). Solid lines indicate aging differences in CA1 PHLPP1. Alternatively CA3 (open triangles) cytoplasmic PHLPP1 showed reduced and stable levels across the lifespan. Animals aged 4 ($n = 4$), 17 ($n = 3$), 28 ($n = 4$), and 37 ($n = 4$) months were used for lifespan graph

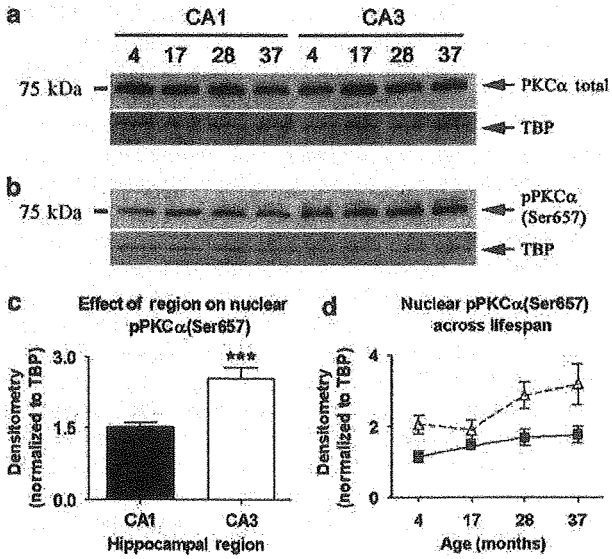

Figure 9 Regional differences in nuclear pPKCα657. (**a** and **b**) Representative blots showing nuclear CA1/CA3 total PKCα and pPKCα657 levels in animals aged 4, 17, 28, and 37 months (top blots) normalized to TBP (bottom blots). (**c**) Mean normalized densitometry averaged across ages ($n = 15$) in CA1 (filled bars) and CA3 (open bars). Significantly higher levels of pPKCα657 are observed in the nucleus of area CA3. (**d**) CA1 (filled squares) and CA3 (open triangles) nuclear pPKCα657 shows no significant aging effects. Animals aged 4 ($n = 4$), 17 ($n = 3$), 28 ($n = 4$), and 37 ($n = 4$) months were used for lifespan graph

Altogether, the results suggest that the regional hippocampal differences in the AKT pathway may contribute to regional differences in neuronal vulnerability to certain stressors, and help protect CA3 neurons from pathology associated with age-dependent disease. In support for these conclusions, the pro-apoptotic protein FOXO3a was higher in area CA1 across lifespan and actively inhibited in pyramidal neuron nuclei of region CA3. Further, nuclear PHLPP1 was higher in CA1

Table 1 Antibody list

Antibody	Concentration	Host	Clonality	Company
TBP	1 : 1000	Mouse	Monoclonal	ABR
β-Actin	1 : 8000	Chicken	Polyclonal	Abcam
p(Thr308)AKT	1 : 1000	Rabbit	Monoclonal	Cell signaling
p(Ser473)AKT	1 : 1000	Rabbit	Polyclonal	Cell signaling
AKT total	1 : 1000	Rabbit	Polyclonal	Cell signaling
FOXO3a	1 : 1000	Rabbit	Polyclonal	ABR
pFOXO3a253 (Immuno-Fluo)	2 μg/ml	Rabbit	Polyclonal	Abcam
FOXO3a (Immuno-Fluo)	1 : 100	Rabbit	Monoclonal	Cell signaling
IGF-1Rα	1 : 200	Rabbit	Polyclonal	Santa Cruz
pIGF-1Rβ	1 : 1000	Rabbit	Monoclonal	Cell signaling
p(Ser370)PTEN	1 : 1000	Rabbit	Polyclonal	Abcam
PTEN total	1 μg/ml	Rabbit	Polyclonal	Abcam
PP2A-A	1 : 1000	Mouse	Monoclonal	Cell signaling
PHLPP1	1 : 200	Goat	Polyclonal	Santa Cruz
PHLPP1 (Immuno-Fluo)	1 : 50	Rabbit	Polyclonal	Cayman
p(Ser657)PKCα	0.5 μg/ml	Rabbit	Polyclonal	Upstate
PKCα total	0.4 μg/ml	Mouse	Monoclonal	Upstate
Neuronal β-tubulin III	1 : 1000	Mouse	Monoclonal	Abcam

AKT, protein kinase B; pAKT, phosphorylated AKT; FOXO3a, forkhead box O3a transcription factor; pFOXO3a, phosphorylated FOXO3a; IGF-1, insulin-like growth factor 1; pIGF-1, phosphorylated IGF-1; pPTEN, phosphorylated PTEN; PHLPP1, PH domain and leucine-rich repeat protein phosphatase 1; PKCα, protein kinase Cα; pPKCα, phosphorylated PKCα; PTEN, phosphatase and tensin homolog; TBP, TATA box binding protein

region and correlated with levels of nuclear pAKT473. To the best of our knowledge, these findings are novel and suggest a new hypothesis to explain the mechanisms for CA1 vulnerability to certain kinds of stressors.

Materials and Methods

Aged animals. NIA Fischer 344/Brown Norway rats were kept in specific pathogen free (SPF) housing and maintained on a 12 h light/dark cycle. Animals were aged 4 ($n=4$), 17 ($n=3$), 28 ($n=4$), and 37 ($n=4$) months, and fed a standard *ad libitum* diet. Animals were killed by CO_2 asphyxiation and decapitated. Hippocampi were removed, separated into CA1 and CA3 sections, and flash frozen in liquid nitrogen. Samples were stored at −80°C until further processing.

Homogenization & nuclear enrichment. Tissues were homogenized using NER nuclear separation kit (PIERCE) according to the manufacturer's instructions. Briefly, frozen samples (~30–40 mg of tissue/sample) were removed from −80°C freezer and immediately placed in a 2 ml glass dounce homogenizer (Kimble–Kontes) containing ice-cold CERI buffer (for cytoplasmic fraction) with 1.5 × protease inhibitors, 1 × EDTA, and 2 × phosphatase inhibitors (PIERCE). Samples were then homogenized using 11 strokes with pestle B and 10 strokes with pestle A, and transferred to 0.5 ml tubes. After sitting on ice for 10 min, samples were briefly vortexed and spun at 16 000 × *g* at 4°C for 5 min. Samples were then placed back on ice and supernatant was collected and saved for cytoplasmic protein analysis. Next, NERI buffer (for nuclear fraction), containing 1.5 × protease inhibitors, 1 × EDTA, and 2 × phosphatase inhibitors (PIERCE) was added to the cell pellet. Samples were vortexed every 10 min for 40 min and spun at 16 000 × *g* at 4°C for 10 min. The supernatant (containing nuclear enriched protein fraction) was collected and stored for later analysis.

Western blot. Protein concentrations were determined using BSA method (PIERCE). Kaleidoscope protein standards (Bio-Rad) and CA1/CA3 samples (20 μg/lane) were loaded on 4–15% gradient gels (Bio-Rad) and run for 1 h at 120 V. Proteins were then transferred to PDVF membranes (Amersham) overnight at 50 V/4°C. Blots were then stained with Ponceau S and photocopied. Blots were washed 5 min in T-BST and subsequently blocked in T-BST (7% milk) for 1 h. Primary antibodies were then applied to blots overnight at 4°C (Table 1), washed three times with TBS, and secondary antibodies applied for 2 h at room temperature. Blots were then developed using ECL Plus Western Blot Detection Kit (Amersham) on biomax film (Kodak). Blots were scanned using 6500 scanner (Bio-Rad), and densitometry determined using UNSCAN IT software (silk scientific).

Immunofluorescence. A 28-month-old Fisher 344/BN rat was anesthetized and perfused with 4% paraformaldehyde. The brain was removed, placed in paraformaldehyde for 1 h, and transferred to 30% sucrose solution for 72 h. After fixation, the brain was embedded in optimal cutting compound and 8 μm coronal sections were made using a cryostat. Slices were collected on Superfrost Plus glass slides (Fisher), and allowed to air dry for 1 h. Slides were then washed with PBS and permeabilized with 0.05% TritonX-100/PBS for 15 min. Slides were washed again in PBS and treated with 1% SDS/PBS for 5 min for mild antigen retrieval. Sections were then washed in PBS, blocked with 20% goat serum (in 1% BSA PBS) for 2 h, and incubated overnight (at 4°C) in 3% goat serum PBS containing rabbit anti-phospho or total FOXO3a + mouse anti-neuronal tubulin III or rabbit anti-PHLPP1. Slides were then washed with PBS and incubated with secondary antibodies (Alexa Fluor goat anti-rabbit 594/goat anti-mouse 488; Invitrogen) for 1.5 h. Slides were then stained with 3% Sudan Black B in 70% ethanol for 10 min to remove lipofuscin autofluorescence. Finally, slides were mounted (Prolong Gold Anti-Fade with DAPI; Invitrogen) and images were taken on an Axiovert 40 CFR fluorescent microscope (Zeiss).

Statistical analysis. Western blot densitometry was analyzed using three way ANOVA (NCSS Statistical Software); data significant at $P<0.05$. *Post hoc* analysis was carried out using Fisher LSD test. All graphs were produced using Prism software (Silk Scientific).

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)

AAV-Mediated Gene Therapy for Retinal Degeneration in the *rd10* Mouse Containing a Recessive PDEβ Mutation

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Purpose. To test AAV-mediated gene therapy in the *rd10* mouse, a natural model of recessive RP caused by mutation of the β-subunit of rod photoreceptor cGMP phosphodiesterase.

Methods. One eye of a cohort of *rd10* mice kept in a dark environment was subretinally injected at postnatal day (P) 14 with 1 μL AAV5-smCBA-PDEβ. The contralateral eye was not injected. The animals were then maintained for 2 weeks in the dark before they were moved to a normal 12-hour light/12-hour dark cycling light environment for visually guided behavioral training. Three weeks after injection, treated *rd10* mice were examined by scotopic and photopic electroretinography and then killed for biochemical and morphologic examination.

Results. Substantial scotopic ERG signals were maintained in treated *rd10* eyes, whereas untreated eyes in the same animals showed minimal signals. Treated eyes showed photopic ERG b-wave amplitudes similar to those of the normal eyes; in untreated partner eyes, only half the normal amplitudes remained. Strong PDEβ expression was observed in photoreceptor outer segments only in treated eyes. Light microscopy showed a substantial preservation of the outer nuclear layer in most parts of the treated retina only. Electron microscopy showed good outer segment preservation only in treated eyes. A visually guided water maze behavioral test under dim light showed significantly improved performance in one eye–treated *rd10* mice compared with untreated mice.

Conclusions. These data demonstrate that P14 administration of AAV5-smCBA-PDEβ can prevent retinal degeneration in *rd10* mice, as reflected by significant structural, biochemical, electrophysiological, and behavioral preservation/restoration. These results serve as a baseline for studying long-term retinal rescue in *rd10* mice. (*Invest Ophthalmol Vis Sci.* 2008;49:4278–4283) DOI:10.1167/iovs.07-1622

Retinal degeneration (RD) is a large family of inherited dystrophies characterized by photoreceptor dysfunction and eventual photoreceptor death. As many as 17 million persons worldwide have vision loss associated with RD, including patients with retinitis pigmentosa (RP), a disease for which no cure exists. A description of autosomal recessive mutations associated with retinal degeneration dates back to the discovery of the "rodless retina" mouse by Keeler in 1924, which later became known as the *rd1* mouse.[1–3] The *rd1* mouse carries mutations in a gene (*Pde6b*) that encodes the β-subunit of rod photoreceptor cGMP phosphodiesterase (PDEβ).[3,4] The biochemical result is a nonfunctional PDEβ and an accumulation of cGMP.[5] Mutations in the human ortholog of *Pde6b* have been linked to autosomal recessive RP.[6,7] In the *rd1* mouse, photoreceptor degeneration begins at 1 week of age, when photoreceptor outer segments first begin to mature.[8] Rods degenerate first, then cones; the culmination is complete ablation of photoreceptors by about 4 weeks of age.[1,2,8–12] Until recently, the *rd1* mouse was considered one of the best models of human autosomal recessive RP; however, because of the rapid rate of photoreceptor cell loss, providing effective, lasting gene replacement therapy has proven difficult. Subretinal injection of adenoviral, retroviral, or adenoassociated viral vectors encoding the PDEβ gene to neonatal *rd1* mice resulted in partial preservation of photoreceptor structure but little, if any, ERG rescue.[13–16] Because it takes at least 1 week for the viral vector–mediated gene to express in the retina and *PDEβ* is expressed in the retina by postnatal day (P)5 to P6, prenatal gene therapy in *rd1* mice may be considered and has been achieved in mice with the RPE65 form of Leber congenital amaurosis.[17] The course of retinal degeneration in humans, however, though often quicker than the course for other mutations, still takes decades. Thus, questions remain as to why the *rd1* mouse has been relatively unresponsive to standard gene replacement therapy[15] and, in particular, whether any element of recessive mutation in PDEβ makes the resultant retinal degeneration so rapid that the mice are refractory to gene replacement therapy in the mature photoreceptor.

A more recently identified mouse strain that exhibits autosomal recessive retinal degeneration, the *rd10* mouse, has a point mutation in exon 13 of the *Pde6b* gene.[18] Recent natural history studies of the *rd10* mouse indicate that it better emulates the slow progression of typical human autosomal recessive RP than the previously described *rd1* mouse.[19,20] Loss of photoreceptors in the *rd10* mouse begins after 2 weeks of age, with peak photoreceptor death occurring at P25.[20] By 5 weeks most photoreceptor cells have been lost.[19,20] This rate of photoreceptor loss is substantially slower than in the *rd1* animal. Importantly, most photoreceptors in the *rd10* retina are lost after the retina has terminally differentiated, whereas

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peak photoreceptor cell death in *rd1* occurs before this developmental stage. In addition, it has been found that rearing *rd10* mice in darkness further slows the rate of degeneration by as much as 4 weeks.[19] Taken together, these findings suggest that retinal degeneration in the *rd10* mouse is more analogous to the human condition and perhaps is better suited than the *rd1* mouse for testing PDEβ gene replacement therapy. Recently, studies using stem cell, antiapoptotic, and antioxidant therapies have shown a measure of retinal rescue in *rd10* mice,[21–23] but the potential for gene therapy has not yet been reported.

Adenoassociated virus (AAV)-mediated gene replacement has already proven to be effective for restoring retinal function and for protecting photoreceptor structure in a number of mouse models of retinal disease.[24–26] This approach has been optimized with the use of AAV serotypes that preferentially target photoreceptors in conjunction with ubiquitously expressed constitutive promoters that drive stronger transgene expression than obtained when using cell-specific promoters.[26] The size of the *Pde6b* cDNA (2.6 kb) and the packaging limitation of AAV vectors (4.5 kb, excluding the required terminal DNA sequences) require the use of a relatively small promoter that directs strong and lasting expression. In this study, an AAV serotype 5 vector (AAV5) containing minimal chicken β-actin promoter/CMV enhancer (smCBA) was used to deliver the *Pde6b* gene to the *rd10* mouse retina. The purpose of this gene replacement strategy was to determine whether retinal degeneration could be delayed in this new murine model of human autosomal recessive PDEβ-based RP, the *rd10* mouse.

Materials and Methods

Animals

C57BL/6J mice and the congenic inbred strain of *rd10* mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at the University of Florida. Except where otherwise indicated, all mice were maintained in the University of Florida Health Science Center Animal Care Services Facilities under a 12-hour light/12-hour dark cycle with less than 15 ft-c environmental illumination. All experiments were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with National Institutes of Health regulations.

Construction of AAV Vectors

AAV5 vectors exhibit higher transduction efficiency in photoreceptors and a faster onset of expression than other AAV serotypes when delivered to the subretinal space (Auricchio A, et al. *IOVS* 2001;42: ARVO Abstract 125)[27] and were, therefore, used for packaging the current vector. Vector plasmids were constructed as previously described.[28] Wild-type murine *Pde6b* cDNA was placed under the control of the ubiquitous, constitutive smCBA promoter[28] to generate pTR-smCBA-PDEβ. Previously, we have shown that the smCBA promoter drives efficient and long-term transgene expression when targeted to photoreceptors through AAV5 (Boye SL, et al. *IOVS* 2006;47:ARVO E-Abstract 852). AAV vectors were packaged and purified according to previously reported methods.[25]

Subretinal Injections

Late-term pregnant *rd10* females were kept in a continuously dark room, except for husbandry at 5 lux or less, and then pups were raised under these same conditions. When the pups were 14 days old, 1 μL AAV5-smCBA-PDEβ (1×10^{10} genome containing vector particles) was subretinally injected into one eye under dim light, and the animals were maintained for 2 more weeks in the same dark environment before they were moved to normal 12-hour light/12-hour dark vivarium cycling room light. The other eye remained uninjected. Subretinal injections were made under direct observation aided by a dissecting microscope under dim light. The injected retinal area was visualized by fluorescein-positive subretinal blebs demarcating the retinal detachment. Such detachments usually resolved within 1 to 2 days. Only animals with minimal surgical complications and initial retinal blebs occupying more than half the retina were retained for further evaluation.[26] Approximately 20 *rd10* mice met these criteria, which allowed at least three animals for each experiment. After all injections, 1% atropine eye drops and neomycin/polymyxin B/dexamethasone ophthalmic ointment were given.

Electroretinography

Three weeks after subretinal injection (P35; 1 week after the move to cyclic light environment), a semiautomated ERG recording instrument adapted for rodent analysis (Jaeger/Toennies) was used for ERG examination. All testing was performed in a climate-controlled and electrically isolated dark room with animals placed on a 37°C warming pad. After overnight dark adaptation, mice were anesthetized by ketamine (72 mg/kg)/xylazine (4 mg/kg) intraperitoneal injection in a dark room under dim red light illumination. Corneas were anesthetized with a drop of 0.5% proparacaine hydrochloride, and the pupils were dilated with 1% atropine and 2.5% phenylephrine hydrochloride. Small contact lenses scaled to mice with gold wire loop electrodes were placed on each cornea with a drop of 2.5% methylcellulose to maintain corneal hydration and to promote conductivity. A silver wire reference electrode was placed subcutaneously between the eyes, and a ground electrode was placed subcutaneously in a hind leg. For light-adapted electroretinography, the animals were put under a background light of 100 cd · s/m^2 for 5 minutes before the recording. Two days after ERG examination, almost all injected *rd10* mice were killed for morphologic and biochemical examination; the exception was one animal with almost 100% initial retinal detachment and minimal injection-related damage that was kept for another 2 weeks in a cyclic light environment for further ERG examination.

Immunocytochemistry for PDEβ Expression

Treated *rd10* mice were killed 2 days after ERG examination for biochemical and morphologic examination. Eyes from treated and untreated *rd10* mice, along with age-matched C57BL/6J mice, were enucleated, and the eyecups and frozen sections were processed as described previously.[29] Retinal sections were permeabilized with 0.1% Triton X-100, rinsed in PBS, blocked in 20% normal goat serum (NGS), and incubated overnight at 4°C in a rabbit polyclonal anti-mouse PDEβ antibody (ABR-Affinity BioReagents, Golden, CO), diluted 1:400 in 20% NGS. This antibody reacts with human and mouse PDEβ protein. After three rinses with 0.1 M PBS, sections were incubated in goat anti-rabbit IgG conjugated with Texas Red (1:300; Molecular Probes, Eugene, OR) and DAPI (1:100; Molecular Probes, Eugene, OR) for 2 hours, followed by 3 rinses with 0.1 M PBS. Sections were then mounted with coverslips before fluorescence photography.

Western Blot Analysis

For PDEβ measurements, eyecups were carefully dissected from treated and untreated *rd10* eyes and age-matched normal C57BL/6J eyes, pooled into separate groups (injected, uninjected, and normal, respectively), and homogenized by sonication in a buffer containing 0.23 M sucrose, 2 mM EDTA, 5 mM Tris-HCl (pH 7.5), and 0.1 mM phenylmethylsulfonyl fluoride. Samples were then centrifuged, and supernatants were collected. Protein concentrations were determined using a protein assay kit (Coomassie Plus; Pierce, Rockford, IL). After the addition of loading buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 200 mM dithiothreitol, 0.02% bromophenol blue), an equal amount (25 μg) of each sample was resolved by SDS-PAGE (10% Tris-glycine gel) and electrotransferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA). The membrane was blocked with 5% horse serum in PBS and incubated overnight with the

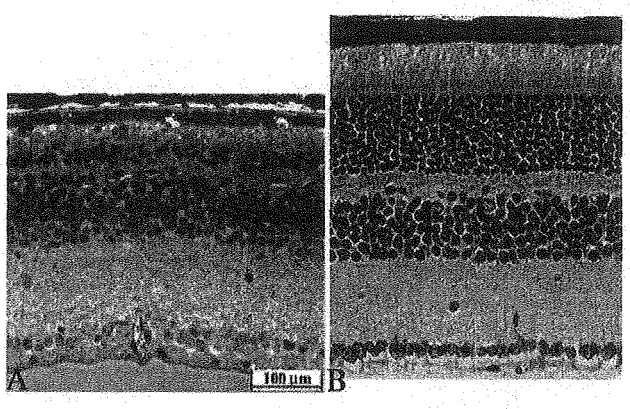

FIGURE 1. Light microscopic images of a 4-week-old *rd10* mouse reared in normal cyclic light environment (*left*) compared with very dim light (*right*). Both images are from equivalent central regions of retina. Only two to three layers of ONL nuclei with residual outer segment material remain in the cyclic light–reared animal. In the dim light–reared animal, a nearly normal complement of 9 to 10 ONL nuclei are evident with clearly improved inner and outer segment morphology.

same PDEβ polyclonal antibody. The blot was then washed three times in PBS containing 0.05% Tween-20 (PBST) and was incubated with an anti-mouse IgG–conjugated alkaline phosphatase secondary antibody for 30 minutes at room temperature. After another wash in PBS, the blot was developed with a color assay using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Treated and untreated *rd10* and normal C57BL/6J samples were compared on the same blot with β-actin as an internal loading control.

Histology and Morphometry

Structural evaluation of the treated and untreated eyes has been described.[26,29] Treated and untreated eyes from *rd10* mutant mice were enucleated, and eyecups were prepared for light and electron microscopic examination.

Visually Guided Behavioral Test

The water maze visually guided behavioral test has been described previously by us.[26] Briefly, 2 weeks after injection, one eye–treated *rd10* mice reared in a dark environment, together with age-matched untreated *rd10* and normal C57 mice, were initially trained in a plastic water tank with a platform positioned in a well-lit room. Training consisted of three blocks of four trials per day for 4 consecutive days. During each trial, the mouse was placed in the water from one of four equally spaced start locations. Behavioral data were acquired as the latency to escape to the platform during the training trials. After training in the well-lit room, the rod function of dark-adapted mice was measured using the same procedure but under very dim light (not detectable with the Datalogging Light Meter, model 401036; Extech Instruments, Waltham, MA).

RESULTS

To maintain *rd10* mice, we initially used lighting conditions that would allow a rate of retinal degeneration sufficiently slow for testing of a gene therapy approach. To that end, we maintained late-term pregnant *rd10* females in a continuously dark room, except for husbandry at 5 lux or less, and then raised the pups under the same conditions. Light microscopic images of retinas from 4-week-old *rd10* reared in a normal 12-hour light/12-hour dark cyclic light environment and from *rd10* mice raised in dim light are compared in Figure 1. Approximately three layers of photoreceptors and minimal outer segments remained in mice reared in vivarium cyclic light, whereas the outer nuclear layer (ONL) was nearly normal in dark-reared *rd10* mice. Thus, only dark-reared *rd10* mice were used to test for therapy.

