Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format.

1. **Grantee Institution:** The Pennsylvania State University

2. **Reporting Period (start and end date of grant award period):** 1/1/2010 - 12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** John Anthony, MPA

4. **Grant Contact Person's Telephone Number:** 814 935 1081

5. **Grant SAP Number:** 4100050904

6. **Project Number and Title of Research Project:** 2. Brain and Behavior in Early Iron Deficiency

7. **Start and End Date of Research Project:** 1/1/2010 - 6/30/2011

8. **Name of Principal Investigator for the Research Project:** James Connor, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 44,116

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Nandar, Wint	Grad Asst	100%	$22,120

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Conner, James	PI	5%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No_____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes____X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert “not funded” in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Novel nutrition therapy for developmental iron deficits	X NIH (The Regents of the Univ of Michigan) ☐ Other federal (specify:__) ☐ Nonfederal source (specify:_)	06/2013	$191,812	$ 0

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:

We are awaiting the summary sheets from the R01 that was submitted. It was not scored so will take substantial revision if we resubmit. We are also planning to submit an R01 for PA-13-155 to the NIH for April 5, 2013 deadline based on the data on the HFE mutant mouse that we generated. We have also crossbred the HFE mutant mouse with the ALS mouse model (SOD1 mutant). We have received funding from the Pape Adams Charitable Foundation for ALS research to pursue this double transgenic mouse to learn the impact of the HFE mutation on ALS.

12. Future of Research Project. What are the future plans for this research project?

We will continue to develop the HFE mutant mouse as a model for human aging and iron overload. We have limited hope and enthusiasm for the rodent model of iron deficiency. This line of research has been replaced by studies on iron deficient non-human primates which is going quite well (NIH R01 grant pending with a score of 14th percentile).

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female			1	
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic			1	
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian			1	
Other				
Unknown				
Total			**1**	

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No_____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____ No_____X_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No_____X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No_____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Our *long-term objective* is to determine *when* and *how much iron to give* to prevent the long-term neurobehavioral sequelae of early iron deficiency (ID). There is the potential for neurotoxicity if excessive iron is given to treat early ID. Thus, the *specific aim* of this study is to determine if larger amounts of iron taken up by brain tissue during aggressive dietary iron treatment result in changes in brain genomic and structural sequelae. We *hypothesized* that aggressive iron intervention during lactation is associated with neurotoxicity.

Strategies: We will assess the early ID in rat pups by cross-fostering of rat pup to mothers given <5 ppm Fe (ID group) or 50 ppm Fe diet (control group). Pups with early ID will receive the 50 ppm (control) or 1000 ppm iron diet (high iron treatment) between P8 and P21. We chose P8 to represent the rodent neuronal equivalency to the intervention being done in the human study at 6 weeks post-partum. The dietary levels of iron to achieve "iron loading" of the brain through this route was estimated from published data where lactating dams were provided from 400-20,000 ppm Fe.

To determine the consequences of aggressive iron repletion, we will measure *brain iron concentration, targeted gene expression profiles for oxidative stress and myelin-associated protein expression* at P21, P90 and 6 months of age. We chose P21 to document the model's acute effects, P90 to document the persistence of the genomic and myelination and 6 months of age to document long-term effects of aggressive iron repletion. Brain iron concentration will be measured with atomic absorption spectrophotometry. The gene expression profile of the brain will targetthe oxidative stress gene and be determined with gene array. We will use RT-PCR to verify the changes from the