Electrophysiological Rescue

At P14, *rd10* mice were subretinally injected with AAV5-smCBA-PDEβ. Three weeks after treatment (1 week after removal of mice to a cyclic light environment), *rd10* mice were examined by dark-adapted and light-adapted electroretinography. Larger dark- and light-adapted ERG responses were evident in vector-treated eyes. When the stimulus intensity was 2.68 cd · s/m^2, the average dark-adapted ERG b-wave amplitudes in PDEβ treated *rd10* eyes were 200 ± 20 μV (Fig. 2A; $n = 3$), which was 37% of the isogenic wild-type mice (544 ± 89 μV; $n = 3$) and approximately threefold higher than in contralateral untreated eyes (70 ± 40 μV; $n = 3$). Paired *t*-test analysis showed significantly smaller dark-adapted b-wave amplitudes in untreated *rd10* eyes compared with C57 eyes ($P < 0.01$). Although statistically not as good as those in normal C57 eyes ($P < 0.05$), dark-adapted b-wave amplitudes were significantly improved in treated *rd10* eyes compared with those in untreated *rd10* eyes ($P < 0.05$). Light-adapted ERG b-wave amplitudes elicited with a flash intensity of 12 cd · s/m^2 were 118 ± 25 μV in normal, 109 ± 23 μV in treated *rd10*, and 66 ± 29 μV in untreated *rd10* eyes (Fig. 2B). Statistical analysis showed similar light-adapted ERG b-wave amplitudes ($P = 0.6$) between normal C57 and treated *rd10* eyes 3 weeks after injection ($n = 6$), whereas a significant difference was found between treated and untreated *rd10* eyes ($P < 0.05$; $n = 6$). Figure 2C–D shows a representative *rd10* mouse 5 weeks after one eye received subretinal vector at P14 (P49, 3 weeks after returning to cyclic light environment). In the untreated *rd10* eye, dark-adapted ERG responses were minimal (Fig. 2C), whereas the light-adapted b-wave amplitudes (Fig. 2D) were approximately 25% of the wild-type controls elicited with flash intensity of 12 cd · s/m^2. In the treated *rd10* eye, approximately 22% of the normal dark-adapted b-wave (Fig. 2C) and 82% of the normal light-adapted b-wave amplitudes elicited with flash intensity of 2.68 cd · s/m^2 (Fig. 2D) persisted. In time domain, the implicit times of the dark- and light-adapted ERG b-waves were approximately 75 ms (2.68 cd · s/m^2) and 45 ms (12 cd · s/m^2), respectively. In the treated *rd10* mouse eye, the implicit time of the dark-adapted b-wave was comparable to that of the wild type mouse; however, the implicit time of the light-adapted b-wave was approximately 60 ms, which is similar to that for the untreated eye but is approximately 15 ms longer than for the normal control. Finally, as additional controls, we tested subretinal AAV5-smCBA-GFP and PBS in *rd10* eyes. No rescue effects were observed in these eyes, indicating rescue is not a consequence of the injection procedure itself (data not shown).

PDEβ Expression

Two days after the final ERG examination, PDEβ expression was assayed by immunohistochemistry of retinal sections in *rd10* eyes. Strong PDEβ staining is evident in the outer segments of treated eyes, similar to that seen in the normal C57 retinas (Fig. 3A). Inner segment staining is weak but detectable in treated eyes. In contrast, no PDEβ expression was observed in any portion of the untreated retina from the same *rd10* mouse. To confirm the identity of this signal, Western blot analysis showed the presence of PDEβ protein in treated *rd10* eyes but not in untreated contralateral eyes (Fig. 3B). We estimated that the level of PDEβ from pooling protein extracts from five treated eyes was slightly less than that seen from a single wild-type eye and, therefore, further estimated that we restored an average of 10% to 15% of the normal level of PDEβ

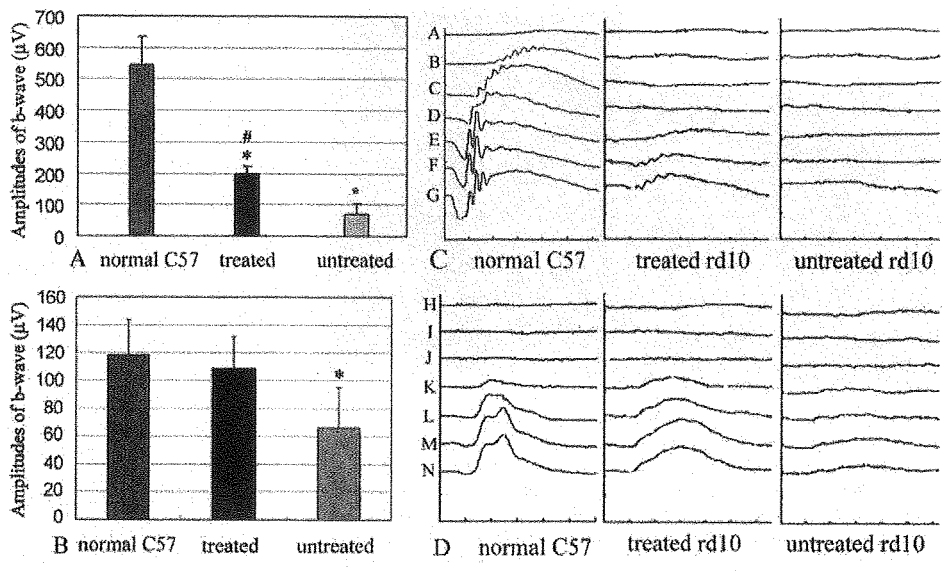

FIGURE 2. Scotopic and photopic ERGs in normal C57 and one eye-treated *rd10* mice. (**A**) Averaged, scotopic b-wave amplitudes at 2.68 cd · s/m^2 flash intensity in normal C57 (*left*), treated (*middle*), and untreated *rd10* (*right*) eyes. (**B**) Averaged photopic b-wave amplitudes at 12 cd · s/m^2 flash intensity in normal C57 (*left*), treated (*middle*), and untreated (*right*) eyes from *rd10* mice 3 weeks after treatment at P14. (**C**) Representative of scotopic ERG waveforms elicited from a set of input single-flash intensities. (A: 0.1 mcd · s/m^2; B: 1.0 mcd · s/m^2; C: 10 mcd · s/m^2; D: 100 mcd · s/m^2; E: 1.0 cd · s/m^2; F: 1.5 cd · s/m^2; G: 2.68 cd · s/m^2) from a normal C57 eye (*left*) and a *rd10* eye 5 weeks after treatment at P14 (*middle*) compared with the untreated eye (*right*) from the same 7-week-old *rd10* mouse. *Y* axis: 250 μV/Division; *X* axis: 25 ms/Div. (**D**) Representative of photopic ERG waveforms from a normal C57 eye (*left*), a treated (*middle*), and an untreated (*right*) eye of an *rd10* mouse 5 weeks after treatment at P14. *Y* axis: 100 μV /Div ; *X* axis: 20 ms/Div. Input flash intensities are H: 1 mcd · s/m^2; I: 10 mcd · s/m^2; J: 100 mcd · s/m^2; K: 1 cd · s/m^2; L: 5 cd · s/m^2; M: 10 cd · s/m^2; N: 12 cd · s/m^2. Symbols and bars represent mean ± SEM. *Significant difference between *rd10*-untreated and *rd10*-treated and between *rd10*-untreated and normal C57 mice. #Significant difference between *rd10*-treated and normal C57 mice.

in treated *rd10* eyes compared with that in age-matched uninjected normal eyes.

Structural Rescue

Vector-treated *rd10* eyes were assessed for the degree of structural rescue that accompanied ERG preservation. In light microscopic images at low magnification, it is apparent that most treated *rd10* retinas maintained a relatively normal ONL, whereas in the untreated eye of the same *rd10* mouse, the ONL contained few and difficult-to-visualize photoreceptor cell bodies (Fig. 4A). Images at higher magnification showed that a typical treated retina retained approximately 30% of its outer segment length compared with the wild-type retina and approximately 60% of its ONL thickness. The best results showed that up to 90% of ONL nuclei and more than 50% of the outer segment length was preserved by treatment (Fig. 4B). In contrast, in the entire untreated eye from the same mouse, at most only one to three rows of ONL nuclei remained, with no outer segments evident in the central retina and only residual outer segment membrane in the periphery (Fig. 4B). Electron microscopic images confirmed that the treated eye contained shortened but normal-appearing outer segments. In the contralateral untreated *rd10* eye, outer segments were absent or only residual structures remained, resulting in the outer limiting membrane (OLM) being nearly opposed to the RPE layer (Fig. 4C). A limited number of electron-dense photoreceptor nuclei remained beneath the OLM in the untreated *rd10* retina (Fig. 4C, lower left).

Rescue of Visually Guided Behavior

To determine whether the observed electrophysiological, biochemical, and structural preservation of the *rd10* retina on

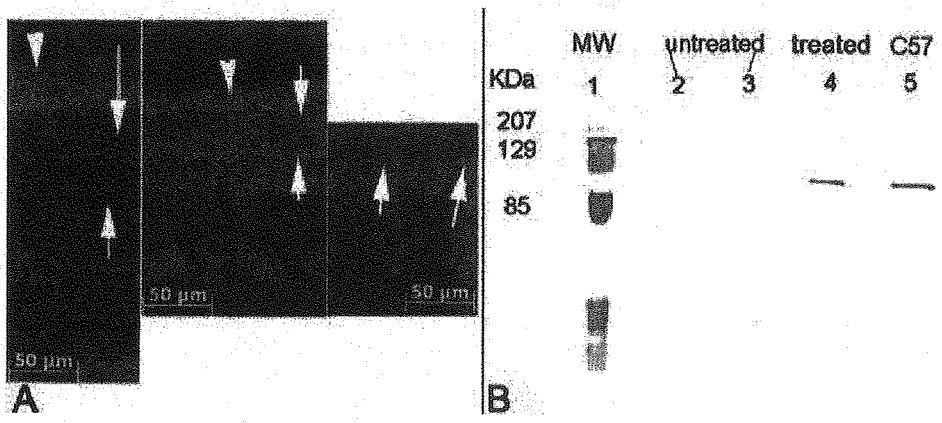

FIGURE 3. Comparison of PDEβ expression in C57BL/6J and *rd10* mice. (**A**) PDEβ immunostaining (*red*) in a 5-week-old uninjected normal C57BL/6J eye (*left*), treated eye (*center*), and untreated eye (*right*) from one *rd10* mouse. Nuclei were stained with DAPI (*blue*). *Arrows*: photoreceptor. *Arrowheads*: outer segments of photoreceptor. (**B**) Western blot showing PDEβ from 5-week-old AAV5-smCBA–PDEβ treated and untreated *rd10* eyes and an untreated age-matched normal C57BL/6J control eye. *Lane 1*: molecular weight marker; *lanes 2* and *3*: five pooled, untreated *rd10* retinas; *lane 4*: five pooled, treated *rd10* retinas; *lane 5*: one normal C57BL/6J retina.

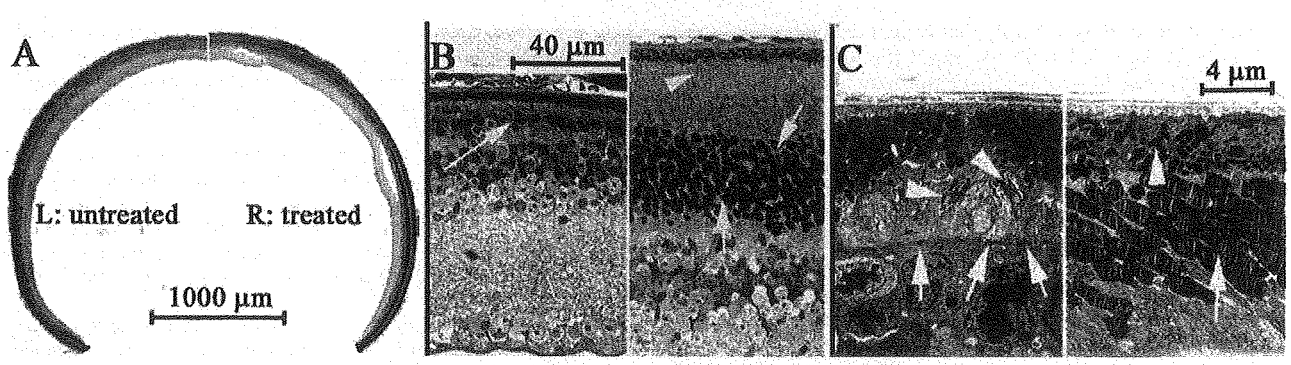

FIGURE 4. Light microscopic (LM) images of treated and untreated eyes from one *rd10* mouse. (**A**) LM images at low magnification showing an untreated (*left*) and a treated (*right*) eye from the same *rd10* mouse. (**B**) LM images from the central retina of both eyes of an *rd 10* mouse 3 weeks after P14 injection with rAAV5-smCBA-PDEβ into one eye. *Left*: untreated eye. *Right*: treated eye. *Arrows*: photoreceptor nuclei. *Arrowhead*: photoreceptor outer segments. (**C**) Electron microscopic images from a 5-week-old *rd10* mouse with one eye treated with AAV5-smCBA-PDEβ at P14. *Left*: untreated eye. *Arrowheads*: residues of outer segments. *Arrow*: outer limiting membrane. *Right*: treated eye. *Arrowhead*: RPE cells. *Arrow*: outer segments.

vector treatment led to improvement in vision that may be useful in a behavioral sense, we tested several *rd10* mice in a visually guided water maze task.[26] After 4 days of training, analysis of times to find the platform under very dim light conditions showed that normal C57 mice averaged 9.7 ± 0.8 seconds, *rd10* mice vector treated in one eye averaged 22.6 ± 4.2 seconds, and untreated *rd10* mice averaged 51.5 ± 1.1 seconds (Fig. 5). Statistical analysis showed significant deterioration on the vision-guided performance task in untreated *rd10* mice ($n = 3$) compared with normal C57 mice ($n = 3$; $P < 0.0001$). Although not as quick as normal C57 mice ($P = 0.0192$), *rd10* mice ($n = 4$) treated in just one eye showed significantly improved vision-guided performance compared with untreated littermates ($P = 0.0003$).

DISCUSSION

The *rd10* mouse is a funduscopically identified RD mouse that has a recessive PDEβ mutation similar to one type of human retinitis pigmentosa.[18] Although there is an early recordable scotopic a-wave, rod photoreceptor degeneration initiates at about P18 and progresses to only two to three ONL nuclei remaining at P30.[19] In fact, photoreceptor outer segments in *rd10* mice are never fully developed when animals are reared in a normal cyclic room light environment. This presents a significant challenge for gene therapy because we estimate it

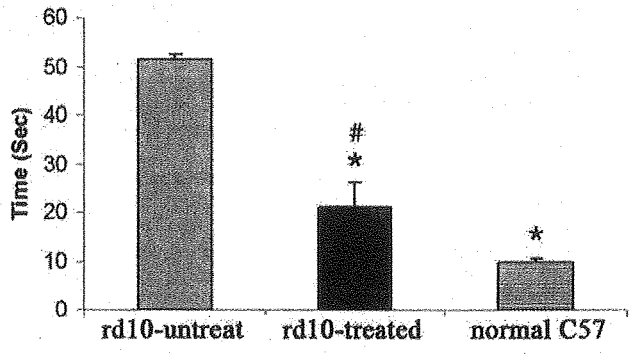

FIGURE 5. Water maze visually guided behavioral test showed the average times to the platform among untreated *rd10* (*left, gray bar*; $n = 3$), treated *rd10* (*middle, black bar*; $n = 4$), and age-matched normal C57 (*right, solid diamonds bar*; $n = 3$) mice. Symbols and bars represent mean ± SEM. *Significant difference between *rd10*-untreated and *rd10*-treated, and *rd10*-untreated and normal C57 mice. #Significant difference between *rd10*-treated and normal C57 mice.

takes 1 to 2 weeks for AAV5-smCBA-PDEβ to express sufficient PDEβ protein for physiological demand in the *rd10* retina, suggesting that the best timing for vector delivery would be P0 to P5 in *rd10* mice. However, mouse eyes at this age have not yet opened and can be easily damaged by any invasive procedure. Transscleral subretinal injection is still possible in neonatal mice. Typically, however, less than 40% of the retina can be transfected, and many injection-related complications have been noted.[13,15,17,27] Successful subretinal injection in this sense requires two elements—a relatively large transfected retinal area that can be reached by transcorneal subretinal injection and minimal injection-related damage—both difficult to achieve in very young mice but more easily achieved in older mice. Hence, the need is apparent to slow degeneration in the *rd10* retina sufficiently to allow more mature animals to be tested while still allowing time for vector to express therapeutic levels of PDEβ protein before degeneration is too far advanced. Fortunately, we confirmed the observation that retinal degeneration could be slowed if *rd10* mice are reared in darkness.[19] The effect of light on accelerating retinal degeneration has been noted for the T4R rhodopsin dog[30] and the T17M rhodopsin mouse.[31] The observation we have made, that light also accelerates degeneration in mutant PDEβ mice, generalizes the phenomenon beyond rhodopsin mutations affecting retinal integrity.

Gene therapy improved dark- and light-adapted ERGs in *rd10* mouse. In particular, the light-adapted b-wave amplitudes in the treated *rd 10* mice were comparable to those of the normal wild-type mice. However, the timing of the light-adapted ERG b-wave of the treated *rd10* eye remains delayed. Further investigation will be necessary to clarify whether the second exposure during ERG examination in P14+5W *rd10* mouse accelerated the b-wave peak delay, whether it was related to the cone degeneration in *rd10* mouse, or both. The general lack of a rescue effect in previous PDEβ gene therapy attempts using the *rd1* mouse[13–15] raises several questions about why the *rd1* mouse did not respond well to gene replacement therapy though the *rd10* mouse, tested here, did. First, is there something specific to recessive PDEβ mutations that confers the property of cryptic dominance when attempting gene replacement therapy, thus preventing more effective gene replacement therapy? Given that we clearly show an upregulation of the PDEβ vector transgene in treated retinas and structural, concomitant with electrophysiological and vision-guided behavioral preservation, this would seem to argue otherwise. However, a cryptically dominant property of the *rd1* mutation not shared by the *rd10* mutation cannot be

eliminated. Second, is it simply that retinal degeneration in the *rd1* mouse is too early and too rapid for AAV-vectored gene therapy to be effective? We believe this may be at least partially the case because slowing the degenerative process in the *rd10* mouse through dark rearing allowed therapy to become more effective. Third, and related to the first two questions, are relatively well-developed photoreceptor outer segments necessary for PDEβ gene therapy to be successful? This may also be partially true. If what is needed for successful PDEβ therapy is a substantial fraction of the normal amount of PDEβ in the presence of other outer segment proteins—perhaps most important the major binding partners of PDEβ, PDEα, and PDEγ—then the expression of vectored PDEβ in dark-reared *rd10* animals with normal outer segments is internally consistent with this hypothesis. Thus, it seems plausible that our ability to preserve photoreceptor structure and function in the *rd10* mouse reflects its slower rate of degeneration, which allows vector-expressed PDEβ to be stably integrated into a normal complement of preexisting outer segment phototransduction components.

Although longer term rescue will clearly be required if gene replacement therapy for PDEβ-based RP is a viable therapeutic option, results reported here using the dark-reared *rd10* mouse demonstrate that relatively complete rescue is possible. This model, therefore, seems well suited for modified gene therapy approaches that may express wild-type PDEβ more rapidly than current vectors and may offer the potential for longer term functional rescue. If achieved, this would represent a hopeful development for one of the more common and aggressive forms of human RP.

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Effects of Donepezil on Verbal Memory After Semantic Processing in Healthy Older Adults

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Objective: To learn if acetylcholinesterase inhibitors alter verbal recall by improving semantic encoding in a double-blind randomized placebo-controlled trial.

Background: Cholinergic supplementation has been shown to improve delayed recall in adults with Alzheimer disease. With functional magnetic resonance imaging, elderly adults, when compared with younger participants, have reduced cortical activation with semantic processing. There have been no studies investigating the effects of cholinergic supplementation on semantic encoding in healthy elderly adults.

Method: Twenty elderly participants (mean age 71.5, SD ± 5.2) were recruited. All underwent memory testing before and after receiving donepezil (5 mg, n = 11 or 10 mg, n = 1) or placebo (n = 8) for 6 weeks. Memory was tested using a Levels of Processing task, where a series of words are presented serially. Subjects were either asked to count consonants in a word (superficially process) or decide if the word was "pleasant" or "unpleasant" (semantically process).

Results: After 6 weeks of donepezil or placebo treatment, immediate and delayed recall of superficially and semantically processed words was compared with baseline performance. Immediate and delayed recall of superficially processed words did not show significant changes in either treatment group. With semantic processing, both immediate and delayed recall performance improved in the donepezil group.

Conclusions: Our results suggest that when using semantic encoding, older normal subjects may be aided by anticholinesterase treatment. However, this treatment does not improve recall of superficially encoded words.

Key Words: memory, donepezil, semantic processing, superficial processing, aging, acetylcholinesterase inhibitor

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People are capable of forming several types of long-term memories, including declarative and procedural. Procedural memories are related to the learning and recall of skills, such as riding a bicycle or tying shoelaces. Declarative memories can be divided into episodic (when, what, and where) and semantic (knowledge). Normal aging is associated with a decline in the ability to form declarative memories,[1] but the reason for this decline is not fully understood. It is possible that the memory decline associated with aging results from cholinergic hypofunction.[2]

Semantic processing of words, as opposed to superficial processing, results in increased free recall of words, with the recall increasing with the depth of processing.[3] Daselaar et al[4] tested 26 young (32.4 SD 1.8 y) and 39 older (66.3 SD 2.0 y) adults for superficial and semantic memory processing using functional magnetic resonance imaging. Daselaar et al found that the semantic task of classifying as living or not living had similar patterns of activation and behavioral performance for both the young and older adults. The younger group, however, showed a greater Blood Oxygenation Level Dependent (BOLD) signal increase in the left hippocampus than did the older group ($P < 0.001$) when the

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Dr Womack was on the speakers' bureau of Pfizer during the course of this study. His compensation was less than $10,000/year.

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semantic task was compared with a nonsemantic task (deciding between upper and lowercase letters). The greater BOLD signal increase for younger adults could be interpreted as underrecruitment in the hippocampal system in older adults.

Using functional magnetic resonance imaging, Logan et al[5] compared superficial and semantic processing of both faces and words in young subjects, age 18 to 31 years, and older subjects, age 61 to 82 years. Logan et al found that when compared with younger subjects, older adults tended to underrecruit the left frontal region (Brodmann's Area 45/47) during superficial incidental tasks. However, with a semantic encoding task (deciding if a word was an animate or inanimate object) the activity seen in the older subjects was almost the same level as that in the young adults. Logan et al infer that frontal resources are recruited with semantic processing, but not spontaneously recruited with superficial processing. That memory performance with semantic processing improves in older adults, but not to the same level of performance as the young adults might suggest that older people have a reduction in semantically induced frontal activation.

Donepezil (an acetylcholinesterase inhibitor) treatment has improved retention of training on aviation tasks in healthy older adults.[6] We wanted to learn if cholinergic supplementation through the use of acetylcholinesterase inhibitors might help the retention of declarative memories in older participants. As semantic encoding helps with the consolidation of declarative memories and cholinergic supplementation might aid semantic processing, we also wanted to learn if cholinergic supplementation enhanced semantic encoding.

The primary goal of this study was to determine if retention of semantically processed words in a Levels of Processing task would differentially increase with donepezil compared with superficial processing due to increased availability of acetylcholine. The 3 secondary goals were to learn if the increased availability of acetylcholine would influence (a) working memory (digit span forwards and backwards), (b) verbal fluency and retrieval [Controlled Oral Word Association Test (COWAT)],[7] and (c) verbal learning [California Verbal Learning Test (CVLT)].[8]

Our primary outcome measures were immediate and delayed recall of semantically processed words compared with superficially processed words. Secondary outcome measures were forwards and backwards digit span, COWAT, semantic categorical fluency performance and recall (immediate and delayed) on the CVLT.

MATERIALS AND METHODS

Research Participants

Our project was approved by the University of Florida Institutional Review Board. Volunteers for the study were required to be between 65 and 85 years old, right handed, have English as their first language and have at least a high school education or its equivalent. All subjects signed an informed consent form before inclusion in the study. After entering the study, participants had to score a 27 or higher on the Mini-Mental Status Examination (MMSE)[9] and pass a physical examination to receive treatment.

Exclusion criteria were uncontrolled hypertension, untreated hyperlipidemia, a history of glaucoma, cardiac surgery, cardiac arrhythmias, mental illness, learning disabilities, substance abuse, head trauma with loss of consciousness, or any history of neurologic deficits. Also excluded were those currently using psychoactive or anticholinergic medications.

Participants were recruited from advertisements placed in the local newspaper. A total of 31 people signed the informed consent form approved by our institutional review board. Subjects were compensated for their travel expenses at $25 per visit. Subjects were compensated for the initial visit even if they did not meet criteria for inclusion in the study.

All participants underwent neuropsychologic testing and a physical examination focusing on general health and neurologic deficits. One person was removed from the study due to a cardiac arrhythmia, 1 due to the presence of an essential tremor, 3 for not meeting minimum MMSE score requirements, and 1 due to age requirements. All participants were informed of the reason for their exclusion from the study and in appropriate situations, were advised to consult their primary care physician.

Five participants chose to discontinue participation in the study. One scheduled a cruise and declined to take any medication while on the cruise. Two other participants removed themselves due to gastrointestinal side effects of the donepezil. One participant developed self-reported tachycardia and removed himself from the study. One changed his mind immediately after signing the consent, but before beginning testing and gave no reason.

The recruiters for the study were blinded to the randomization. The testers for the study were blinded to the randomization until after scoring was completed. Participants were randomized by one of the authors who did not participate in testing (G.P.C.). Randomization was performed to match for age and sex, with no attempt made to match for other demographic variables or baseline test performance.

Baseline testing for all participants consisted of the WAIS-R Vocabulary and Information subtests, National Adult Reading Test (NART),[10] Boston Naming Test (BNT),[11] MMSE,[9] and the Geriatric Depression Scale (GDS).[12]

The demographics of participants who completed the study are shown in Table 1A.

Table 1B shows the P values of independent t tests between the different groups in the study. Comparisons are shown between the placebo (P) group and the donepezil (D) as well as between finishers of the study (placebo and donepezil groups combined) and noncompleters (NC). Owing to concerns about differences in average scores for performance between the donepezil and placebo group, although not significant at the

TABLE 1.

(A) Participant Demographics	Placebo	Donepezil	Noncompleters*
Participants	8	12	4
Sex	3M, 5F	5M, 7F	1M, 3F
Age (y)	73.1 (6.0)	70.6 (4.6)	72 (1.4)
Years of education	15.0 (2.6)	15.7 (2.8)	14.25 (2.6)
WAIS-R Vocabulary Scaled Scores	12.5 (3)	14.75 (2.5)	10.75 (2.7)
WAIS-R Information Scaled Scores	12.2 (1.8)	13.5 (2.1)	11.5 (1.7)
NART estimated VIQ	112.4 (6.9)	118.1 (7.2)	108.3 (12.5)
BNT	57.1 (2.6)†	58.4 (1.9)	54.5 (1.3)
MMSE	29.6 (0.5)	29.2 (1.0)	29.2 (0.95)
Geriatric Depression Scale	2.2 (3.6)‡	1.1 (1.3)	5.5 (2.1)

Means and SDs for the placebo, donepezil, and noncompleters groups are shown.
*Although 5 subjects quit the study, only 4 completed neuropsychologic tests.
†One subject had no BNT score.
‡One subject in the placebo group scored an 11 (mild depression) on the Geriatric Depression Scale at Session 1, this changed to a 7 (normal) on Session 2 and a 6 on Session 3.

(B) Statistical Comparisons	P vs. D	F vs. NC	D vs. NC
Age (y)	0.32	0.78*	0.57
Years of education	0.60	0.44	0.395
WAIS-R Vocabulary Scaled Scores	0.09	0.65	**0.017**
WAIS-R Information Scaled Scores	0.19	0.19	0.17
NART estimated VIQ	0.12	0.13	**0.072**
BNT	0.24	**0.008**	**0.002**
MMSE	0.26	0.84	0.89
Geriatric Depression Score	0.32	**0.007**	**0.00**

P values are given for 2-tailed *t* test for equality of means. No correction for multiple comparisons was made. *P* values less than 0.05 are shown in bold.
*Levene test for equality of variance between the 2 groups was significant at the $P = 0.05$ level for this test ($P = 0.022$), and equal variances were not assumed. Equal variances were assumed otherwise.
BNT indicates Boston Naming Test; P, placebo group; D, donepezil group; F, finishers (placebo and donepezil groups); NC, noncompleters.

$P = 0.05$ level, comparisons were made between the donepezil group and the noncompleters.

Donepezil Dosing

Acetylcholinesterase inhibitors are available as Food and Drug Administration approved treatments for Alzheimer disease. The main medications in this class are donepezil (Aricept), rivastigmine (Exelon), and galantamine (Razadyne). We selected donepezil as it was the only one of these medications available at the start of the trial with once a day dosing as well as being highly selective for muscarinic acetylcholine synapses and generally well tolerated.

Donepezil dosing was initially set at 5 mg/d increasing after 7 days to 10 mg/d. The first participant to receive the donepezil tolerated the medication. The next 3 participants who received donepezil complained of gastro-intestinal side effects and self-discontinued the study. As a result, the target dose was then changed to 5 mg/d for 6 weeks. All subjects enrolled on the 5 mg/d dose completed the study. All subjects enrolled in the placebo arm also completed the study. The subject given 10 mg/d performed within the 95% confidence intervals for the performance of subjects receiving 5 mg on the Levels of Processing task.

Study Design

This study was a double-blind, randomized, placebo-controlled parallel group, repeated measures study. A schematic diagram of the study is shown below (Fig. 1). Subjects were provided 42 tablets of either donepezil or placebo. The pills were overencapsulated to maintain the double-blind nature of the study.

Apparatus

The stimulus words were taken from Battig and Montague.[13] These words were selected to be matched for frequency[14] and familiarity.[15] There were no differences

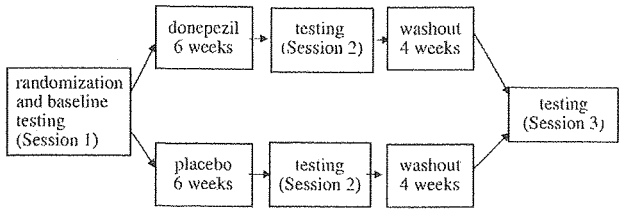

FIGURE 1. Overall study flow. Subjects are randomized to either placebo or donepezil on the first day of testing and underwent baseline testing (Table 1A and 1B) as well as the first of 3 sets of tests (Session 1). The second set of tests (Session 2) occurred after 6 weeks of "treatment." The third set of tests (Session 3) occurred after 4 weeks of washout.

(at the $P = 0.05$ level) in frequency or familiarity between semantically and superficially processed words. The words were presented using 72-point Tahoma font with PowerPoint with an LCD computer monitor on a desk at a comfortable viewing distance from the subject.

Levels of Processing Tests

The Levels of Processing test, first described by Craik and Lockhart[16] and later modified[17] involves the performance of 2 separate linguistic tasks. In the "superficial" encoding task, the subject is presented single words in a serial order on a computer screen and asked to count the number of consonants in each word and repeat that number verbally. In the "semantic" encoding task, the subject is presented words in the same manner and asked to rate a word for pleasantness on a scale from −5 (extremely unpleasant) to +5 (extremely pleasant) and to repeat their rating aloud. The superficial task discourages semantic processing by focusing attention on the appearance of the word, and the semantic task encourages semantic processing.

The test is organized into 4 blocks of 6 words each. At the start of a block of words, the task instructions (either consonant counting or pleasantness assessment) are displayed on the computer screen. After each response the space bar was pushed and the next word was immediately displayed. Four parallel forms of the test with different word lists (1 list per day of testing) were used. The starting task alternated between consonant counting (C) and assessing pleasantness (P) during the 3 testing sessions. Thus, if the sequence of tasks for Session 1 was CPCPCP, then for the next session the task would be PCPCPC. Each word was used only once for all 3 sessions combined.

Participants were asked immediately after completion of the test to recall all words (either superficially or semantically processed) that were previously shown on the computer screen (immediate recall). After approximately 20 minutes, they were asked again to recall the words (delayed recall). Delayed recognition in which 24 targets and 24 foils were presented was also assessed.

Repeated Testing

Subjects were tested 3 times. Premedication tests are labeled Session 1, maintenance dose tests (6 wk after start of medication) are labeled Session 2, and washout tests (4 wk after ending medication) are called Session 3.

Subjects had parallel versions in each session of the CVLT,[8] digit span forward and backward, COWAT,[7] and a semantic (or category) fluency task.[18] Three different versions of the CVLT were used, with 2 of them matched with the original for word frequency. The COWAT used the letters FAS, CFL, and GSR for the 3 versions employed in this fluency test. Categories used for the category fluency test were animals, fruits and vegetables and a shopping list. The MMSE and GDS were also tested in each session.

Statistical Methods

A repeated measures analysis of variance (ANOVA) was used for the initial analysis, with treatment the between subject factor (donepezil vs. placebo) and immediate and delayed recall performance the within subject factor (comparing Sessions 1 to 2) with a significance criteria set at $P < 0.05$. Planned post-hoc analysis of immediate recall and delayed recall for both superficial and semantic processing was carried out with paired t tests to compensate for the high interindividual variance and small intraindividual changes in performance in memory seen with test performance.[19]

RESULTS

Primary Outcome Variables

In the Levels of Processing task, when donepezil treatment is compared with placebo, delayed recall of semantically processed words was the only test to approach significance comparing Sessions 1 to 2 using an ANOVA [$F(1,18)$ $P = 0.049$].

Test results of Session 2 were compared with Session 1 using paired t tests. These results are shown in Table 2. Immediate recall and delayed recall of semantically processed words in the donepezil group was significantly different between these trials ($P = 0.018$ and $P = 0.001$, respectively).

Ceiling effects were seen on recognition of semantically processed words for both placebo (11.6 words on average) and donepezil (11.7 words) out of a maximum of 12 words and no further analysis of the recognition task was performed.

TABLE 2. Session 2 Versus Session 1 Performance Comparing Placebo Versus Donepezil Groups

	Average Difference Between Session 2 and Session 1		*P* Values of Differences Between Sessions 2 and 1	
Test	**Placebo**	**Donepezil**	**Placebo**	**Donepezil**
Immediate recall				
Superficial	1.13 (2.3)	0.25 (2.2)	0.208	0.704
Semantic	0.5 (2.3)	2.08 (2.6)	0.563	0.018
Delayed recall				
Superficial	0.38 (1.2)	0.17 (1.8)	0.402	0.761
Semantic	0.50 (2.4)	2.4 (1.7)	0.582	0.001

Evaluation of donepezil versus placebo effects. The average differences and SDs of the differences between Sessions 2 and 1 tests are shown by test for placebo and donepezil. Paired t test (2-tailed) results for each test for placebo and donepezil are shown.

TABLE 3. Probability Values Comparing Changes in Immediate and Delayed Recall for Placebo and Donepezil Treatments

	Session 1 (Pretreatment) to Session 3 (Postwashout)	
	Placebo	Donepezil
Immediate recall		
Superficial	0.073	0.795
Semantic	0.544	0.012
Delayed recall		
Superficial	0.142	0.884
Semantic	0.460	0.006

Paired *t* test were used to compute *P* values for changes between Session 1 and Session 3 for donepezil and placebo groups.

The placebo group had no significant change in performance on any recall test. Superficial processing in the donepezil group did not show significant change for either immediate recall or delayed recall. Paired *t* tests were also performed comparing Sessions 1 to 3 for recall and demonstrated a significant improvement in the donepezil group with semantic encoding in both immediate and delayed recall (Table 3).

Shown below (Figs. 2A, B) are graphs of the average number of words recalled in the Levels of Processing task in the delayed condition for Sessions 1, 2, and 3.

Secondary Outcome Variables

There were no significant differences between Sessions 2 and 1 in the COWAT or semantic fluency tasks for either the donepezil or placebo groups using an ANOVA $F(1,18)$ $P \leq 0.05$. Similarly, there was no difference in forwards or backwards digit span, total words recalled in 5 trials or words recalled in delayed free recall in the CVLT.

Postwashout Testing

Post-hoc analysis on the Levels of Processing test compared performance of delayed recall of superficial and semantic processing between Sessions 1, 2 and 3 (Table 3 and Figs. 2A, B).

Estimated effect sizes (Cohen *d*) are 1.23 for Sessions 1 to 2 and 0.989 for Sessions 1 to 3 comparing superficially processed words to semantically processed words for the donepezil group with delayed recall.

Compliance, Tolerability, and Adverse Effects

Discontinuation in the initial group with a target dose of 10 mg was consistent with previous Phase 2/3 trials, that of digestive system distress. Our completion rate for this study after single 5 mg/d doses were started (100%) is slightly better than the completion rate of phase 2/3 trials of donepezil with dosages of 5 mg/d (83%). This might be due to higher motivation of the volunteers. One subject who completed the study commented that she had occasional leg muscle cramps when she swam, a complaint seen by 5% of those using donepezil.[20]

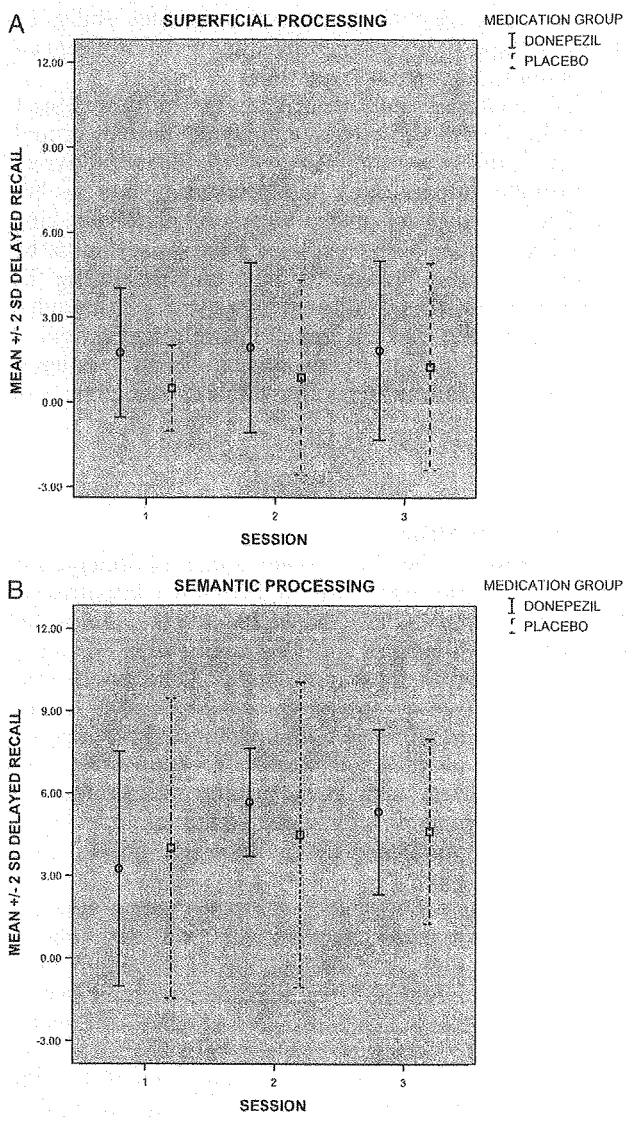

FIGURE 2. Delayed recall for Sessions 1, 2, and 3 of donepezil and placebo. Delayed recall is tested approximately 20 minutes after presentation of words. Figure 2A shows the number of words recalled after superficial processing on placebo and donepezil. Figure 2B is the same as Figure 2A, but for delayed recall of semantically processed words. Session 1 is before giving placebo or donepezil. Session 2 is performed 6 weeks later before discontinuation of placebo or donepezil. Session 3 is performed 4 weeks after Session 2, or postwashout. In each pair of means the donepezil mean is on the left and the placebo mean is on the right.

An alternative explanation for the discontinuation in the 10 mg group is their neuropsychologic performance status. The donepezil-treated group and the noncompleters group show differences at the $P = 0.05$ level for the WAIS-R, NART estimated VIQ, BNT, and GDS, with average scores for all tests lower in the noncompleter group (Table 1B). Having higher neuropsychologic

performance at baseline may result in greater ability to tolerate donepezil, however, our study was not designed or powered to answer this question.

Two adverse events occurred in the study. A subject on placebo developed paroxysmal atrial fibrillation and informed us after returning from a trip. She was advised to immediately discontinue her medication and see her physician. A donepezil subject informed us after finishing her medication that she was prescribed hyoscyamine by her primary care physician for mild diarrhea during the 6 weeks of medication. Although hyoscyamine is intended to act peripherally as an anticholinergic medication, it also has centrally acting cholinergic effects. Both patients' data were included in the study without reassignment of group.

DISCUSSION

Verbal Processing

Our results differ from recent studies of donepezil in healthy adults and those with mild cognitive impairment. Beglinger et al[21] studied 26 healthy adults randomized into 2 arms (14 donepezil and 12 placebo) with 14 days of medication and 14 days of washout. Participants were chosen for average to low-average initial cognitive performance to avoid ceiling effects and to increase the likelihood of detecting cognitive improvement. No improvement was seen in cognitive performance in the donepezil group, with speed, attention and short-term memory worsening in the donepezil group, compared with placebo.

In contrast to Beglinger's study, we had no selection criteria for maximum performance and our subjects scored higher than national averages for the NART, BNT, and the WAIS-R vocabulary and information subtests. The relationship between baseline intelligence and response to donepezil has not been elucidated and further studies are necessary to determine if baseline intelligence might be an important factor in our results.

Our study was also longer in duration than Beglinger's, 6 weeks, rather than 2 weeks. Although Mihara et al[22] found that steady state levels were obtained after approximately 2 weeks of daily dosing in young male volunteers, longer times might be needed for steady state levels in older subjects. Thus, 2 weeks of donepezil might not have resulted in adequate steady state levels. We did not find any decrease in cognitive performance in the donepezil arm of the study. Finally, Beglinger's study did not use semantic encoding as a task. These different results suggest that cholinergic supplementation and semantic encoding may be requirements for improved recall.

Support for this possibility comes from a study by Barnes et al,[23] who found increased nicotinic receptor density in rat hippocampus and neocortex after infusion for 35 days with donepezil or galantamine using an implanted pump. Long-term potentiation was increased in both rat medication groups compared with saline control. The increased long-term potentiation was correlated with the increased nicotinic receptor binding sites. We wonder if the decreased cholinergic innervation seen with normal aging is partially reversed in the neocortex and hippocampus due to acetylcholine esterase inhibitors and that this reversal, combined with the cortical components of semantic encoding acts to increase the recall of semantically processed words.

Grön and coworkers[24] studied the effects of 5 mg/d of donepezil in 30 young (23.6 y SD 2.47) male medical students for 30 days using a double-blind parallel group design. These investigators found significant improvements in a repetitive verbal recall paradigm (word list learning). We, however, did not find any significant improvement for total words recalled, either immediate or delayed on the CVLT (List A), which is similar to the repetitive verbal recall task used by Grön. In our study, subjects improved with semantic processing but not on word list learning, unlike Grön's subjects, suggesting that younger subjects might be more likely to spontaneously semantically encode words than do older subjects. This possibility will have to be tested in future studies.

Digit Span

We wanted to learn if digit span (forward and backward) would improve on donepezil, however, we did not find a statistically significant effect. Our results agree with Grön and coworkers, who also did not find statistical significance. Drachman and Leavitt[25] found that physostigmine did not alter digit span in healthy young adults. When treated with physostigmine, healthy older adults (64 to 82-y old) were found to have a trend toward improved digit recall and free-recall of words. However, when these experimental participants were compared with the control group no significant differences were found.[26] Similarly, Freo et al[27] found that physostigmine did not alter accuracy in a working memory task of faces for either young or older adults. Davis et al[28] found that physostigmine improved performance for long-term memory, but not short-term verbal memory. However, maximal performance on the memory test was limited for entry into the study with 20 volunteers rejected and only 19 accepted into the drug phase of the study based on a screening test.

COWAT and Semantic Fluency

We had also expected that the COWAT and semantic fluency would improve with increased availability of acetylcholine due to either activation of the lexical semantic networks or improvement in attention. However, differences between the placebo and donepezil group's performance were not found to be significant.

Postwashout Performance

After 4 weeks of washout (Session 3), when compared with baseline (Session 1), the subjects in the donepezil group still demonstrated a benefit in recalling semantically processed words, both for immediate and delayed recall. There are several possible explanations for this persistent improvement in semantic memory. The plasma half-life for

elimination of donepezil in the elderly was found to be 104 hours by Ohnishi et al[29] in 6 elderly volunteers of age 65 to 82. Five or 6 half lives are usually considered adequate for restoration of the medication-free baseline state. The 4 weeks we used for a washout period is slightly over 6 half lives and should have been adequate for full restoration of acetylcholinesterase. However, the restoration of acetylcholinesterase in red blood cells is longer than the plasma elimination half life and is approximately 130 to 180 hours.[30] Thus, the 4-week washout might have been 4 to 5 elimination half lives, not 6 half lives. This interval might have not been adequate and might explain the persistence of improved performance.

Our washout results are also consistent with a study of patients with Alzheimer disease who underwent a 3-week washout and did not return to their predonepezil baseline. However, patients with a 6-week washout period did experience a down turn in performance during the washout period. This result was seen for both 5 mg and 10 mg/d treatments.[31]

A second possibility to explain the persistent treatment effect is that the increased levels of acetylcholine enhanced brain plasticity, altering the systems that mediate declarative memory and that these alterations persisted when the donepezil was withdrawn. The study by Barnes et al[23] suggests that plasticity is increased with donepezil, although there was no washout period in their study.

A third possibility, although not statistically significant, is that the slightly higher performance of the donepezil group on the NART and the WAIS-R vocabulary scores reflects this group's increased ability to rehearse or learn, resulting in an increased practice effect. This argument, however, also suggests that their performance should also increase from Sessions 2 to 3, which did not occur.

Limitations and Future Directions

Although the results of our study are promising, there are several limitations. The number of subjects is small. The differences in neuropsychologic performance between the donepezil group and the noncompleters might explain the differences we have seen in the donepezil group, with poorer tolerability of the donepezil in the lower performing group resulting in selective drop out of lower performers. However, there were also differences in dosing between the noncompleters (all at 10 mg/d) and the completers (only 1 at 10 mg/d and the rest at 5 mg/d).

To understand if the improved semantic encoding we observed is altered by age, both young and older subjects should be studied in the future. We also need to study a more normally distributed population to learn if intelligence influenced the retention of semantically processed words. Furthermore, to learn if the gains induced by donepezil treatment are long lasting, subjects need to be tested after a longer washout period. Finally, we remain uncertain as to the means by which donepezil aids semantic encoding and functional imaging might provide some insight on this last point.

Summary

A small increase in immediate and delayed recall for semantically processed words was seen in healthy elderly adults who were given 5 mg of donepezil/day for 6 weeks when compared with healthy elderly adults who were given a placebo. When compared with the placebo group, the donepezil group retained an increase in recall after 4 weeks of washout.

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BRAIN RESEARCH

Research Report

Effects of the hypnotic drug zolpidem on cell proliferation and survival in the dentate gyrus of young and old rats

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ABSTRACT

Sleep loss/disruption has been shown to suppress adult hippocampal neurogenesis. Whether the administration of hypnotic drugs, by promoting sleep, especially in older subjects, who typically exhibit poor sleep, has a beneficial effect on neurogenesis parameters is unknown. We examined the effects of zolpidem, a widely prescribed nonbenzodiazepine hypnotic, on cell proliferation and survival in the dentate gyrus of young (~2 1/2 months) and old (~13 months) male Sprague–Dawley rats. Zolpidem (5, 10 or 20 mg/kg, i.p.) or vehicle was administered twice daily, at the beginning and middle of the sleep period, for either 2 days (acute study) or 21 days (chronic study). Proliferation and cell survival were measured by staining for Ki67 or 5-bromo-2′-deoxyurdine (BrdU), respectively. Acute administration of zolpidem produced a suppression of cell proliferation, which attained statistical significance only in the aged animals. The magnitude of the suppressive effect was larger in the hilus than in the subgranular zone (SGZ). In contrast, chronic administration of zolpidem produced little or no effect on proliferation in either age group, despite marked differences in basal proliferation levels between the two age groups. Similarly, there was little change in cell survival following chronic zolpidem administration in young versus old animals. A slight reduction of cell survival in the granular cell layer (GCL)/SGZ was observed in young animals and a slight augmentation in aged animals. To the extent that zolpidem improves sleep, these data suggest little or no benefit of hypnotic drug treatment on neurogenesis parameters in young or old rats.

1. Introduction

Impaired cognitive ability is a common feature of aging in humans, and is attributable to any of a variety of factors and their interactions, including, genetic, environmental, dietary, and behavioral. Sleep loss or insomnia is also a complaint of many elderly, and in most cases this is simply considered a natural component of the aging process. Whereas insomnia was long considered a minor complaint or incidental symptom, in recent years it has emerged as an important etiological factor in many disease processes. Consistent with this, recent evidence from our lab (and others) suggests that sleep loss/disruption may play an important causal role in diminished cognitive capacity, at least in part, by suppressing the process of new brain cell production (hippocampal neurogenesis) (Guzman-Marin et al., 2003, 2005, 2007; Tung et al., 2005). Animal studies have shown that hippocampal neurogenesis is critical for some important aspects of learning

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Abbreviations: ANOVA, analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; GCL, granular cell layer; DG, dentate gyrus; SGZ, subgranular zone

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and memory (Shors et al., 2001; Leuner et al., 2006; Winocur et al., 2006; Zhang et al., 2008), and it is known that the hippocampus plays an important role in episodic memory in humans (Nadel and Moscovitch, 1997; Rosenbaum et al., 2008). We also know from animal studies that both sleep quantity/quality and neurogenesis show significant age-related declines (Kuhn et al., 1996; McDonald and Wojtowicz, 2005; Mendelson and Bergmann, 1999; Kirov and Moyanova, 2002; Bondolfi et al., 2004).

One response to sleep complaints has been a dramatic increase in the number of prescriptions written for hypnotic drugs. Americans filled over 40 million prescriptions in 2005 — an increase of nearly 60% over the previous five years (Saul, 2006). This number alone is not alarming, but the fact is that we are in uncharted waters. For example, in comparison with the clinical and preclinical information regarding antidepressant medications, the data about hypnotic drugs is meager, and derived mostly from healthy, young subjects. This problem is particularly critical for the elderly both because they constitute the major consumer group for hypnotics and because their often compromised overall health status makes them especially susceptible to any potentially harmful drug effects.

The overarching hypothesis for the present research is that sleep loss may lead to cognitive decline especially in the elderly, in part, because of a suppression of hippocampal neurogenesis. Based on this, we examined the effect of acute and chronic administration of the most prescribed hypnotic drug zolpidem on hippocampal cell proliferation and cell survival in young adult and aged rats. We predicted that aged animals would derive a larger benefit from zolpidem in this hippocampal process because, in general, they exhibit more sleep loss/disruption than young animals. However, it is possible that despite improving certain aspects of sleep amount and architecture, these drugs may *exacerbate* rather than mitigate the decline in cell proliferation/neurogenesis typically seen in aged rodents (Kuhn et al., 1996; Bondolfi et al., 2004; McDonald, and Wojtowicz, 2005). Finally, to our knowledge, this is the first study to examine the effects of a major hypnotic drug on the processes of hippocampal cell proliferation and survival.

2. Results

2.1. *Effect of zolpidem on body weight*

All groups of rats treated with zolpidem, either acutely (2-day) or chronically (21-day), had significantly reduced body weight or gained less when compared to their respective vehicle control group ($p<0.001$). Furthermore, there was no significant difference ($p>0.05$) between the dose levels of zolpidem used in a particular experiment and the magnitude of effect on body weight. In the acute studies, mean percent changes in baseline body weight after treatment were +6% (vehicle), −2% (zolpidem 10 mg/kg) and −3% (zolpidem 20 mg/kg) in the young rats, and +1% (vehicle), −4% (zolpidem 10 mg/kg) and −4% (zolpidem 20 mg/kg) in the aged rats. In the chronic studies, mean percent changes in baseline body weight after treatment were +31% (vehicle), +11% (zolpidem 5 mg/kg) and +16% (zolpidem 10 mg/kg) in the young rats, and −1% (vehicle) and −9% (zolpidem 5 mg/kg) in the aged rats. In this study, the age rats could not tolerate the 10-mg/kg dose of zolpidem.

2.2. *Effects of acute zolpidem administration on cell proliferation in young adult rats*

Proliferating cells in the DG were identified by staining for the endogenous marker, Ki67 (see Fig. 1). The overall number of proliferating cells in the DG of young adult rats was not significantly altered following zolpidem administration for 2 days (vehicle=10570±426, $n=6$; zolpidem 10 mg/kg=8942±1083, $n=6$; zolpidem 20 mg/kg=9524±836, $n=8$). Similarly, there were no significant differences in the level of cell proliferation in the SGZ (vehicle=8056±398; zolpidem 10 mg/kg=7094±803; zolpidem 20 mg/kg=7481±744) or hilus (vehicle=2514±129; zolpidem 10 mg/kg=1848±293; zolpidem 20 mg/kg=2043±142), although the effect in the hilus approached statistical significance ($p=0.084$).

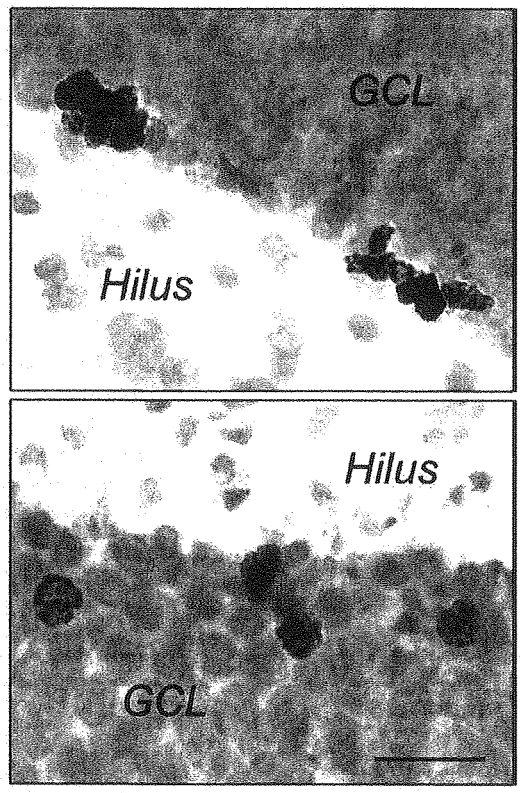

Fig. 1 – Photomicrographs (1000× magnification) showing Ki67 labeling (cell proliferation) and BrdU labeling (cell survival) in the dentate gyrus of the hippocampus in young adult rats. Immunoreactive cells appear brown in cresyl violet-stained sections. The top panel depicts two clusters of newly-generated Ki67+ cells at the border between the granule cell layer (GCL) and the hilus, in the subgranular zone. The bottom panel depicts several surviving BrdU+ cells in the GCL of the dentate gyrus. Scale bar=20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. *Effects of acute zolpidem administration on cell proliferation in aged rats*

As shown in Fig. 2, there was a large suppression (~30–45% decrease) of cell proliferation in the DG of aged rats following zolpidem administration for 2 days (vehicle = 2707 ± 325, n = 5; zolpidem 10 mg/kg = 1862 ± 220, n = 5; zolpidem 20 mg/kg = 1442 ± 246, n = 4). This effect was statistically significant only at the higher dose (20 mg/kg). When the level of proliferation was examined in the hilus, there was a significant decrease of 50% or more in proliferation at both dose levels (vehicle = 1294 ± 172; zolpidem 10 mg/kg = 641 ± 136; zolpidem 20 mg/kg = 550 ± 146). Cell proliferation, however, was not significantly altered in the SGZ (vehicle = 1414 ± 244; zolpidem 10 mg/kg = 1222 ± 99; zolpidem 20 mg/kg = 893 ± 108).

One of the interesting aspects of these data in aged rats is the marked reduction in the basal level of cell proliferation (~75%) as compared to young adult rats (see values above). Also, the DG of the aged rats appears to show a much larger drug response than that of the young adult rats.

2.4. *Effect of chronic zolpidem administration on cell proliferation and survival in young adult rats*

In this study, the dose levels of zolpidem were reduced from those used in the acute studies (as a precaution) to ensure drug tolerability over the course of the experiment. When cell proliferation in young adult rats was examined at the end of the 21 day regimen of zolpidem administration (twice daily), no significant change in overall cell proliferation was found in the DG (vehicle = 8071 ± 393, n = 10; zolpidem 5 mg/kg = 8686 ± 528, n = 5; zolpidem 10 mg/kg = 7469 ± 251, n = 5). There was, however, a significant reduction (~27%) in cell proliferation in the hilus at the 10 mg/kg dose (vehicle = 2042 ± 135; zolpidem 5 mg/kg = 1730 ± 125; zolpidem 10 mg/kg = 1495 ± 104), whereas no significant change in proliferation was seen at either dose level in the SGZ (vehicle = 6029 ± 288; zolpidem 5 mg/kg = 6955 ± 408; zolpidem 10 mg/kg = 5974 ± 279).

In addition to studying cell proliferation, the survival of proliferating cells in the DG was examined by staining for BrdU (see Fig. 1). The number of surviving newly-generated cells (labeled at the beginning of the experiment) in the DG of young adult rats was not significantly altered following 21 days of zolpidem administration (vehicle = 5106 ± 663, n = 4; zolpidem 5 mg/kg = 3849 ± 156, n = 4; zolpidem 10 mg/kg = 5061 ± 289, n = 4). Moreover, there was no statistically significant change in cell survival in either the GCL/SGZ (vehicle = 3615 ± 451; zolpidem 5 mg/kg = 2574 ± 139; zolpidem 10 mg/kg = 3450 ± 246) or the hilus (vehicle = 1491 ± 239; zolpidem 5 mg/kg = 1269 ± 62; zolpidem 10 mg/kg = 1602 ± 240), although the effect in the GCL/SGZ approached statistical significance (p = 0.084).

2.5. *Effect of chronic zolpidem administration on cell proliferation and survival in aged rats*

In this study, the aged animals could only tolerate a 5 mg/kg dose administered twice daily. There was no significant change in overall cell proliferation in the DG at the end of a 21 day regimen of zolpidem administration (vehicle = 2206 ± 108, n = 16; zolpidem = 2172 ± 92, n = 16). Similarly, no significant change in cell proliferation was found either in the SGZ (vehicle = 970 ± 68; zolpidem = 1070 ± 61) or the hilus (vehicle = 1236 ± 73; zolpidem = 1102 ± 66).

Finally, no significant alterations were seen in the rate of cell survival in the DG (vehicle = 1300 ± 50, n = 15; zolpidem = 1313 ± 60, n = 16), or the GCL/SGZ (vehicle = 553 ± 36; zolpidem = 612 ± 46) and hilus (vehicle = 747 ± 44; zolpidem = 702 ± 40), over the 21 day period of zolpidem administration in aged rats.

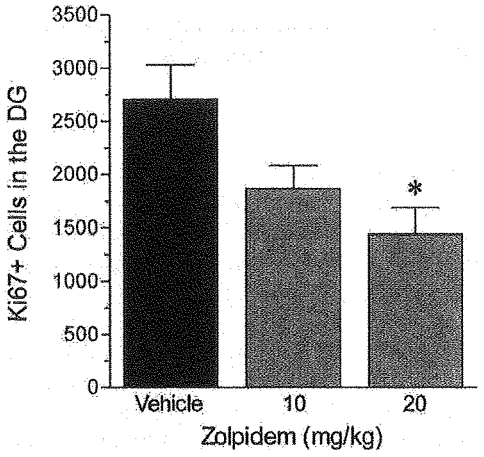

Fig. 2 – Effect of acute zolpidem administration on the number of Ki67+ cells in the dentate gyrus of aged rats. Zolpidem (10 or 20 mg/kg, i.p.) or drug vehicle (saline plus 0.1% Tween-80) was administered twice daily, at 6-hour intervals, for 2 days. Rats were perfused 18 h after the last injection. Values are means ± SEM (n = 5 for vehicle control and zolpidem 10 mg/kg; n = 4 for zolpidem 20 mg/kg). *p < 0.05 vs. vehicle control by one-way ANOVA and post-hoc Bonferroni's multiple comparison test.

3. Discussion

To examine the hypothesis that hypnotic drugs, by improving sleep, would enhance adult hippocampal neurogenesis, we selected zolpidem, the most widely used hypnotic drug, for study. Zolpidem is a nonbenzodiazepine hypnotic that acts via the benzodiazepine sites of γ-aminobutyric acid$_A$ (GABA$_A$) receptors with a rapid onset and short duration of action. The drug is extensively metabolized in both animals and humans, with no active metabolites (Durand et al., 1992). In rats, the biological half-life of zolpidem is about 42 min (Lau et al., 2002). The drug is primarily effective in facilitating sleep onset and sleep continuity and is less effective in increasing total sleep time. Compared to benzodiazepine hypnotics, zolpidem displays weak anxiolytic, anticonvulsant and muscle relaxant properties, and therefore is considered to be hypnoselective (Depoortere et al., 1986). In addition, unlike conventional benzodiazepine hypnotics, zolpidem increases EEG slow-wave activity (1–4 Hz) in rats, thus more closely resembling normal sleep (Depoortere et al., 1986; Wisor et al. 2006). Moreover, the drug only minimally affects rapid-eye-movement (REM) sleep (Depoortere et al., 1986; Mailliet et al., 2001).

Despite these positive influences on sleep, it is clear that acute administration of zolpidem can exert a suppressant

effect on hippocampal cell proliferation. This appears to be particularly prominent in aged animals where the suppression was approximately 30–40% whereas in young adults, the decrease was approximately 10–15%, at equivalent dose levels. In both young and old animals, the suppressant effects were of a larger magnitude in the hilus as compared to the SGZ. The latter may indicate a more powerful effect on gliogenesis (predominantly in the hilus) than on neurogenesis (predominantly in the SGZ) (Cameron et al., 1993; Bondolfi et al., 2004; Steiner et al., 2004). The broader physiological significance of this difference is unknown. What would be the functional effect of a change in cell proliferation that is limited to the hilus?

In contrast to these suppressant effects on cell proliferation of acute administration of zolpidem, chronic administration produced little or no effect in either age group. This may reflect the fact that tolerance can develop to the sedative-hypnotic effects of zolpidem during chronic dosing in rats (Voss et al., 2003; Ebert et al., 2008), and thereby could have influenced the hippocampal responses to zolpidem. In humans, however, there is little evidence that tolerance develops to zolpidem with long-term use (Holm and Goa, 2000).

Although not statistically significant, there was a trend toward a differential effect on cell survival following chronic administration of zolpidem in young versus old animals. The lower dose of zolpidem produced a general decrease of about 25% in the DG of young animals, while the higher dose had little or no effect. When the low dose was administered to aged animals there was very little change in cell survival. If anything, there was a tendency toward an increase (~11%) in cell survival in the GCL/SGZ and a decrease (6%) in the hilus. Two aspects of these data deserve further comment. First, the decreased cell survival in young animals administered zolpidem may have implications for the drug's effect on learning and memory during chronic use. Second, the slight augmentation of cell survival in aged animals would be consistent with our hypothesis. We proposed that hypnotic drugs, by improving the quality and continuity of sleep in aged animals (which typically display reduced and/or disturbed sleep), may exert a beneficial effect on neurogenesis parameters. In contrast, administration of hypnotic drugs to young animals (which already display optimal levels of sleep) would not be expected to improve either sleep quality or quantity, and may, in fact, cause a deterioration of normal sleep. Such an effect may be associated with a reduction in cell survival in young animals, as seen in the present study. Confirmation of the differential effects of zolpidem on the sleep patterns and amount in these two specific age groups of male rats remain to be carried out.

Finally, our zolpidem data are of interest in view of the emerging role of GABA in the regulation of adult hippocampal neurogenesis (Ge et al., 2007)). Recent studies have shown that neural progenitors express $GABA_A$ receptors (Mayo et al., 2005), and that newborn granule cells receive exclusively GABAergic synaptic stimulation (via ambient GABA) during the first two weeks of development, which may serve as an excitatory signal (Overstreet Wadiche et al., 2005; Tozuka et al., 2005; Karten et al., 2006). Because zolpidem is a positive allosteric modulator of the $GABA_A$ receptor, its chronic administration might be expected to influence neurogenesis. However, in both young and old animals, we observed no significant change in hippocampal cell proliferation or survival following chronic zolpidem treatment. These results may be partly explained by the fact that immature granule cells (including neural progenitors) in the dentate gyrus are relatively insensitive to zolpidem, due to maturational differences in the expression of $GABA_A$ receptor subunits (Overstreet Wadiche et al., 2005; Karten et al., 2006). Thus, newborn granule cells lack the α_1 subunit (Overstreet Wadiche et al., 2005), whereas zolpidem preferentially interacts with α_1 subunit-containing $GABA_A$ receptors (Sanger et al., 1994). However, by potentiating $GABA_A$ receptor-mediated inhibition in mature neurons, chronic zolpidem treatment would be expected to suppress activity-dependent adult neurogenesis, although this presumed effect was not observed in the present study.

The lack of effect of chronic zolpidem treatment on hippocampal neurogenesis (particularly in the aged animals) may be related to the fact that lower dose levels of the drug were used in the chronic versus acute experiments. Alternatively, the ability of GABAergic agents, such as zolpidem, to modulate hippocampal neurogenesis may be related to the behavioral state of the animal. In the present study, zolpidem was administered to rats specifically during their normal sleep period, as opposed to their active, waking period.

In a related study, we also found no change in hippocampal cell proliferation in either young or old rats following prolonged anesthesia (8 h) with isoflurane and propofol (Tung et al., 2008). These general anesthetics also act to potentiate GABAergic transmission in the brain. The volatile anesthetic isoflurane largely interacts with the α_1 subunit, whereas the intravenous anesthetic propofol mainly interact with the β subunits of the $GABA_A$ receptor complex (Jenkins et al., 2002; Bali and Akabas, 2004). The ability of other, more specific, GABAergic drugs to modulate adult hippocampal neurogenesis in vivo remains to be determined.

4. Experimental procedures

4.1. *Animals*

Adult, male Sprague–Dawley rats ages 2 and 12–13 months were obtained from Taconic Farm (Germantown, NY) and Harlan Industries (Indianapolis, IN), respectively. The animals were housed in an AAALAC-approved animal facility under controlled lighting (12-h light/12-h dark cycle, light on at 09:00 h) and temperature (22±1 °C, 40–50% relative humidity) conditions, in standard polycarbonate cages with free access to food and water. All experiments were conducted in accordance with NIH animal care guidelines and with the approval of Princeton University Institutional Animal Care and Use Committee. All precautions were taken to minimize any animal pain or discomfort. Animals were allowed to habituate to the new environment for 1–2 weeks. One day after arrival, rats were gently handled twice daily for three days, to reduce possible stress during subsequent handling procedures (e.g., drug injections). Three days prior to the experiment, the animals were housed in individual polycarbonate cages. At the time of initial drug treatment, the mean body weight of the young rats was 317±7 g (n=40), compared to 510±6 g (n=46) for the group of aged rats.

Please cite this article as: Takase, L.F., et al., Effects of the hypnotic drug zolpidem on cell proliferation and survival in the dentate gyrus of young and old rats, Brain Res. (2009), doi:10.1016/j.brainres.2008.12.049

4.2. *Zolpidem and BrdU administration*

Zolpidem tartrate (a gift from Sanofi-Synthelabo, Montpellier, France) was dissolved in sterile 0.9% NaCl (saline) plus 0.1% Tween-80. Drug solutions were prepared fresh daily, and doses are expressed as the salt. An equivalent volume of drug vehicle (2 ml/kg body weight) served as the control in these experiments. To evaluate effects on hippocampal proliferation and cell survival, zolpidem (5, 10 or 20 mg/kg) or drug vehicle was administered i.p. at light onset and again 6 h later. This dual dose regimen was used to try to maintain a hypnotic drug effect over the entire 12-h light phase, when the preponderance of sleep occurs in the rat. The doses of zolpidem were chosen based on previous literature demonstrating the hypnotic efficacy of the drug in rats (Edgar et al., 1997; Mailliet et al., 2001; Wisor et al., 2006). Additionally, direct behavioral observations confirmed that zolpidem exerted a rapid (<5 min) and reliable sedative-hypnotic effect (complete suppression of motor activity and assumption of a recumbent posture) in both young and old rats, with no evidence of tolerance to this action following repeated drug injections. In the acute studies, zolpidem was administered for 2 days and in the chronic studies it was administered for 21 days. To label newly generated cells in the hippocampus, rats received a single i.p. injection of 5-bromo-2′-deoxyuridine (BrdU; 200 mg/kg), 24 h prior to the initiation of chronic zolpidem or vehicle treatment. The BrdU (Sigma-Aldrich Company, St. Louis, MO) was dissolved in sterile saline (containing 0.007 N NaOH) and given in a volume of 10 ml/kg body weight. The effect of chronic zolpidem administration on the subsequent survival of the newborn cells was determined at the end of the study. All animals were sacrificed 18 h after the last drug treatment at light onset (09:00 h), as described below.

4.3. *Animal perfusion*

Rats were deeply anesthetized with chloral hydrate (1000 mg/kg, i.p.) and perfused transcardially with cold physiological saline (containing 10 IU heparin/ml), followed by paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4). Brains were removed, postfixed in paraformaldehyde, cryoprotected with 30% sucrose (in 0.1 M PBS), and then sectioned on a microtome.

4.4. *Immunohistochemistry*

Frozen coronal sections (40-μm thick) were cut throughout the entire hippocampus and every 12th section was then processed for BrdU (cell survival) and Ki67 (cell proliferation) using a slide-mounted immunoperoxidase technique (Fornal et al., 2007).

For BrdU staining, sections were boiled in citric acid, digested with trypsin, denatured with hydrochloric acid, and then incubated with a mouse monoclonal antibody raised against BrdU (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) for 48 h at 4 °C. For Ki67 staining, sections were boiled in citric acid and then incubated with a mouse monoclonal Ki67 antibody (NCL-Ki-67-MM1; Novocastra Laboratories Ltd) for 48 h at 4 °C. Following primary antibody incubation, sections were incubated with a biotinylated horse anti-mouse IgG and with avidin–biotin complex (Vector Laboratories, Burlingame, CA), and then reacted with 3,3′-diaminobenzidine (DAB) to visualize labeled cells. Sections were then counterstained with cresyl violet, dehydrated and coverslipped with DPX mountant.

All slides were analyzed blind with respect to treatment using an Olympus BX-60 light microscope. In every 12th section, BrdU+ and Ki67+ cells (stained brown) were counted bilaterally in the DG at 400× magnification. Cell counts for each animal were summed across all sections and then multiplied by 12 to obtain an estimate of the total number of labeled cells in the DG. In addition, labeled cells were also counted separately in the granular cell layer (GCL)/subgranular zone (SGZ) and in the hilus. Cells located within two cell body widths from the border of the GCL were considered to be in the SGZ; cells located more distally were considered to be in the hilus.

4.5. *Statistical analysis*

Data are expressed as means ± SEM. For statistical comparisons between treatment groups, either a one-way analysis of variance (ANOVA) was used, followed by *post hoc* Bonferroni's multiple comparison test, or, where appropriate, an unpaired (two-tailed) t test. In all cases, a probability value of $p < 0.05$ was considered statistically significant.

Acknowledgments

We gratefully acknowledge the generous financial support from The McKnight Brain Institute of the University of Florida for making this work possible. We also thank Sanofi-Synthelabo for kindly providing the zolpidem for this study and Jessica Barson for her excellent technical assistance. L.T.F was a recipient of a postdoctoral fellowship from the Brazilian government (CNPq, process 200574/2006-0).

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Please cite this article as: Takase, L.F., et al., Effects of the hypnotic drug zolpidem on cell proliferation and survival in the dentate gyrus of young and old rats, Brain Res. (2009), doi:10.1016/j.brainres.2008.12.049

Progress report: Development of nanoencapsulated orexin (Nanorexin^{TM}) as a potential therapeutic for age-related memory loss

The MBI awarded $25,000 to formulate nanoencapsulated orexin A, establish dosing, assess toxicology, and conduct a scaled-down assessment of behavioral efficacy treating memory-impaired aged rats. Because the operational definition of impaired aged rats is the 25% of a sample population with lowest memory performance, 32 aged rats must be purchased to obtain 2 groups of 4 impaired rats, one to receive Nanorexin and one to receive a control formulation.

The project is on budget and has achieved several milestones although the priority need to secure salary support delayed progress. Formulation and analysis of Nanorexin was completed by Nanotherapeutics in late October and the compound is ready for behavioral testing. The PI, Dr. Talton, and Dr. Foster met with Animal Care Facility veterinarians and staff on Dec. 18 to discuss integration and implementation of the housing, drug dosing procedures, and behavioral testing for the initial test groups. A consensus was reached to substitute intranasal application of Nanorexin in aqueous solution for the complex and labor-intensive aerosolized powder inhalation procedures. These can be done in the animal home cage rooms where spontaneous spatial alternation behavioral testing can also be accommodated. Running-wheel cages will be used to monitor potential Nanorexin effects on general activity and sleep-wake cycles. An IACUC protocol for the drug and behavioral testing has been submitted and is under review. Memory-impaired rats pre-screened with the assistance of Dr. Foster's laboratory and the Behavioral Core Facility will be transfered to the Nanorexin testing protocol. The first rats have been purchased for ARML screening, and drug testing will begin as soon as IACUC approval is in place.

It was decided that it was not practical to conduct thorough dosing and toxicology studies before behavioral testing indicates efficacy. These studies are costly and labor-intensive, and Nanotherapeutics has several new large projects that command their resources.

A first-year graduate student in the UF College of Medicine Masters in Biotechnology Program has joined the project as a member of the PI's lab, and is interested in adopting several experiments to complete the laboratory research requirements for his degree. David Demosthenes has a Masters degree in Entrepreneurship and a Bachelor of Science in Finance from UF, plus a good background in biological sciences. He is enthusiastic about gaining experience with basic science and biotechnology professionals working on solutions for age-related neurobiological problems, and is focused on becoming able to launch similar biotech enterprises himself. He contributed to the development of the Nanorexin IACUC protocol and the technical details of the drug testing, and has become quite knowledgeable about orexin signaling in the brain. We look forward to his continued participation as preclinical development proceeds.

Michael A. King, Ph.D.

Annual Report-McKnight Brain Research Foundation
Sponsored Institutes and Research Programs
(Include activity of all McKnight supported faculty and trainees)
Report Period: January 30, 2009

Some gift agreements require both Institute reports and Chair reports. If applicable, please clearly state whether a particular response relates to a Chair or Institute.

Any capitalized terms used on the template are intended to have the same meaning as the term is defined in the Gift Agreement.

1. Summary of scientific achievements since last report

After this award was made, it became clear that one of the principle investigators –Dr. Brent Reynolds- was being actively recruited to the University of Florida, first through the Department of Pharmacology, then through the Department of Neurosurgery. When Dr. Reynolds accepted an appointment to the Department of Neurosurgery, we decided to hold off on beginning the proposed studies until his laboratory and personnel could be moved to Gainesville. He arrived in the Spring of 2008, and shortly thereafter the first disbursement from the award were made. Because of this delay, we have no significant progress to report at this time.

2. Publications in peer reviewed journals

NA

3. Publications (other)

NA

4. Presentations at scientific meetings

NA

5. Presentations at public (non-scientific) meetings or events

NA

6. Awards (other)

NA

7. Faculty. Please include abbreviated CV with publications for previous 12 months

Attached.

8. Trainees
Post doctoral
Pre-doctoral
Other

NA

9. Clinical/translational programs
New programs
Update on existing clinical studies

NA

10. Technology transfer
Patents applications
Revenue generated from technology

NA

11. Budget update (last year's budget and actual results - with an explanation of material variances)
Status of matching funds, if applicable
Projected budget for coming year
Extramural funding

NA

12. Educational programs focusing on age related memory loss
Scientific
Public

NA

13. Collaborative programs with other McKnight Institutes, institutions and research programs

NA

14. Collaborative program with non McKnight Institutes, institutions and research programs

NA

15. Briefly describe plans for future research and/or clinical initiatives

NA

16. If applicable, please provide endowment investment results for the report period.

NA

17. Where any funds used for a Prohibited Purpose during the report period?

No

18. Do you recommend any modification to the Purpose or mandates in the Gift Agreement?

No

19. Did all activities during the report period further the Purpose?

NA

20. Please describe any negative events (loss of personnel, space, budget, etc.) that occurred during the report period and the possible impact on carrying out the Gift Agreement.

The only negative event was the delay necessitated by moving Dr. Reynold's laboratory to Gainesville from Australia.

21. Please provide any general comments or thoughts not covered elsewhere – a response is not required. Please respond only if you would like to add something not otherwise covered elsewhere.

NA

22. Signature, date and title of person submitting the report

Eric D. Laywell, Ph.D.

Assistant Professor

Department of Anatomy & Cell Biology

College of Medicine

University of Florida

Curriculum Vitae

Name: Eric Dion Laywell
Birthdate: 10/27/64
Marital Status: Married (Francee)
Children: one son (Matthias, 9); two daughters (Savannah & Natalie, 6)

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Undergraduate: University of Michigan
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1995-1997

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Assistant Professor (Research)
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2004-present

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Laboratory Instructor in Dental Gross Anatomy (1989) and Medical Gross Anatomy at the University of Tennessee, Memphis (1989-93; 1997-98).

Laboratory Instructor in Medical Neuroanatomy at the University of Tennessee, Memphis (1992- 93, 97-98).

Laboratory Instructor in Medical Gross Anatomy at the University of Utah (1994 & 1995).

Medical Neuroscience at the University of Florida (2003).

Biomedical Engineering Gross Anatomy at the University of Florida (2005 & 2006).

Medical Gross Anatomy at the University of Florida (2006-present)

Papers:

Levkoff, L.H., Marshall, G.P. II, Ross, H.R., Calderia, M., Reynolds, B.A., Cakiroglu, M., Mariani, C.L., Streit W.J., & **E.D. Laywell** (2008) Bromodeoxyuridine inhibits cancer cell proliferation in vitro and in vivo. *Neoplasia* 10:804-16.

Ross, H.R., Levkoff, L.H., Marshall, G.P. II, Calderia, M., Steindler, D.A., Reynolds, B.A., & **E.D. Laywell** (2008) Bromodeoxyuridine induces senescence in neural stem and progenitor cells. *Stem Cells* 26:3218-27.

Marshall, G.P. II, Cakiroglu, M., Steindler, D.A., & **E.D. Laywell** (2008) Subventricular zone microglia possess a unique capacity for massive in vitro expansion. *GLIA* 56:804-16.

Zheng T., Marshall, G.P. II, Chen, K.A., & **E.D. Laywell** (2009) Transplantation of neural stem/progenitor cells into developing and adult CNS.

Annual Report-McKnight Brain Research Foundation
Sponsored Institutes and Research Programs
(Include activity of all McKnight supported faculty and trainees)
Report Period: January 1 – December 31, 2008

Some gift agreements require both Institute reports and Chair reports. If applicable, please clearly state whether a particular response relates to a Chair or Institute.

Any capitalized terms used on the template are intended to have the same meaning as the term is defined in the Gift Agreement.

1.) Summary of scientific achievements since last report

I.) The antioxidant response element (ARE) regulates the expression of many neuroprotective antioxidant enzymes and scavengers which contribute to the endogenous defense against oxidative stress. We have continued to characterize gene products and small molecules that can regulate the ARE.

(A) *Gene products that activate the ARE.* After a variety secondary assays, we narrowed down our hit list from the 15,000 cDNA screen to one single cDNA. Upon overexpression, this particular cDNA potently induces the neuroprotective enzymes NQO1 and HO-1. We validated that it is required for full effect of the ARE model activator *tert*-butylhydroquinone, indicating that we have identified a new essential component in the ARE pathway. Overexpression through gene delivery or activating this protein by small molecules may have beneficial antioxidant effects.

(B) *Gene products that repress ARE activation.* Our initial high-throughput small interfering RNA (siRNA) screen used pools of four siRNAs against the same target and was associated with significant noise. Furthermore, we found that the pooling of several siRNAs may also have contributed to lack of hit reproducibility. However, one target, upon reduction of gene dosage by siRNAs, appears to reproduce. We have obtained individual siRNAs and validation is in process. During the past year we also acquired a siRNA library targeting the druggable genome with quadruplicate coverage where all siRNAs are arrayed individually. ARE activation of several siRNAs against the same target would increase the confidence in on-target effects of our hits. Major emphasis was placed on preparing stock solutions of the library and preparing screening sets by spotting the library into 384-well assay plates.

(C) *Small molecule ARE activators.* In collaboration, we have characterized a fatty acid oxidation product that can activate the ARE. A manuscript has been submitted for publication. Our marine discovery efforts to identify novel ARE activators from the ocean have yielded enough preliminary data to apply for (and obtain) NIH grant funding. We have been studying algae extracts for cancer prevention, but also test extracts for their ability to activate the ARE in neural cell

lines related to neuroprotection and memory loss. This project is still at an early stage.

II.) Through other grant-mechanisms we discovered a new anticancer agent from the marine environment, largazole. We found that this compound inhibits cancer cell growth by inhibiting a class of enzymes, termed histone deacetylases (HDACs). Interestingly, aberrant HDAC activity has been linked to a variety of other diseases, including memory loss. We would like to pursue further studies with this compound with respect to memory loss.

2.) Publications in peer reviewed journals

3.) Publications (other)

Luesch, H., Liu, Y. "Genome-Wide Overexpression Screen for Activators of Antioxidant Gene Transcription" In: Advanced Protocols in Oxidative Stress I, Series: Methods in Molecular Biology 477, Humana Press, 2008.

ATTACHED

4.) Presentations at scientific meetings

Luesch, H. "From Marine Natural Products to Gene Discovery: Integrative Approaches in Chemical Biology and Functional Genomics" University of North Florida, Jacksonville, FL, March 28, 2008 (Invited Speaker).

Luesch, H. "From Marine Natural Products to Target Proteins: Integrative Approaches in Chemical Biology" The 22nd Naito Conference on Chemical Biology (I), Sapporo, Japan, September 9–12, 2008 (Invited Speaker).

5.) Presentations at public (non-scientific) meetings or events

6.) Awards (other)

7.) Faculty. Please include abbreviated CV with publications for previous 12 months

Other 2008 papers not supported by McKnight:

Ying, Y.; Liu, Y.; Byeon, S. R. ; Kim, H.; **Luesch, H.**;* Hong, J.* "Synthesis and Activity of Largazole Analogues with Linker and Macrocycle Modification" *Org. Lett.* **2008**, *10*, 4021–4024.

Taori, K.; Paul, V. J.; **Luesch, H.*** "Kempopeptins A and B, Serine Protease Inhibitors with Different Selectivity Profiles from a Marine Cyanobacterium, *Lyngbya* sp." *J. Nat. Prod.* **2008**, *71*, 1625–1629.

Ying, Y.; Taori, K.; Kim, H.; Hong, J.;* **Luesch, H.*** "Total Synthesis and Molecular Target of Largazole, a Histone Deacetylase Inhibitor" *J. Am. Chem. Soc.* **2008**, *130*, 8455–8459.

Matthew, S.; Schupp, P. J.; **Luesch, H.*** "Apratoxin E, a Cytotoxic Peptolide from a Guamanian Collection of the Marine Cyanobacterium *Lyngbya bouillonii*" *J. Nat. Prod.* **2008**, *71*, 1113–1116.

Gunasekera, S. P.; Ross, C.; Paul, V. J.; Matthew, S.; **Luesch, H.** "Dragonamides C and D, Linear Lipopeptides from the Marine Cyanobacterium Brown *Lyngbya polychroa*" *J. Nat. Prod.* **2008**, *71*, 887–890.

Matthew, S.; Ross, C.; Paul, V. J.; **Luesch, H.*** "Pompanopeptins A and B, New Cyclic Peptides from the Marine Cyanobacterium *Lyngbya confervoides*" *Tetrahedron* **2008**, *64*, 4081–4089.

Kwan, J. C.; Rocca, J. R.; Abboud, K. A.; Paul, V. J.; **Luesch, H.*** "Total Structure Determination of Grassypeptolide, a New Marine Cyanobacterial Cytotoxin" *Org. Lett.* **2008**, *10*, 789–792.

Taori, K.; Paul, V. J.; **Luesch, H.*** "Structure and Activity of Largazole, a Potent Antiproliferative Agent from the Floridian Marine Cyanobacterium *Symploca* sp." *J. Am. Chem. Soc.* **2008**, *130*, 1806–1807.

8.) Trainees
Post doctoral: Yanxia Liu (since 2006)
Pre-doctoral
Other

9.) Clinical/translational programs
New programs
Update on existing clinical studies

10.) Technology transfer
Patents applications
Revenue generated from technology

11.) Budget update (last year's budget and actual results - with an explanation of material variances)
Status of matching funds, if applicable
Projected budget for coming year
Extramural funding

ARE-related grants (not for age-related memory loss but for protection from other oxidative stress induced injury/disease):

Scientist Development Grant, American Heart Association (Florida Affiliate), H. Luesch, "Protection from Stroke-Induced Brain Damage through Modulation of Gene Expression" 7/1/2006–6/30/2009, Direct cost $240,000, Total cost $264,000.

1R21CA133681-01A1 (NIH/NCI), H. Luesch, "Activation of the Cancer Preventive Nrf2-ARE Pathway by Seaweed" 7/1/2008–6/30/2010, Total direct cost, Direct cost $272,807, Total cost $357,447.

12.) Educational programs focusing on age related memory loss
Scientific
Public

13.) Collaborative programs with other McKnight Institutes, institutions and research programs

14.) Collaborative program with non McKnight Institutes, institutions and research programs

15.) Briefly describe plans for future research and/or clinical initiatives

We will pursue two major avenues to achieve neuroprotection.

I.) Consistent with the originally proposed studies, we will continue to discover ways to activate the ARE and thereby increase the cellular antioxidant status.

A. We will continue to validate gene products from our cDNA screen that positively regulate the ARE. We will transfer our top ARE activating gene in adenoassociated viral vector (AAV) and test for ARE activation *in vivo* (rat brain). We also intend to initiate structure-function studies by mutagenesis.

B. Using our new siRNA library with quadruplicate coverage of the druggable genome, we will execute screens with transcriptional readout (ARE reporter screen) to identify new negative regulators of the ARE. Furthermore we will optimize screens to directly measure antioxidant proteins in a high-throughput fashion.

C. Algae extracts/fractions that can activate the ARE reporter in tissue culture will be evaluated for the induction of ARE-regulated antioxidant proteins in cellular models and possibly also *in vivo* at the end of 2009.

II.) Our discovery of the HDAC inhibitor largazole is prompting us to test this compound also for the prevention of memory loss, since epigenetic gene silencing due to aberrant HDAC activity has been linked to memory loss. We intent to identify collaborators for this endeavor.

16.) If applicable, please provide endowment investment results for the report period.

17.) Where any funds used for a Prohibited Purpose during the report period?

18.) Do you recommend any modification to the Purpose or mandates in the Gift Agreement?

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Chapter 26

Genome-Wide Overexpression Screen for Activators of Antioxidant Gene Transcription

Hendrik Luesch and Yanxia Liu

Abstract

Genome-wide screens have proven powerful in associating gene products with certain phenotypes or signal transduction pathways, and thus are valuable tools to ascribe gene function. These genomic screens can be extended to discover genes/proteins that attenuate oxidative stress-induced damage, which is implicated in aging, neurodegenerative disorders, and other diseases. One mechanism by which humans protect themselves from oxidative stress is through an endogenous stress response that leads to transcriptional activation of the antioxidant response element (ARE). The ARE is located in the 5′-flanking regions of many phase II detoxification and antioxidant enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and regulates the expression of these genes. Increasing the levels of antioxidant enzymes without causing oxidative stress can potentially counteract degeneration and may be therapeutically useful. On a genomic scale, ARE activators can be identified by screening expression cDNA libraries in a high-throughput amenable reporter gene assay. Further validation of putative hits requires testing of cDNAs for their ability to upregulate the expression of endogenous ARE-regulated genes on the transcript and protein levels, and their ability to protect cells from oxidative insults. General screening procedure and subsequent hit validation are discussed in detail.

Key words: Oxidative stress, Genome-wide screen, cDNA library, Antioxidant response element, Reporter gene assay, Neuroprotection, Cytoprotection.

1. Introduction

Oxidative stress contributes to aging and age-related neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, and Huntington's disease *(1, 2)*. The ARE, a *cis*-acting enhancer element found in the 5′-flanking regions of numerous

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genes including those encoding phase II detoxification enzymes, mediates the transcriptional activation of downstream genes in cells exposed to oxidative stress *(3)*. The activation of the ARE has neuroprotective effects; oxidative damage induced death of neuroblastoma cells, astrocytes, or neurons is attenuated or prevented *(4, 5, 6)*. Oxidative stress-independent ARE activation could provide a therapeutic approach to neurodegeneration by promoting the beneficial upregulation of neuroprotective enzymes without the detrimental effects of uncontrolled oxidative stress.

It is impossible to rapidly identify positive ARE regulators in traditional small-scale or one-gene-at-a-time studies. Here, we describe an approach to find genes involved in protection from oxidative stress by genome-wide high-throughput screening of thousands of expression cDNAs spatially arrayed in 384-well plates with an ARE reporter gene assay *(7)*. Putative hits need to be rigorously validated in secondary assays; examples are described in greater detail in this chapter as well.

2. Materials

2.1. Equipment

1. Dispense instrument (e.g., WellMate from Matrix Technologies Corp., Hudson, NH).
2. SpectraMax M5 or CLIPR luminescence plate reader (Molecular Devices, Sunnyvale, CA).
3. Real-time PCR system model 7300 (Applied Biosystems, Foster City, CA).
4. Gel Logic 2200 imaging system (Kodak, Rochester, NY).

2.2. Cell Culture and Transfection

1. IMR-32 neuroblastoma cells (ATCC, Manassas, VA).
2. Dulbecco's Modified Eagle Medium (DMEM) (1X) liquid (high glucose) (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT) and 1% Antibiotic-Antimycotic (Invitrogen).
3. OPTI-MEM® I Reduced Serum Medium (Invitrogen).
4. FuGENE 6 (Roche, Indianapolis, IN).
5. siLentFect (Bio-Rad, Hercules, CA).
6. Versene 1:5000, liquid (Invitrogen).
7. CMV-*GFP* (green fluorescent protein) plasmid (Clontech, Palo Alto, CA).

2.3. Genome-wide Screening for ARE Activators

1. cDNA library can be obtained from Origene Technologies (Rockville, MD) or Mammalian Gene Collection (MGC; e.g., ATCC). Alternatively, pre-spotted (Assay-Ready) MGC cDNA screening sets are available from Open Biosystems (Huntsville, AL).
2. *NQO1*-ARE luciferase reporter construct *(8)*.
3. Expression cDNA encoding constitutively active phosphatidylinositol 3-kinase (PI3K), PI3K* *(9)* (*see* **Note 1**).
4. Bright-Glo™ luciferase assay system (Promega, Madison, WI).
5. Dispense instrument (WellMate from Matrix Technologies Corp., Hudson, NH).
6. Luminescence plate reader (SpectraMax M5 or CLIPR from Molecular Devices, Sunnyvale, CA).
7. 384-well solid white flat bottom tissue culture (TC)-treated microplates (Corning Incorporated, Corning, NY).

2.4. Hit Confirmation

1. β-Galactosidase plasmid (e.g., actin-*lacZ*).
2. *N*-acetyl cysteine (Sigma, St. Louis, MO).
3. Gal-Screen® assay system (Applied Biosystems, Foster City, CA).
4. Bright-Glo™ luciferase assay system (Promega).
5. 24-well TC-treated plates (Nunc, Rochester, NY).
6. 96-well solid white flat bottom TC-treated microplates (Corning Incorporated).
7. Vector control (e.g., pCMV6-XL4; OriGene Technologies).

2.5. Real-Time PCR

1. RNeasy Kit (Qiagen, Valencia, CA).
2. Oligo$(dT)_{12-18}$ Primer (Invitrogen).
3. 10 mM dNTP Mix, PCR Grade (Invitrogen).
4. RNaseOUT™ Ribonuclease Inhibitor (Invitrogen).
5. SuperScript™ II Reverse Transcriptase (Invitrogen).
6. Human *NQO1* and *GAPDH* TaqMan® Gene Expression Assays (Applied Biosystems).
7. TaqMan® Universal PCR Master Mix (Applied Biosystems).
8. DEPC-treated sterile water.
9. 7300 Real-Time PCR System (Applied Biosystems).
10. 6-well TC-treated plates (Nunc).

2.6. Immunoblot Analysis

1. Protein lysis buffer: PhosphoSafe extraction reagent (Novagen, Madison, WI).
2. Sample buffer: NuPAGE® LDS Sample Buffer (Invitrogen).

Buffers (*see* **Note 2**)

3. Running buffer: NuPAGE® MES SDS Running Buffer (Invitrogen).
4. Transfer Buffer: NuPAGE® Transfer Buffer (Invitrogen) with 10–20% Methanol (Fisher Scientific, Fair Lawn, NJ).
5. Wash buffer: Tris Buffered Saline, with Tween® 20 (TBS-T, pH 8.0, Sigma).
6. Blocking buffer: 5% (w/v) BSA (Sigma) or 5% nonfat dry milk in TBS-T.

PAGE Gels

1. NuPAGE® Novex® 4–12% Bis-Tris Mini Gels (Invitrogen).

Antibodies

1. Primary antibodies: anti-NQO1 (Abcam, Cambridge, MA), anti-β-actin (Cell Signaling, Beverly, MA).
2. Secondary antibodies: Anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody (Cell Signaling); Anti-goat IgG, HRP-linked antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Detection

1. SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).
2. X-ray film (Kodak) or Imaging System (e.g., Gel Logic 2200; Kodak).

Others

1. BCA protein assay kit (Pierce), PVDF membrane (Millipore, Billerica, MA or Invitrogen), filter paper (Pierce), cell scrapers (Nunc), 6-well TC-treated plates (Nunc).

2.7. Protection Assay

1. Hydrogen Peroxide (H_2O_2, Sigma).
2. CellTiter-Glo® Luminescent Cell Viability Assay (Promega).
3. 24-well TC-treated plates (Nunc).
4. 96-well solid white flat bottom TC-treated microplates (Corning Incorporated).

3. Methods

In genome-wide screens, each plate should contain a positive and negative control. Constitutively active phosphatidylinositol 3-kinase (PI3K), PI3K* *(9)*, which is known to activate the ARE in IMR-32 cells *(10)*, can be used as a positive control; empty vector is a suitable negative control. However, instead of relying on a single well (or even quadruplicate wells) as the negative control, the average luminescence readout of the plate can be used as background control in genome-wide screening (**Fig. 26.1**). This is acceptable since only relatively few cDNAs will exert an effect

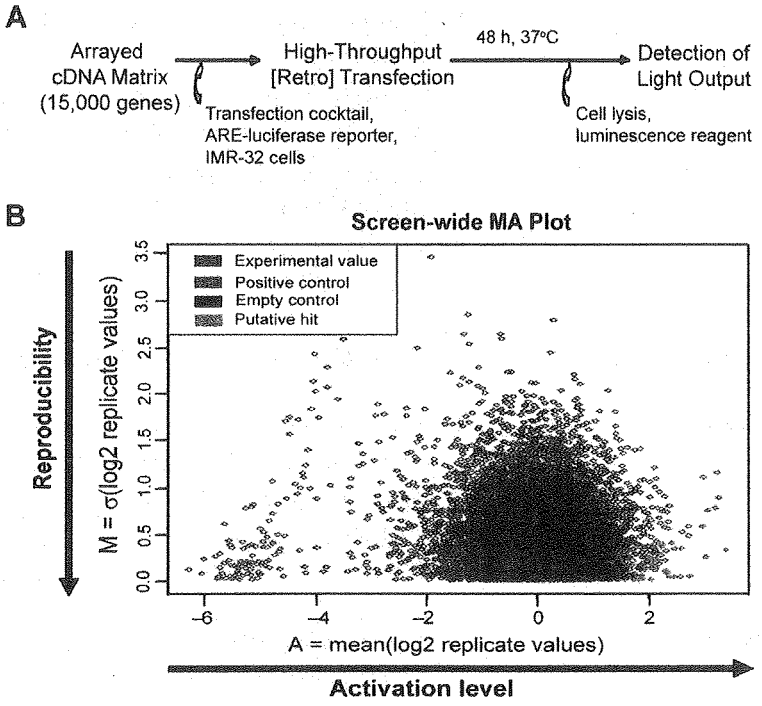

Fig. 26.1. Genome-wide cDNA overexpression screen for ARE activators. (**A**) General high-throughput screening procedure. Approximately 15,000 expression cDNAs, normalized and arrayed in 384-well plates, were transfected into IMR-32 human neuroblastoma cells along with an ARE-luciferase reporter construct. Following 48 h incubation, luciferase activity was assessed by measuring luminescence output per well. (**B**) Screen-wide MA plot. The screen was carried out in duplicate and M (a measure of screen-to-screen variation; σ = standard deviation) plotted as a function of A (a measure of the mean ARE activation from both screens). The cDNAs that strongly activated the ARE in a reproducible manner were investigated further (indicated in green; lower right corner). (Copyright 2007 National Academy of Sciences, U.S.A.; reproduced from reference 7 with permission).

in the ARE reporter gene assay when using a random cDNA collection. However, empty vector (or a cDNA that has no effect) should be used as negative control for confirmation and validation studies.

ARE activators, identified by high-throughput genome-wide cDNA library screening, should be subjected to thorough validation, accounting for well-to-well differences in transfection efficiency by cotransfecting another reporter gene construct that is not responsive to ARE activators (**Fig. 26.2A**). To ensure that the cDNAs do not induce oxidative stress, which would also lead to ARE activation, ARE activity is determined in the presence of excess antioxidant (e.g., *N*-acetyl cysteine). It is then necessary to correlate the reporter assay data with the induction of endogenous target genes. One enzyme that is highly responsive to ARE activation and also plays an important role in the protection from

oxidative stress is NQO1. NQO1 prevents the reduction of quinones, which causes the production of radical species; it has also been associated with Alzheimer's disease *(11)*. Further validation of the identified ARE activators is carried out by real-time PCR and Western blotting for NQO1 gene products (**Fig. 26.2B, C**). Since the objective is to find neuroprotective cDNAs, one needs to ultimately demonstrate that the cDNAs can attenuate oxidative stress-induced damage or cell death. Upon overexpression of neuroprotective cDNAs, cell viability in the presence of hydrogen peroxide is expected to be increased (**Fig. 26.2D**).

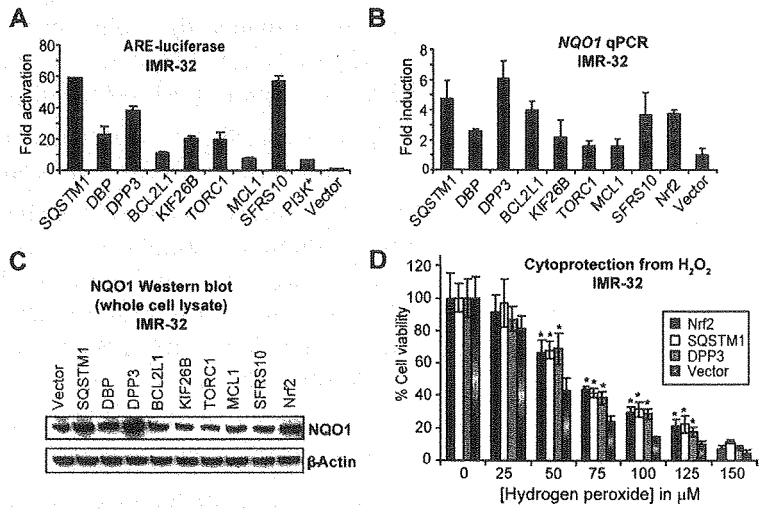

Fig. 26.2. Confirmation and validation of putative screening hits in IMR-32 cells. (**A**) Transcriptional ARE activation in IMR-32 cells. IMR-32 cells were cotransfected with cDNAs, the ARE-luciferase reporter, and actin-*lacZ* for normalization in 24-well plates. Luminescence was detected 48 h later; normalized values are given ($n = 6$). Eight cDNAs showed equal or higher activity than the positive control, PI3K*. (**B**) Effect of cDNA overexpression in IMR-32 cells on *NQO1* transcript levels as analyzed by quantitative real-time PCR (qPCR). The cDNAs were introduced by lipofection and then total RNA was isolated 48 h later, reverse-transcribed to cDNA, and subjected to TaqMan analysis ($n = 3$). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression was used as internal control for normalization. Overexpression of several cDNAs increased *NQO1* levels to the same extent as Nrf2 overexpression. (**C**) Induction of NQO1 upon cDNA overexpression in IMR-32 cells as analyzed by Western blot analysis. cDNAs were transfected using lipofection, and proteins were isolated 48 h later, resolved by SDS-PAGE, and subjected to Western blot analysis for NQO1. SQSTM1 and DPP3 induced NQO1 most strongly and to a comparable extent as Nrf2. A representative blot ($n = 4$) is shown. (**D**) Nrf2, SQSTM1, and DPP3 mediate protection from oxidative stress in vitro. In 24-well plate format using siLentFect, IMR-32 cells (180,000) were transfected with corresponding cDNAs and with vector control (0.5 μg). After 48 h, cells were treated with various concentrations of hydrogen peroxide ($n = 4$). Cell viability was assessed 6 h later using the CellTiter-Glo® assay kit (Promega). Overexpression of Nrf2, SQSTM1, or DPP3 resulted in a shift of the IC_{50} for hydrogen peroxide against IMR-32 cells. * Indicates statistical significance compared to vector of $p < 0.05$. (Copyright 2007 National Academy of Sciences, U.S.A.; reproduced with minor modifications from reference 7 with permission).

3.1. Reporter Gene Assay-Based Identification of ARE Activators

3.1.1. High-Throughput Genome-Wide Screening

1. cDNAs are arrayed in 384-well plates (**Fig. 26.1A**); each well contains pre-spotted 62.5 ng of cDNA (*see* **Note 3**). The plates are stored at −80°C.
2. The cDNA-containing plates and Opti-MEM are equilibrated to room temperature (*see* **Note 4**).
3. Transfection mixture (*see* **Note 5**) is prepared based on the ratio of 3 (μL, FuGENE 6) to 1 (μg, cDNA and reporter gene). In a sterile bottle, Opti-MEM is added first, then FuGENE 6 is added, gently mixed by inversion (~five times), and then ARE-luciferase reporter construct is added, again followed by repeated inversion to allow gentle mixing (*see* **Notes 6** and 7). For one 384-well plate, the transfection mixture contains the following: Opti-MEM (7.68 mL; 20 μL/well), ARE-luciferase reporter construct (19.2 μg; 50 ng/well), FuGENE 6 (129.6 μL; 0.3375 μL/well).
4. 20 μL of transfection mixture is dispensed into each well and incubated for 30 min (*see* **Note 8**).
5. During the incubation period, cells are prepared (*see* **Note 9**). The medium is carefully removed from the tissue culture dishes by aspiration and 2 mL of Versene is added to each 10-cm dish to detach IMR-32 cells. The cells are spun down and resuspended in 20% FBS-containing DMEM at a density of 400,000 cells/mL.
6. Around 20 μL of the resuspended cells is dispensed into each well (8000 cells/well).
7. The cells are incubated in a cell culture incubator (37°C, 5% CO_2) for 48 h (**Fig. 26.1A**).
8. Detection reagent (Bright-Glo) is prepared immediately before use by mixing the lysis buffer and luciferase substrate and equilibrated to room temperature. Assay plates are equilibrated to room temperature (~10 min).
9. Around 40 μL of Bright-Glo is dispensed into each well.
10. The plate is mixed on a plate shaker for ~1 min and the luminescence is read within 5 min after adding Bright-Glo (*see* **Note 10**).
11. The fold activation over plate average is calculated for each well (**Fig. 26.1B**).
12. cDNAs that showed high activation in duplicate assays are selected for confirmation studies.

3.1.2. Hit Confirmation

1. This study is performed in a 24-well plate format.
2. Transfection mixture (*see* **Note 5**) is prepared in a sterile microcentrifuge or Falcon tube (*see* **Note 6**). Each tube (for four wells: two for *N*-acetyl cysteine treatment, two for solvent control) contains the following: Opti-MEM (1 mL; 250 μL/well),

cDNA or CMV empty vector or CMV-*PI3K** (6.24 μg; 1.56 μg/well), ARE-luciferase reporter construct (2.5 μg; 625 ng/well), actin-*lacZ* (800 ng; 200 ng/well; for normalization), and FuGENE 6 (28.62 μL; 7.155 μL/well).

3. The transfection mixture is incubated for 30 min (up to 1 h) at room temperature.
4. The same method as described above (*see* **Section 3.1.1**) is used to prepare cells at a density of 800,000 cells/mL in 20% FBS-containing DMEM (*see* **Note 9**).
5. Resuspended cells (1 mL) are added to each tube and tube content is mixed by inverting or tapping.
6. The mixture is transferred to a 24-well plate (each tube's content is split into four wells).
7. The plate is incubated in a cell culture incubator (37°C, 5% CO_2).
8. 12 h after transfection, cells are treated with excess of antioxidant, *N*-acetyl cysteine (1 mM final concentration), or solvent (DMEM).
9. The cells are incubated in the cell culture incubator for another 36 h.
10. Bright-Glo (0.5 mL; the same volume as the medium volume) is added to half of the wells (*N*-acetyl cysteine treated and solvent treated), well content mixed by pipetting or on a plate shaker, and transferred to a 96-well white solid bottom plate to analyze luciferase activity by luminescence.
11. Gal-Screen system (0.5 mL; the same volume as the medium volume) is added to the other half of the wells. The mixture is homogenized by pipetting or on a plate shaker and incubated for approximately 60–90 min, then transferred to a 96-well white solid bottom plate. Luminescence is read as a measure of β-galactosidase activity, which correlates with transfection efficiency.
12. The ARE activity (=luciferase activity) is normalized for transfection efficiency (=β-galactosidase activity) by determining the ratio of both activities.
13. The cDNAs, which activate the ARE to the same extent with or without antioxidant are used for the validation study (**Fig. 26.2A**).

3.2. Validation Study

1. Overexpression of cDNAs in IMR-32 cells
   1.1 Transfection is performed in 6-well plate format. The transfection mixture (*see* **Note 5**) is prepared in a sterile microcentrifuge or Falcon tube. Each tube (for each well) contains the following: Opti-MEM (1.0 mL), cDNA or CMV empty vector (1.6 μg),

3.2.1. Induction of Endogenous ARE-Regulated Genes on Transcription Level (Real-Time PCR)

CMV-*GFP* (0.4 μg, to monitor transfection efficiency), siLentFect (4.0 μL, based on the ratio of 2:1, *see* **Note 11**). The transfection mixture is incubated for 30 min at room temperature. Cells are prepared at a density of 600,000 cells/mL in 20% FBS-containing DMEM (*see* **Note 9**).

1.2 Resuspended cells (1.0 mL) are added to each tube, and tube content is mixed by inverting or tapping.

1.3 The mixture is transferred to a 6-well plate.

1.4 Cells are incubated in a cell culture incubator (37°C, 5% CO_2) for 48 h.

2. Real-time PCR assay

2.1 Total RNA extraction – This step should be performed according to the handbook of the RNeasy kit (*see* **Note 12**).

2.2 First strand cDNA synthesis – First strand cDNA is synthesized by using 2 μg of total RNA according to the manual for SuperScript™ II Reverse Transcriptase (Invitrogen).

2.3 Real-time PCR

2.3.1 The PCR reaction mixture is prepared for each sample separately as follows (*see* **Note 6**). For 96-well plate assays, 25 μL reactions are prepared for each well. Each reaction contains either 1.25 μL human *NQO1* or human *GAPDH* (as endogenous control for normalization) TaqMan Gene Expression Assay (20X), 11.25 μL of the mixture of first strand cDNA (10–100 ng) and sterile H_2O, and 12.5 μL of TaqMan Universal PCR Master Mix (2X). Assays are carried out in triplicate.

2.3.2 Quantitative PCR is monitored on the ABI 7300 system according to the manufacturer's instructions for TaqMan analysis.

2.3.2 Relative *NQO1* transcript levels in cDNA-transfected cells versus vector control-transfected cells are determined (**Fig. 26.2B**).

3.2.2. Induction of Endogenous ARE-Regulated Genes on Protein Level (Immunoblot Analysis)

1. Overexpression of cDNAs in IMR-32 cells
The same method as described in **Section 3.2.1** (Real-time PCR) is used.
2. Western blotting

2.1 Cell lysates (*see* **Notes 12** and **13**) are collected according to the lysis buffer manual.

2.2 Protein concentrations are measured using a BCA protein assay kit.

2.3 Protein samples (20–50 μg) are separated by gel electrophoresis at 200 V for ~40 min.

2.4 Proteins are transferred from the separating gel to a PVDF membrane at 30 V for 1–2 h at room temperature.

2.5 The PVDF membrane is incubated in 50 mL blocking buffer for 1 h at room temperature on a rocking shaker.

2.6 The membrane is incubated with primary antibody of a 1:500 (anti-NQO1) or 1:1000 (anti-β-actin) dilution in blocking buffer at 4°C overnight on a rocking shaker.

2.7 The primary antibody is then removed and the membrane washed three times for 15 min each with 50 mL TBS-T.

2.8 The membrane is then incubated with secondary antibody of a 1:2000 dilution in blocking buffer at room temperature for 1 h.

2.9 The secondary antibody is removed and the membrane washed three to five times for 15 min each with TBS-T.

2.10 The signal is detected with SuperSignal West Femto Maximum Sensitivity Substrate (or other substrates for HRP) with X-ray film or imaging system (**Fig. 26.2C**).

3.3. Protection Assay

Overexpression is performed in 24-well plate format. The same transfection method as described above (*see* **Section 3.2.1**) is used. The transfection mixture (for each well) consists of the following: Opti-MEM (250 μL), cDNA or CMV empty vector (0.5 μg), CMV-*GFP* (0.1 μg, to monitor transfection efficiency), and siLentFect (1.2 μL).

3.3.1. Overexpression of cDNAs in IMR-32 Cells

3.3.2. Oxidative Stimulation

After transfection, cells are placed into the incubator for 48 h to allow cDNA overexpression and consequent induction of ARE-regulated target genes (*see* **Note 14**). Cells are treated with various concentrations of H_2O_2 for 6 h in quadruplicate (*see* **Note 15**).

3.3.3. Cell Viability Assay

1. CellTiter-Glo® reagent is prepared shortly before use and 500 μL added to each well (*see* **Note 16**).
2. The content is mixed for 2 min on a plate shaker to induce cell lysis.
3. The product mixtures are transferred to 96-well plates (200 μL per well) and plates incubated at room temperature for 10 min to stabilize the luminescence signal.
4. Luminescence is recorded and viability calculated for each well (**Fig. 26.2D**).

4. Notes

1. cDNAs encoding other known ARE activators such as Nrf2 (>200-fold activation) can be used alternatively. However, reliable detection of *PI3K** (5- to 10-fold activation) as a hit would guarantee that other weak activators could be detected as well.
2. Alternatively, buffers can be made according to standard recipes or obtained from other commercial suppliers.
3. cDNA amount can be reduced as desired. For example, assay-ready plates from Open Biosystems contain 35 ng of DNA per well.
4. The assay plates are centrifuged for 1–2 min at 1000 g to make sure that the DNA is on the bottom of the wells. Approximately 30 min are needed to equilibrate cDNA-containing assay plates and Opti-MEM to room temperature.
5. All transfection mixtures can be prepared either as described in this protocol or, alternatively, by pre-adding DNA into the tube, and then adding Opti-MEM followed by transfection reagent. This would not affect the transfection efficiency for IMR-32 cells.
6. Extra amount is prepared due to volume loss occurring between each dispensing or pipetting step.
7. For library screening, plates and dispenser should be kept as sterile as possible and the time calculated carefully. For example, two people may be needed when dealing with larger plate numbers (e.g., 30+). One person may then dispense the transfection mixture into the wells while the second person starts with the preparation of cells.
8. The transfection mixture could be incubated from 20 min to >1 h (depending on cell type and transfection reagent). Usually, 30–45 min is advised.
9. The confluency of the IMR-32 cells mentioned in this protocol should be 50–80%.
10. All the luminescence readings are performed within 5 min after adding Bright-Glo. The half-life of Bright-Glo is approximately 30 min in IMR-32 cells. Other luciferase detection reagents with more stable signal and from different suppliers may be used.
11. To transfect IMR-32 cells, the ratio of FuGENE 6 (μL) to DNA (μg) amount is 3:1. For siLentFect, the ratio is 2:1. The ratio should be optimized before starting the experiment by cotransfecting CMV-*GFP* to monitor transfection efficiency. Transfection efficiency varies depending on cell type and transfection reagent.

12. The rinse step is optional and depends on cell types. To remove medium from IMR-32 cells, the plate should be tilted and residual medium removed by pipetting instead of rinsing with PBS. IMR-32 cells attach only loosely to the plate and may otherwise be washed away easily.
13. Other lysis buffers can be used. PhosphoSafe or other phosphatase inhibitor containing lysis buffers retain the phosphorylation status of the proteins, potentially allowing the investigation of phosphorylation changes upon cDNA overexpression.
14. Optimal time points may be cDNA dependent. Generally 48–72 h should be suitable.
15. Optimal treatment time must be determined empirically. Longer (chronic) exposure times and other oxidative insults such as mitochondrial complex I inhibitors (e.g., rotenone) or complex II inhibitors (e.g., 3-nitropropionic acid) may also be considered.
16. This reagent measures ATP content as an indicator of cell viability. Other cell viability reagents such as MTT (Promega), AlamarBlue (Invitrogen), or sulforhodamine B (Sigma) may also be suitable. Dynamic range, which depends on the combination of cell line and detection reagent, should be considered in the reagent selection.

Acknowledgments

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1. Summary of scientific achievements since last report

Our project consists of a new drug clinical trial in humans and for this past year our efforts reflect the regulatory processes involved with such a project. Please see **timetable** below describing our efforts toward initiating this clinical trial. Upon funding from the McKnight Brain Research Institute, our first priority was to identify a company that could provide the compound resveratrol for the study. Based on recommendations from colleagues, we contacted the company DSM Inc, a dietary supplement company. This company agreed to provide resveratrol for the study provided the FDA was not involved. Thus, we sought out FDA approval to receive exempt status to conduct the study in parallel with our discussions with DSM. Unfortunately, the FDA exempt application was rejected and, the FDA indicated an "investigational new drug" (IND) application was needed for the project. Since DSM is a nutrient company, they would not support an IND application. We then contacted Pharmascience, a company that manufactures resveratrol and is actively involved in the IND process. After two months of deliberation, this company rejected the proposal as they stated it presented a conflict of interest with a study they were currently supporting. In our third attempt, we contacted the company Orengus Pharmaceutical Inc, who eventually agreed to provide the product after several conference calls, pending approval of a written contract (i.e., Letter of Agreement). To achieve this goal, we enlisted the help of the UF Licensing and Technology Office. The correct wording that would protect all interested parties was a critical element of this contract, and negotiations spanned between August to December 2008. The contract was finalized in January 2009, and all parties have accepted the terms of agreement.

Timetable of events

Date	Action	Outcome
2//08	Inquiry to FDA about resveratrol of a dietary supplement	Recommendation to submit as exempt protocol
3/08	Contact **DSM Inc** for providing resveratrol compound	DSM agrees to provide product and assist in FDA exempt status
5-6/08	Submission to FDA to place project into exempt status.	FDA rejected exempt status. FDA requires "investigational new drug" (IND) application.

6/08	DSM Inc does not agree to provide support for an IND application	Search for new company to provide resveratrol product
6/08	Contact Pharmascience to provide resveratrol product	Company is interested and requires a proposal sent to executive committee to be reviewed in one month
7/08	**Pharmascience Inc.** rejects proposal because of a conflict of interest	Search for new company to provide resveratrol product
7/08	**Orgenus Pharmaceutical Inc.** agrees to review the proposal and agrees to support and FDA IND application	Orgenus Pharmaceutical Inc agrees to provide resveratrol for our study pending approval of a Letter of Agreement by both parties
8-12/08	UF licensing and DSR collaborate to draft a contract to Orgenus.	Letter of Agreement is circulated 4 times for clarification of wording and 3 conference calls are held to discuss details of contract
1/09	Contract accepted by UF and Orgenus	FDA IND application is being drafted

2. Publications in peer reviewed journals – No publications have resulted from this project

3. Publications (other) – No other publications have resulted from this project

4. Presentations at scientific meetings – No scientific presentations have resulted from this project

5. Presentations at public (non-scientific) meetings or events – Two public presentations have been made by the Co-PI's. One presented at Oak Hammock in 1/09 entitled, "Healthy Aging and products of the future" and another presented in 10/08 entitled "Nutraceuticals for the elderly".

6. Awards (other) – None

7. Faculty. Please include abbreviated CV with publications for previous 12 months

Dual-PI Todd Manini

1. Clark BC, **Manini TM**. Sarcopenia ≠ Dynapenia. Journals of Gerontology: Biological and Medical Sciences. 63(8) 829-834, 2008.
2. **Manini, TM** and BC Clark. Blood flow restricted exercise and skeletal muscle health. Invited review article in preparation for *Exercise and Sports Sciences Review*. In Press, 2008.

3. **Manini, TM** and M Pahor. Physical activity and maintaining physical function in older adults. Invited review article for *British Journal of Sports Medicine*. 43(1): 28-31, 2009.
4. Pahor M, **Manini TM**, Cesari M. Sarcopenia: clinical evaluation, biological markers and other evaluation tools. Journal of Aging and Health, In Press, 2008.
5. Marzetti E, Lawler JM, Hiona A, **Manini TM**, Seo AY, Leeuwenbergh C. Modulation of age-induced apoptotic signaling and cellular remodeling by exercise and caloric restriction in skeletal muscle. Free Radical Biol Med. 2008 Jan 15;44(2):160-8.
6. Clark BC, **Manini TM**, Hoffman RL, Russ DW. Restoration of voluntary muscle strength following 3-weeks of cast immobilization is suppressed in women compared to men. Archives of Physical Medicine and Rehabilitation. 90(1): 178-80, 2009.
7. Delmonico MJ, Zmuda JM, Taylor BC, Cauley JA, Harris TB, **Manini TM**, Schwartz A, Li R, Roth SM, Hurley BF, Bauer DC, Ferrell RE, Newman AB. Association of the ACTN3 Genotype and Physical functioning with age in older adults. Journals of Gerontology: Biological and Medical Science. In Press, 2008.
8. **Manini TM**, Everhart JE., Patel KV, Schoeller DA., Cummings S, Mackey D, Bauer DC, Simonsick EM., Colbert LH, Visser M, Tylavsky F., Newman AB, Harris TB. Activity energy expenditure and mobility limitation in older adults: Differential associations by sex. American Journal of Epidemiology, In Press.
9. Cesari M, Onder G, Zamboni V, **Manini TM**, Shorr RI, Russo A, Bernabei R, Pahor M, Landi F. Physical function and self-reported health status as predictors of mortality – Results from longitudinal analysis in the ilSIRENTE Study. BMC Geriatrics. In Press, 2008.

Dual-PI Steve Anton

1. Williamson, D.A., Martin, C.K., **Anton, S.D.**, York-Crowe, E., Han, H., Redman, L., & Ravussin, E. for the Pennington CALERIE Team. (2008). Is caloric restriction associated with development of eating disorder symptoms? results from the CALERIE trial. *Health Psychology 27*(1 suppl.), S32-S42.
2. **Anton, S.D.**, Martin, C.K., Heilbronn, L.K., York-Crowe, E., Han, H., Redman, L.M., Ravussin, E., & Williamson, D.A. (2008). Psychosocial and Behavioral Pre-Treatment Predictors of Weight Loss Outcomes in a Calorie Restriction Trial. *Eating and Weight Disorders, 13(1), 30-7*
3. **Anton, S.D.**, Morrison, C.D., Cefalu, W.T., Martin, C.K., Coulon, S., Han, H., White, C.L., Geiselman, P., & Williamson, D.A. (2008). Effects of Chromium Picolinate on Food Intake, Satiety, and Food Cravings in Overweight Female Carbohydrate Cravers. *Diabetes Technology and Therapeutics*,10(5), 405-12.
4. Redman, L.M., Rood, J. **Anton, S.D.**, Champagne, C., Smith, S.R., & Ravussin, E. (2008). Calorie restriction and bone health in young overweight individuals. *Archives of Internal Medicine, 168(17), 1859-66*
5. Hofer, T., Fontana, L., **Anton, S.D.**, Weiss, E. P., Villareal, D. T., Malayappan, B., & Leeuwenburgh, C. (2008). Long-term effects of caloric restriction or exercise on DNA and RNA oxidation levels in white blood cells and urine in humans. *Rejuvenation Research,* 11(4), 793-9.
6. Newton, R. L., Milsom, V., Nackers, L., & **Anton, S.D.** (2008). Supporting behavior changes in overweight patients: A guide for the primary care physician. *Journal of Clinical Outcomes Management.* 15, 536 – 544.
7. Chung, H.Y., Cesari, M., **Anton, S.D.**, Marzetti, E., Giovannini, S., Seo, A.Y., Carter, C., Yu, B.P., & Leeuwenburgh, C. (2009). Molecular Inflammation: Underpinnings of Aging and Age related Diseases. *Ageing Research Reviews,* 8(1), 18-30.

8. Martin, C.K., Coulon, S.M., Markward, N. Greenway, F., & **Anton, S.D.** (2009). The association between energy intake and viewing television, distractibility, and memory for advertisements. *American Journal of Clinical Nutrition*, 89(1), 37-44.
9. Martin, C.K., Han, H., Coulon, S.M., Allen, H.R., Champagne, C., & **Anton, S.D.** (2009). A novel method to remotely measure food intake of free-living individuals in real time: the remote food photography method.
British Journal of Nutrition. 101(3), 446-56
10. Newton, R. L., Han, H., **Anton, S.D.,** Martin, C.K., Stewart, T.M., Lewis, L. et al. (in press). An Environmental Intervention to Prevent Excess Weight Gain in African American Students: A Pilot Study. *American Journal of Health Promotion.*
11. Martin, C.K., Han, H., **Anton, S.D.**, Greenway, F.L., & Smith, S. (in press). Effect of valproic acid on food intake, physical activity, and hormones: Results of a randomized controlled trial. *Journal of Psychopharmacology.*
12. **Anton, S.D.**, Han, H. York-Crowe, E. Martin, C.K. Ravussin, E., & Williamson, D.A (in press). Effect of calorie restriction on appetite. *Journal of Human Nutrition and Dietetics.*
13. Sacks, F.M., Bray, G.A., Carey, V.J., Smith, S.R., Ryan, D.H., **Anton, S.D.** et al. (in press). Randomized trial comparing fat, protein, and carbohydrate consumption of diets for weight loss. *New England Journal of Medicine.* 27 (10) ,1242-9
14. Park, M.J., Lee E.K., Kim, M.S., Sung, B., Lee L., **Anton, S.D.** et al. (in press). The Anti-Inflammatory Effect of Kaempferol in Aged Kidney Tissues: The Involvement of Nuclear Factor $_{K}$B via Nuclear Factor-Inducing Kinase/IkB Kinase and Mitogen-Activated Protein Kinase Pathways. *Journal of Medicinal Food.*
15. Chung, J.H., Kim M., Kim, D.H., **Anton, S.D.**, Choi, J.S., Park K.Y., Rhee, S.H., Yu, B.P., & Chung, H.Y. (in press). PPAR expression by a short-term feeding of zingerone in aged rats. *Journal of Medicinal Food.*

Book Chapters
1. Perri, M. G., Foreyt, J. P., & **Anton, S.D.** Preventing weight gain after weight loss. In G. A. Bray and C. Bouchard (Eds.), Handbook of obesity treatment: Clinical applications (3rd ed.) New York: Marcel Dekkar, Inc., pp. 249 – 268.
2. **Anton, S.D.**, Exner, A., Newton, R. L. (in press). Intentions are not Sufficient to Change Behavior: Strategies that Promote Behavior Change and Healthy Weight Management. In F. Columbus (Ed.), New Perspectives on Knowledge, Attitudes and Practices in Health. New York: Nova Science Publishers, Inc.

8. Trainees
Post doctoral
Pre-doctoral – Ida Kellison, 5th year doctoral student in Clinical and Health Psychology
Other

9. Clinical/translational programs – Our study reflects a translation of information on animal models to humans. This is the first trial to study the effects of resveratrol supplementation on memory performance in humans
New programs
Update on existing clinical studies – Please see item 1.

10. Technology transfer – We have a contract in place with Oregnus Pharmaceutical Inc that protects UF's right to any intellectual property that results from this clinical trial.
Patents applications - None

Revenue generated from technology - None

11. Budget update (last year's budget and actual results - with an explanation of material variances) – No expenses have occurred during this time.
Status of matching funds, if applicable
Projected budget for coming year – We anticipate data collection to begin the third quarter of 2009 with approximately half of the participants to be completed with the study (please see timeline below). Therefore, half the budget will be used in 2009 and the other half in 2010.
Extramural funding - None

12. Educational programs focusing on age related memory loss
Scientific – This project has provided the opportunity to mentor a pre-doctoral student from the Department of Psychology on treatments for Age-related memory loss. Her training experiences to data have involved: preparation of IRB materials; preparation of FDA IND application; development of MRI protocol specific to study

13. Public - None

14. Collaborative programs with other McKnight Institutes, institutions and research programs - None

15. Collaborative program with non McKnight Institutes, institutions and research programs

The project is jointly tied to the Claude D. Pepper Center, a Center grant funded from by the National Institute on Aging. We have recently received funding from the Claude D. Pepper Center to conduct additional MRI's of muscle to evaluate lipid content and oxidative capacity in the trial. We are collaborating with investigators from the University of Washington to implement a new radio-frequency coil to evaluate phospocreatine recovery rates in estimating resphorylation of ATP by mitochondria. This collaboration will allow us to determine whether resveratrol supplementation effects oxidative capacity of muscle. More importantly, funding from the McKnight Brain Foundation provided the study infrastructure in which to make the proposal competitive. Therefore, we are maximizing the infrastructure of this study to assess other important physiological processes that may be affected by resveratrol supplementation.

Briefly describe plans for future research and/or clinical initiatives

We intend to use these pilot data as a cornerstone for proposing external funding that will investigate whether resveratrol can improve memory performance in the elderly. As mentioned above this is the first trial in older adults and thus we are hopeful that these data will allow us to plan a larger trial of resveratrol supplementation. There was a recent request for proposals interventions for age-related memory loss by the National Institute on Aging and with these pilot data we would highly competitive for such an award.

16. If applicable, please provide endowment investment results for the report period. – Not applicable

17. Where any funds used for a Prohibited Purpose during the report period? No

18. Do you recommend any modification to the Purpose or mandates in the Gift Agreement? No

19. Did all activities during the report period further the Purpose?

Yes, these regulatory activities are an essential part of conducting a clinical trial involving an investigational drug in humans. The development of a contract with a pharmaceutical company and FDA IND application are time-consuming processes, but these are needed to conduct a clinical trial that protects the intellectual rights of UF and most importantly ensures the safety of human subjects.

20. Please describe any negative events (loss of personnel, space, budget, etc.) that occurred during the report period and the possible impact on carrying out the Gift Agreement.

The major negative events with this past years' activity are related to the unanticipated long timeline for which to complete the necessary regulatory compliances. This is partially due to the novel nature of the study being among the first to evaluate the effects of resveratrol in humans.

21. Please provide any general comments or thoughts not covered elsewhere – a response is not required. Please respond only if you would like to add something not otherwise covered elsewhere.

While our efforts have yet to yield scientific information on the effect of resveratrol on memory performance in older adults, we have accomplished a major goal in building collaboration with a pharmaceutical company. Below you will find our timeline for the future regulatory processes and the start of data collection.

Timetable for regulatory compliance and completion of data collection	2009				2010		
	Q 1	Q 2	Q 3	Q 4	Q 1	Q 2	Q 3
FDA investigational new drug approval	→						
IRB application		→					
Recruitment/enrollment			↔	↔			
Specific Aim 1: data collection/processing				↔	↔	↔	
Specific Aim 2: data collection/processing				↔	↔	↔	
Manuscript and grant preparation						↔	↔

We anticipate 6 months of additional regulatory compliance efforts that will involve FDA IND and IRB applications. Recruitment will begin in the third quarter of the 2009.

22. Signature, date and title of person submitting the report.

Todd Manini, Ph.D.

Assistant Professor

Title

"Do different neurons age differently?"
Direct single-cell genomic profiling of neuronal subtypes in a memory forming network as a function of aging

Principal Investigator: Leonid L. Moroz, Ph.D., Departments of Neuroscience, the Whitney Laboratory

Lay Summary of Progress:

Progress in aging research related to mechanisms of memory loss, specifically its therapeutics, might not come as fast as the public anticipates and wishes. The major obstacle is that not many people (including scientists!) really appreciate or even grasp the technical difficulty of measuring the emerging molecular complexity of the brain where every neuron is unique and uses thousands of different signal transduction components – many of which can be effected by aging. Thus, integrative and fundamental research, especially focusing upon the logic of gene regulation in individual neurons of memory-forming circuits following memory consolidation and loss, are needed. This is the major objective of our research program. Last year we showed that different cholinergic neurons in model memory circuits age differently, and this aging process involves more that 4,000 gene products based on our single-neuron microarray results. Initially we thought we were finding a large number of gene products even for already known complex process. Then we realized, and reported during the last meeting, that microarrays are able to grab only a small fraction of molecular events in these cells. Thus, we decided to validate the data with our novel approach for unbiased gene expression profiling using 2nd generations of sequencing technologies. The protocols we developed using a SOLiD (ABI) platform are sufficient to work with virtually any neuron and allow us to identify and quantify >99% of all gene expression products. Furthermore, we can now profile even individual synapses. The results have exceeded our expectations of the complexity of genomic machinery in a neuron; we find more than 200,000 unique gene products present in a given neuron with the vast majority being novel non-coding RNAs, antisense and splice forms. All these groups are affected by aging and work is now in progress to annotate these components as a function of age related memory loss. Thus, for the first time we were able to grab the entire genomic blueprint of identified living neurons in memory circuits. I would like to stress that such an approach allows prediction of molecular logics that support the coordinated activity of the entire genome in a neuron.

Specifically, there are three major breakthroughs for this year.

First, we found there are subsets of transposon-like mobile elements and transposases that are unregulated following the aging process which might induce irreversible modification of a neuron's genome.

Second, we found that DNA methylation and related epigenetic modification differentially happens in aging neurons, changing neuronal fate, plasticity and related functions in memory forming circuits.

Third, we developed an approach to monitor all these epigenetic changes from single functionally identified neurons in memory circuits with single-nucleotide resolution at the level of the entire operational genome in a cell.

As a result, we can now identify master regulatory elements in every neuron of a memory circuit following ARML. Currently, we are testing this approach and plan to apply it to the process of normal aging. Moreover, this approach leads us to a new view of the logic that supports the orchestrated activity of multiple genes in living neurons. This logic incorporates similar principles discovered for evolutionary dynamics following adaptive modification

within populations. Thus, we are planning to implement modern tools of system biology to reconstruct and model these processes.

Relevance of Findings to Memory Loss in Aging:

The complexity of events associated with age-related memory loss (ARML) cannot be overestimated. Mechanisms under investigation range from global hormonal and oxidative stress of an organism (or various brain regions) to precise neuron-specific changes in synaptic strength, associated local protein synthesis, and dynamics of selected molecules in cellular microdomains. The problem is further complicated by the enormous diversity of neurons in the CNS and even synapses within the same network or one neuron. Large scale single-neuron analysis is not only challenging but mostly impractical for any model currently used in ARML. We simply do not know whether all neurons and synapses age differently or if some neurons (or synapses) are more resistant to aging than others. What is happening in any given neuron while it undergoes "normal" aging? What are the genomic changes that make the aging apparently irreversible? How realistic are therapeutic strategies not only to slow down but reverse this process? What would be the balance between neuron-specific vs global therapy in aging? Evidently, the foundation for the development of all new therapeutic approaches for ARML is a mission to provide a complete molecular/genomic inventory of events and mechanisms in all neuronal types and synapses of memory-forming networks as a function of aging. It might look unrealistic and overambitious not only in the hands of any individual laboratory or institute but for the entire neuroscience community. However, **it is an achievable goal today**, as we have proved for the simplest (2-3 neurons) memory-forming network, and now we plan to expand it to more finicky neurons and neural circuits in the aging mammalian brain.

Now we are developing novel approaches to monitor activity of virtually ***ALL*** **genes and their regulatory elements in virtually any single neuron** as the first step to move to mammalian brain. I hope that it would be possible to initiate this program for mammalian neurons as collaborative efforts between three McKnight Brain Institutes. I suggested it during our last meeting in March, but now it can be done at an entirely new level. Also we will continue to analyze molecular events in all functional classes of cells in a simpler memory-forming neural circuit of *Aplysia* –an opportunity that can not be achieved in any other model available for experimental neuroscience. Once identified in a simpler model system the targeted mRNA homologs and their encoded proteins can be analyzed in mammalian systems – an approach that proved to be highly efficient in modern neuroscience. Taking into account the relatively short life cycle of *Aplysia californica* (240-300 days), the use of the proposed model opens unlimited possibilities.

Progress toward Completing Publication and Scientific Presentation of Findings:

Publications:

Moroz, LL. et al (2009). The sequencing of the *Aplysia* californica genome: the model for single-cell and real time genomics. ***Nature*** in revision.

Moroz, L.L. (2009). *Aplysia*. Quick Guide. ***Current Biology***, in revision

Mikhailov, K.V. et al (2009). Cell differentiation preceded multicellularity in early metazoan evolution: *Gastrea* verus *Synzoospore* theory in postgenomic age: ***BioEssays***, in press.

Cummins S.F. et al (2009). Candidate chemosensory receptor subfamilies differentially expressed in the chemosensory organs of the mollusc *Aplysia*. ***Genome Biology*** *in press*

Vázquez-Acevedo, N., Reyes-Colón, D., Ruiz-Rodríguez, E., Rivera, N., Rosenthal, J., Kohn, A., **Moroz, L.L.**, Sosa, M. (2009). Cloning and immunoreactivity of the 5-HT1Mac and 5-HT2Mac receptors in the central nervous system of the freshwater prawn *Macrobrachium rosenbergii*. ***Comparative Neurology***, in press,

Lee YS, et al. (2008). Transcriptome analysis and identification of regulators for long-term plasticity in *Aplysia kurodai*. **Proc Natl Acad Sci U S A**. Nov 25;105(47):18602-18607.

Panchin, Y., **Moroz, L.L.** (2008) Molluscan mobile elements similar to the vertebrate Recombination-Activating Genes. ***Biochem. Biophys. Res. Commun.*** 369(3):818-823.

Wu., Y., Yang, C.J., **Moroz, L.L.**, Tan, W. (2008) Nucleic Acid Beacons for Long-Term Real-Time Intracellular Monitoring. ***Analytical Chemistry*** 80(8):3025-3028.

Bishop, C.D., Pires, A., Norby, S.W., Boudko, D., **Moroz, L.L.**, Hadfield, M.G.(2008). Analysis of nitric oxide-cyclic guanosine monophosphate signaling during metamorphosis of the nudibranch *Phestilla sibogae* Bergh (Gastropoda: Opisthobranchia). ***Evolution & Development*** 10(3): 288-299.

Hatcher, N.G., Zhang, X., Stuart, J.N., **Moroz, L.L.**, Sweedler, J.V., Gillette, R. (2008). 5-HT and 5-HT-SO(4), but not tryptophan or 5-HIAA levels in single feeding neurons track animal hunger state. ***Journal of Neurochemistry*** 104(5):1358-1363.

Presentations at Scientific meetings: 22 presentations (lectures and posters):

Progress toward Completing Acquisition of Extramural Funding to Continue/Expand this Project:

Funded:

"**Molecular Engineering Approach to Study the Long Term Synaptic Plasticity**"
Funding Agency: NIH, R01: Research Grant;
PIs: Moroz, L.L., J. Ju
Effective Date: March, 2008 – March , 2012
This project will develop novel bioengineering and imaging technologies for molecular analysis of RNAs in synapses.

"**Sequencing of the *Aplysia* genome**"
Agency: NIH, 2005-2008
PIs: Leonid L. Moroz, Eric Kandel, Eric Lander
NIH approved proposal (staring date: Winter 2006) to sequence *Aplysia* genome as a model in cellular and system neuroscience. The sequencing will be performed using facilities of one of the Broad Institute with involvement of UF and Columbia University for the *Aplysia* genome annotation and neuronal transcriptome analysis.

"**Genomic Bases of Evolution of Homologous Neurons & Neuronal Circuits**"
Agency: ***NSF***; August 2008-August 2010
PI: Leonid L Moroz;
This project proposes to understand how genomic changes in the organization of specific neurons within central pattern generators lead to adaptive evolutional modifications of neural circuits underlying locomotion.

"**Instrumentation for Coupled Experimental-Computational Neuroscience and Biology Research**"
Agency: NSF: Period: 07/01/08-07/01/09
Co-PIs: J. Fortes and L.L Moroz;
Equipment acquisition for computational biology and genomics, digital gene expression profiling and statistical analysis of genome-scale datasets

Submitted/Pending:

Nitric Oxide signaling in classical conditioning: from genes to behavior
Agency: NIH, R01, 2009-2014
PIs: Leonid L. Moroz

Epigenomic dissection of memory-forming circuits: Toward single-cell epigenomics
Agency: NIH, R21, 2009-2014
PIs: Leonid L. Moroz

System biology of living neurons in memory-forming circuits
Agency: NIH, Transformative R01, 2009-2014
PIs: Leonid L. Moroz, J.Ju, E.R. Kandel

"Genomic Dissection of Circuit Dynamics"
Agency: NIH, R21, June 2009-May 2011
PI: L.L. Moroz

Annual Report-McKnight Brain Research Foundation
Sponsored Institutes and Research Programs
(Include activity of all McKnight supported faculty and trainees)
Report Period: 10/3/08-12/31/08

Some gift agreements require both Institute reports and Chair reports. If applicable, please clearly state whether a particular response relates to a Chair or Institute.

Any capitalized terms used on the template are intended to have the same meaning as the term is defined in the Gift Agreement.

1. Summary of scientific achievements since last report

As a recently hired faculty member in the McKnight Brain Research Foundation in the Department of Neuroscience, my time and financial support thus far have been committed to establishing my laboratory and planning/writing for grant applications.

2. Publications in peer reviewed journals

None yet

3. Publications (other)

None yet

4. Presentations at scientific meetings

None yet...plan to present research at future SFN meetings and MBI-sponsored events

5. Presentations at public (non-scientific) meetings or events

None yet

6. Awards (other) none yet

7. Faculty. Please include abbreviated CV with publications for previous 12 months

See attached (all publications are pre-hire)

8. Trainees – currently advertising
   Post doctoral
   Pre-doctoral
   Other

9. Clinical/translational programs
   New programs
   Update on existing clinical studies

   n/a

10. Technology transfer
    Patents applications

Revenue generated from technology

n/a

11. Budget update (last year's budget and actual results - with an explanation of material variances)
Status of matching funds, if applicable
Projected budget for coming year
Extramural funding

12. Educational programs focusing on age related memory loss
Scientific
Public

None yet

13. Collaborative programs with other McKnight Institutes, institutions and research programs

Currently establishing lab but am developing relationships and planning future collaborations with other faculty in the MBI-UF

14. Collaborative program with non McKnight Institutes, institutions and research programs

None yet

15. Briefly describe plans for future research and/or clinical initiatives

Plans included examining the biological function of cilia on neurons. I plan to explore the roles of these structures during development and in the aging brain. I'm interested in how cilia develop and what their function is on neurons. Further, I would like to explore what happens to these structures in aged animals. I'm also planning a genetic strategy to 're-activate' master genes used during early development (eg underlying learning and memory) in aged cortical neurons as a method of 'rejuvenating' old neurons.

16. If applicable, please provide endowment investment results for the report period.

n/a

17. Where any funds used for a Prohibited Purpose during the report period?

no

18. Do you recommend any modification to the Purpose or mandates in the Gift Agreement?

no

19. Did all activities during the report period further the Purpose?

yes

20. Please describe any negative events (loss of personnel, space, budget, etc.) that occurred during the report period and the possible impact on carrying out the Gift Agreement.

none

21. Please provide any general comments or thoughts not covered elsewhere – a response is not required. Please respond only if you would like to add something not otherwise covered elsewhere.

22. Signature, date and title of person submitting the report.

Matthew Sarkisian, Assistant Professor, Department of Neuroscience

Curriculum Vitae

Matthew Robert Sarkisian, Ph.D.

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BIRTHPLACE:

New York, New York
February 18, 1973

EDUCATION:

B.S., Biological Sciences, May 12, 1995
Clemson University
Clemson, SC 29632

Ph.D., Physiology and Neurobiology, December 14, 2001
University of Connecticut
Storrs, CT 06269

POSITIONS HELD:

Children's Hospital, Harvard Medical School, Boston, MA
May 1994-August 1994
May 1995-August 1997
Position: Research Technician (advisor: Gregory L. Holmes, M.D., currently Chief of Neurology and Professor of Medicine and Pediatrics at Dartmouth Medical School)

University of Connecticut, Storrs, CT
August 1997 - December 2001
Position: Graduate Student (advisor: Joseph J. LoTurco, Ph.D., Professor in the Dept of Physiology & Neurobiology)

Yale University School of Medicine, New Haven, CT
February 2002- January 2008
Position: Post-Doctoral Fellow (advisor: Pasko Rakic, MD, PhD, Duberg Professor of Neurobiology and Neurology, Chairman, Dept of Neurobiology, Director, Kavli Institute of Neuroscience, 2008 Kavli Laureate)
February 2008 – October 2008
Position: Associate Research Scientist

University of Florida, Gainesville, FL
October 2008-present
Position: Assistant Professor in Dept of Neuroscience, Investigator in McKnight Brain Institute

RESEARCH AWARDS & HONORS:

1996: George M. Savoy Junior Fellowship (awarded by the Savoy Foundation and Eastern Association of Electroencephalographers)
1999-2001: Univ of Connecticut Predoctoral Fellowship (awarded by UConn Neurosciences Steering Committee)
2004-2005: James Hudson Brown-Alexander Brown Coxe Postdoctoral Fellowship (awarded by Yale Univ School of Medicine).
2006-2007: Eric W. Lothman Training Fellowship (Epilepsy Foundation of America Distinguished Postdoctoral Award)

TEACHING EXPERIENCE:

Undergraduates mentored:
UConn: Samarjit Rattan, Justin Oborski, Mikhail Frenkel, Laurie Cosker, Bethany Goughenour
Yale: Christopher Bartley

Fall 1999 - University of Connecticut - Teaching Assistant for PNB263W: Investigations in Neurobiology. This was an advanced undergraduate laboratory course designed to teach basic concepts and techniques in neurobiology in addition to promoting independent research projects. Responsibilities included overseeing the lab, grading quizzes and formal lab reports.

Summer 1998-2000: UConn Mentor Connection "Brain Power": Mentorship of high school students in neuroscience research.

SERVICE TO DEPARTMENT OF NEUROSCIENCE/MCKNIGHT BRAIN INSTITUTE:

2008- Member of Cell and Tissue Analysis Core (CTAC) Advisory Board

INVITED TALKS:

10/2005 Yale Univ., Dept of Neurobiology
01/2007 Univ. of Montreal, Center for the Study of Brain Diseases
03/2007 Queens College, CUNY, Dept of Psychology
08/2007 Univ. of Louisville, Dept of Molecular, Cellular and Craniofacial Biology
09/2007 GlaxoSmithKline, King of Prussia, PA
10/2007 Rutgers University, Dept of Cell Biology and Neuroscience
03/2008 Medical College of Georgia, Institute of Molecular Medicine and Genetics
03/2008 Robert Wood Johnson Medical School, Dept of Neuroscience and Cell Biology
04/2008 Children's Memorial Research Center, Northwestern Univ, Dept of Pediatrics
05/2008 Univ. of Florida, McKnight Brain Institute, Dept. of Neuroscience

PUBLICATIONS (Peer-reviewed):

1. Tandon P., Liu Z., Stafstrom C.E., **Sarkisian M.**, Werner S.J., Mikati M., Yang Y., Holmes G.L. Long-term effects of excitatory amino acid antagonists NBQX and MK-801 on the developing brain. Developmental Brain Research 1996; 95: 256-262.

2. Liu Z., Stafstrom C.E., **Sarkisian M.**, Tandon P., Yang Y., Hori A., Holmes G.L. Age-dependent effects of glutamate toxicity in the hippocampus. Developmental Brain Research 1996; 97: 178-184.

3. Neill J.C., Liu Z., **Sarkisian M.**, Tandon P., Yang Y., Stafstrom C.E., Holmes G.L. Recurrent seizures in immature rats: effect on auditory and visual discrimination. Developmental Brain Research 1996; 95: 283-292.

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24. Town T, Breunig J[†], **Sarkisian M.R.**[†], Spilianakis C, Ayoub AE, Liu X, Ferrandino A, Gallagher AR, Li MO, Rakic P, Flavell RA. The *stumpy* gene is required for mammalian ciliogenesis. PNAS 2008; 105: 2853-2858. [†]co-contributors

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26. Hashimoto-Torii K, Torii M, **Sarkisian M.R.**, Bartley CM, Shen J, Radtke R, Gridley T, Sestan N, Rakic P. Interaction between Reelin and Notch signaling regulates neuronal migration in the cerebral cortex. Neuron 2008; 60: 273-284.

* co-first author publication

REVIEW ARTICLES (Peer-reviewed):

1. Holmes GL, Chevassus-Au-Louis N, **Sarkisian MR**, and Ben-Ari Y. Consecuencias de la epilepsia refractaria durante el desarrollo (Consequences of refractory epilepsy during development). Revista de Neurología 1997; 25: 749-753.

2. **Sarkisian MR.** Overview of the current animal models for human seizure and epileptic disorders. Epilepsy& Behavior 2001; 2: 201-216.

3. **Sarkisian MR**, Bartley CM, Rakic P. Trouble making the first move: interpreting arrested neuronal migration in the cerebral cortex. Trends in Neurosciences 2008; 31: 54-61.

COMMENTARIES:

1. **Sarkisian MR.** Animal models for human seizure and epileptic activity. Reply. Epilepsy & Behavior 2001; 2: 506-7.

BOOK CHAPTERS:

1. **Sarkisian M**, Tandon P, and Holmes GL. Long-term effects of kindling on learning and memory in the developing brain. In: Kindling 5. M.E. Corcoran, S.L. Moshé (Eds). Plenum Press, New York, 1998: 27-34.

2. Holmes GL, **Sarkisian M**, Ben-Ari Y., and Chevassus-Au-Louis N. Effects of recurrent seizures in the developing brain. In: Childhood epilepsies and brain development A. Nehlig, J. Motte, S.L. Moshé, Plouin P (Eds). John Libbey, London, England. 1999: 263-276.

3. Holmes GL, Khazipov R, Liu Z, **Sarkisian MR**, Stafstrom C.E. Behavioral consequences of status epilepticus in the immature brain. In: (in press) 2005

4. Rakic P, Hashimoto-Torii K, **Sarkisian MR**. Genetic determinants of neuronal migration in the cerebral cortex. In: Cortical development: genes and genetic abnormalities. G. Bock, J. Goode (Eds). Wiley, Chichester (Novartis Foundation Symposium) 2007: 288; 45-58.

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1. **Sarkisian MR**: Atlas of Prenatal Rat Brain Development. J. Altman, S. Bayer, CRC Press, 1995. In: Journal of Epilepsy 9: 302, 1996.

2. **Sarkisian MR**: The Neuron. I.B. Levitan, L.K. Kaczmarek, Oxford University Press, 1997. In: Journal of Epilepsy 11: 394, 1998.

ABSTRACTS:

1. **M.R. Sarkisian**, J.C. Neill, Z. Liu, M. Mikati, R. Paine, and Gregory L. Holmes. Effects of kainic acid-induced seizures on memory; developmental effects. Epilepsia. 36(Suppl. 3): S42, 1995.

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3. J.C. Neill, M.A. Mikati, Z. Liu, R. Paine, **M. Sarkisian**, and G.L. Holmes. Effects on auditory discrimination of phenobarbital and nimodipine treatment for status epilepticus. Soc Neurosci Abstr 21:778, 1995.

4. **M. Sarkisian**, P. Tandon, C. Stafstrom, Z. Liu, Y. Yang, A. Hori, J. Neill, A. Bolanos, and G.L. Holmes. Effects of repeated kainic acid seizures in the immature rat: a decrease in seizure intensity and long-term consequences. Electroencephalography and Clinical Neurophysiology 99: 10P, 1996.

5. P. Tandon, Z. Liu, C.E. Stafstrom, **M. Sarkisian**, S. Werner, Y. Yang, and G.L. Holmes. Long-term effects of excitatory amino antagonists NBQX and MK-801 on the developing brain. Electroencephalography and Clinical Neurophysiology 99: 12P, 1996.

6. P. Tandon, **M. R. Sarkisian**, C.E. Stafstrom, Y.Yang and G.L. Holmes. Repeated exposure to kainic acid in developing rats does not cause repeated seizures, neuronal loss, or behavioral deficits later in life. Epilepsia 37(Suppl. 5): 66, 1996.

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8. Z. Liu, C.E. Stafstrom, Y.Yang, **M. Sarkisian**, A.Hori, P. Tandon and G.L. Holmes. A focal lesion in the mesial temporal lobe of newborn rats results in hippocampal electroencephalographic seizures later in life. Epilepsia 37 (Suppl. 5): 49, 1996.

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42. Town T, Breunig J, **Sarkisian MR**, Spilianakis C, Ayoub AE, Liu X, Ferrandino AF, Li MO, Rakic P, Flavell, RA. The *Stumpy* gene is required for mammalian ciliogenesis and hindbrain development. Soc Neurosci Abstr 2008.

PROFESSIONAL AFFILIATIONS:

Member of the Society for Neuroscience (1998-)
Member of the American Epilepsy Society (2000-2002, 2006-2007)
Member of the New York Academy of Sciences (2008-)

Annual Report-McKnight Brain Research Foundation
Sponsored Institutes and Research Programs
(Include activity of all McKnight supported faculty and trainees)
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1. Summary of scientific achievements since last report

Title: Microglia as Therapeutic Targets in Age-Related Memory Loss.

Amount awarded: $50,000

Principal Investigator: Wolfgang J. Streit, Ph.D.

Key Personnel: Heiko Braak, MD, Co-Investigator, J.W. Goethe University, Frankfurt, Germany
Qing-Shan Xue, Ph.D., Postdoctoral Fellow, UF

Lay Summary of Progress: The objective of our study was to determine whether age-related memory loss (ARML) in humans was accompanied by structural deterioration of the cells that comprise the brain's immune system, called microglia. In order to gauge the extent of ARML, we used the Braak staging system which is based on a semi-quantitative assessment of degenerating neuronal structures, termed neurofibrillary tangles and neuropil threads. This system stages subjects from 0 to VI. In order to limit our analysis to subjects with mild cognitive impairment (MCI) and to exclude subjects with Alzheimer's disease (AD) and/or dementia, we only examined brains up to stage III. Cases that are stage IV and higher are almost always AD subjects. Our investigation has yielded the following results and conclusions thus far: 1) Structural deterioration of microglia occurs invariably whenever neurodegenerative changes are present, that is, in subjects that are Braak stage I, II, and III. 2) Degenerating microglia are found in the immediate vicinity of degenerating neuronal structures, suggesting that the two are closely interconnected. 3) Microglial degeneration precedes neuronal degeneration, suggesting that neurons degenerate because they lose supporting glial cells. 4) The preservation of microglia should be the therapeutic goal of future interventions aimed at preventing ARML.

Progress Toward Completing Specific Aims:
Our Specific Aims were as follows:
Aim 1: To perform light microscopic post-mortem histopathological studies to determine the state of microglial integrity in the hippocampus of humans with MCI. We will

correlate the degree and extent of microglial deterioration with the extent of MCI as determined by the staging system of Braak & Braak. Selection of appropriate samples from the University of Frankfurt brain bank will be made in accordance with the criteria provided on the neuropathology of cognitively normal elderly.
Aim 2: To perform electron microscopy on human brain samples in order to analyze the ultrastructure of microglial senescent changes, as well as their relationship to synaptic connections.

In essence, specific aim 1 has been completed, and we have shown that microglial deterioration (also known as microglial dystrophy) is prevalent in any brain containing neurodegenerative changes. We were able to do this in human archival brain tissue using double immunohistochemical staining for both microglia (Iba1 antibody) and for hyperphosphorylated tau protein (AT8 antibody), which is a component of the paired helical filaments that comprise neurofibrillary tangles. An example of such staining is shown in **Figure 1.** Importantly, none of the MCI subjects we studied contained any amyloid protein, thus ensuring exclusion of any case that could have been diagnosed as early stage Alzheimer's disease (diagnosis of Alzheimer's requires presence of both neurofibrillary degeneration and amyloid plaques).

Specific aim 2 has not yet been completed due to a lack of resources for doing expensive electron microscopy (we had applied for $100,000 but were awarded $50,000).

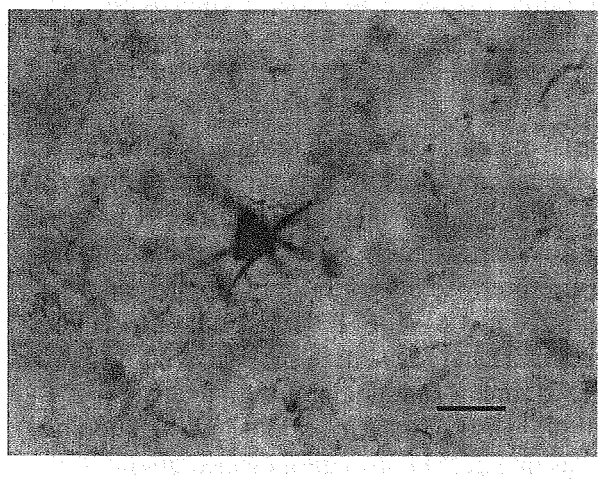

Figure 1. Double immunohistochemical staining for microglia with Iba1 (brown) and with AT8 antibodies (black) to show microglia and neurodegenerative changes in the entorhinal cortex of a 88-year-old subject at Braak stage I. A prominent neurofibrillary tangles is seen in the center and several smaller neuropil threads are scattered throughout. The processes of microglial cells are fragmented indicating ongoing degeneration. Scale bar: 50 µm

In addition to examining primarily brain tissue from elderly individuals, we also obtained tissue samples from a rare case of a young 21-year-old subject who, upon post-mortem examination, was found to have minimal neurodegenerative changes and classified as being Braak stage I. Using the same double-staining procedure, we were able to determine that an area of minimal tau pathology in the hippocampus of this young subject (Braak stage I) was accompanied by selective microglial fragmentation, whereas a non-pathological young control subject (Braak stage 0) revealed intact ramified microglial cells in the same brain region (**Figure 2**).

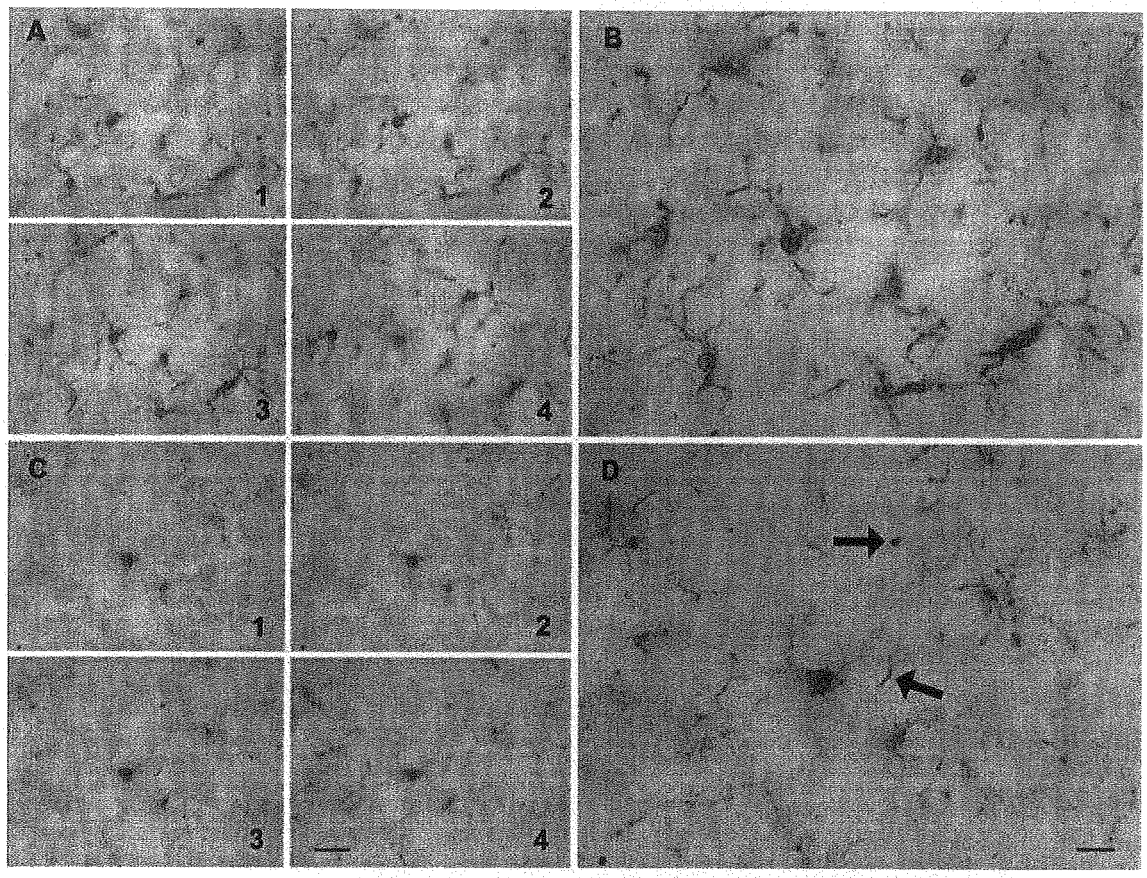

Figure 2. Microglial degeneration can occur independent of age and is evident in cases of young subjects with minimal tau pathology. Double-label immunohistochemistry for microglia (Iba1) and tau (AT8) is shown in the hippocampus of two young subjects with no (B) or minimal (D) tau pathology (Braak stage 0, 22-year-old; Braak stage I, 21-year-old). Panels A and C show focus series of four individual micrographs taken 10-15 µm apart, and reassembled into composite images in B and D. Note the difference in microglial morphology in the two young subjects, one of which shows minimal tau pathology evident as neuropil threads (arrows in D). Microglia in B show normal ramified appearance but are fragmented in D. Scale bars: 20 µm (A,C); 10 µm (B,D)

To investigate the temporal relationship between microglial deterioration and neurofibrillary degeneration, we took advantage of the predictability of the spread of tau pathology inherent to Braak staging, namely, that neurodegeneration proceeds predictably from the allocortex (entorhinal and hippocampal areas) to the isocortex. By performing simultaneous analysis of microglial morphology in both the entorhinal cortex and middle temporal gyrus in the same coronal section through the temporal lobe at increasing Braak stages, we were able to determine the extent of microglial dystrophy in regions already showing neurodegeneration as well as in regions that would have developed neurodegeneration if the subject had lived longer. The results, shown in **Figure 3**, demonstrate that microglial cells begin their structural deterioration before

neurofibrillary pathology sets in. This is particularly evident during Braak stage III where widespread microglial cytorrhexis is coincident with extensive tau pathology in the entorhinal region but also present in the middle temporal gyrus which at this stage does not yet show neurodegenerative changes (Figsures 3G,H). These observations strongly suggest that microglial degeneration precedes the onset of neurofibrillary pathology and therefore support the hypothesis of a causal relationship between the loss of microglial structural integrity and the onset of neurodegeneration.

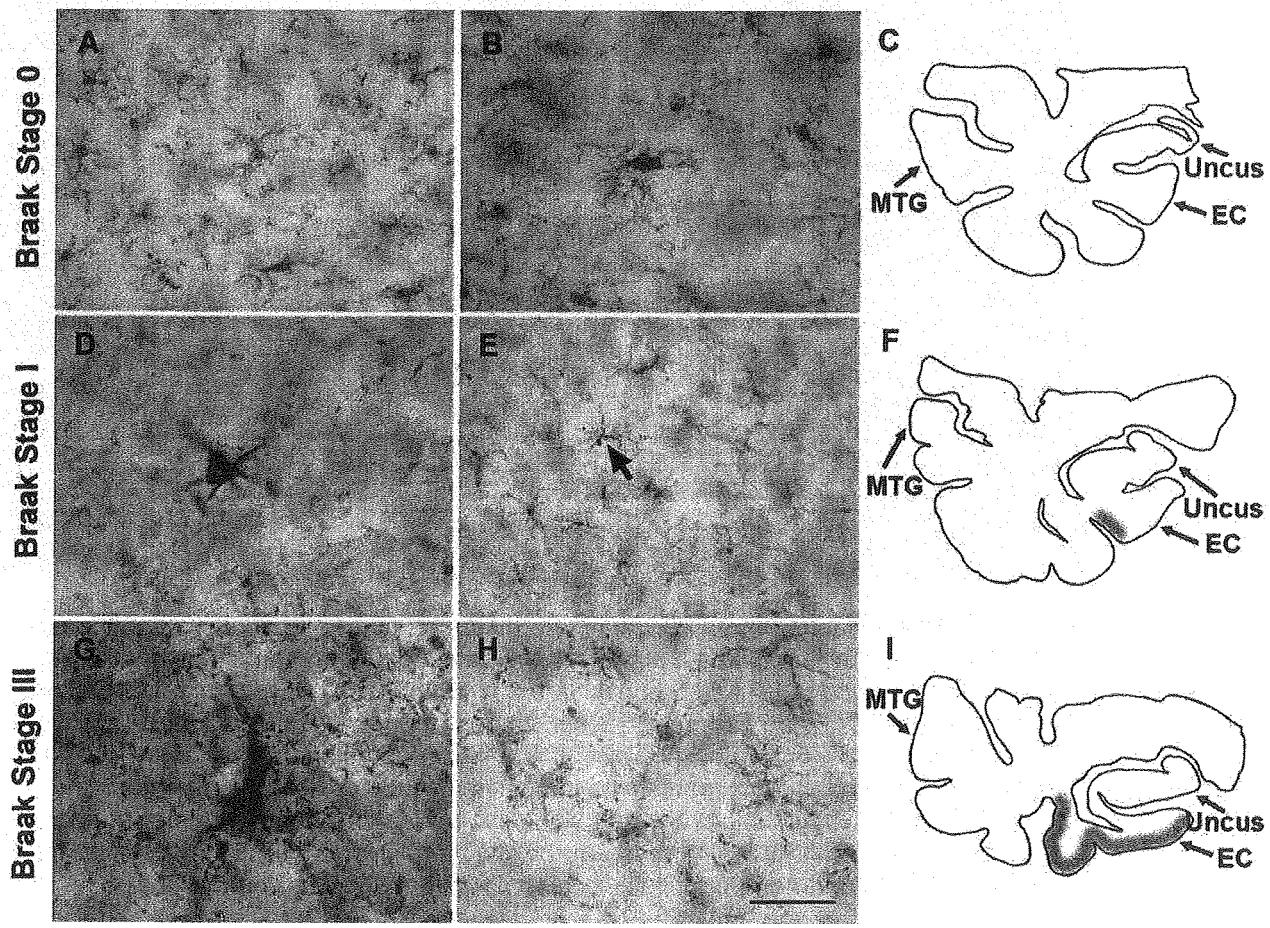

Figure 3. Microglial fragmentation precedes the spread of tau pathology in the temporal lobe. Double-label immunohistochemistry for microglia (Iba1) and tau (AT8) is shown in three elderly subjects with tau pathology increasing from stage 0 to stage III. Camera lucida drawings of the actual sections are shown in panels C, F, and I indicating for orientation the uncus, as well as both sampling areas in the EC and middle temporal gyrus (MTG); areas of tau pathology are shaded in orange. Representative micrographs of the EC (A, D,G) and MTG (B, E, H) reveal microglia (brown) and tau pathology (black) at the different stages. Normal ramified microglia are evident at stage 0 in both EC and MTG in the absence of tau pathology (A,B); mostly fragmented microglia are seen in association with a neurofibrillary tangle and neuropil threads in D, whereas mostly ramified and only a single fragmented cell (arrow) are present in E during stage I; severe microglial fragmentation and loss of discernable cell shape is colocalized with extensive tau pathology in G; microglial processes are fragmented also in H in the absence of neurodegeneration, but cells retain recognizable contours. Scale bar: 50 µm (A,B,D,E,G,H).

2. Publications in peer reviewed journals

None

3. Publications (other)

None

4. Presentations at scientific meetings

None

5. Presentations at public (non-scientific) meetings or events

None

6. Awards (other)

None

7. Faculty. Please include abbreviated CV with publications for previous 12 months

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BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Wolfgang J. Streit, Ph.D.	Professor
eRA COMMONS USER NAME	
wstreit	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
College of Charleston, Charleston, SC	B.S.	1980	Biology
Medical University of South Carolina, Charleston,	Ph.D.	1985	Pathology/Neuropathol

A. Positions and Honors

Positions:

1985 to 1986: Post-doctoral Fellow, Max Planck Institute for Psychiatry, Martinsried, Germany.

1986 to 1989: Staff Scientist, Max Planck Institute for Psychiatry, Martinsried, Germany.

1989 to 1994: Assistant Professor, University of Florida, Department of Neuroscience, Gainesville, FL.

1994 to 1999: Associate Professor (with tenure), University of Florida, Department of Neuroscience,
Gainesville, FL.

1999 to present: Professor, University of Florida, Department of Neuroscience, Gainesville, FL.

Honors:
1990: Robert Feulgen Prize of the International Association of Histochemists.
1993 to present: *Ad hoc* member, various NIH Study Sections.
1998 to 1999: Temporary Member of MDCN-2 Study Section, NIH.
1999 to 2003: Full Member, NINDS Initial Review Group Subcommittee A.
Editorial Boards: *GLIA* (1994-2005); *The Journal of Histochemistry and Cytochemistry* (1995-2005); *Journal of Neuroscience Research* (since 1997); *Experimental Neurology* (1998-2002); *Brain Pathology* (1999-2006); *International Journal of Developmental Neuroscience* (since 2002); *Journal of Neuropathology and Experimental Neurology* (2002 – 2005); *Journal of Neuroinflammation* (2004-present)

B. Selected peer-reviewed publications (2008)

Frank, S., Burbach, G.J., Bonin, M., Walter, M., STREIT, W.J., Bechmann, I., Deller, T. TREM2 is upregulated in amyloid plaque-associated microglia in aged APP23 transgenic mice. GLIA 56:1438-1447, 2008

Lopes, K.O., Sparks, D.L., STREIT, W.J. Microglial dystrophy in the aged and Alzheimer's disease brain is associated with ferritin immunoreactivity. GLIA 56:1048-1060, 2008.

Chiu, C., Luo, D., STREIT, W.J., Harrison, J.K. CX3CL1 and CX3CR1 in the GL261 murine model of glioma; CX3CR1 deficiency does not impact tumor growth or infiltration of microglia and lymphocytes. J. Neuroimmunol. 198:98-105, 2008

Levkoff, L.H., Marshall II, G.P., Ross, H.H., Calderia, M., Reynolds, B.A., Cakiroglu, M., Mariani, C.L., STREIT, W.J., Laywell, E.D. Bromodeoxyuridine inhibits cancer cell proliferation *in vitro* and *in vivo*. Neoplasia 10:804-816, 2008

STREIT, W.J., Miller, K.R., Lopes, K.O., Njie, E. Microglial degeneration in the aging brain- bad news for neurons? Frontiers in Bioscience 13:3423-3438, 2008

8. Trainees

Post doctoral

1. Qing-Shan Xue, PhD

2. Hua Yao, PhD

Pre-doctoral

1. Kelly Miller

2. Emalick Njie

9. Clinical/translational programs
New programs
Update on existing clinical studies

N/A

10. Technology transfer
Patents applications
Revenue generated from technology

N/A

11. Budget update (last year's budget and actual results - with an explanation of material variances)
Status of matching funds, if applicable
Projected budget for coming year
Extramural funding

See attached financial report

12. Educational programs focusing on age related memory loss
Scientific
Public

N/A

13. Collaborative programs with other McKnight Institutes, institutions and research programs

N/A

14. Collaborative program with non McKnight Institutes, institutions and research programs

J.W. Goethe University, Frankfurt, Germany

15. Briefly describe plans for future research and/or clinical initiatives

Discover causes of microglial dystrophy and develop preclinical studies for treatment.

16. If applicable, please provide endowment investment results for the report period.

N/A

17. Where any funds used for a Prohibited Purpose during the report period?

No

18. Do you recommend any modification to the Purpose or mandates in the Gift Agreement?

No

19. Did all activities during the report period further the Purpose?

Yes

20. Please describe any negative events (loss of personnel, space, budget, etc.) that occurred during the report period and the possible impact on carrying out the Gift Agreement.

N/A

21. Please provide any general comments or thoughts not covered elsewhere – a response is not required. Please respond only if you would like to add something not otherwise covered elsewhere.

22. Signature, date and title of person submitting the report.

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Wolfgang J. Streit, Ph.D.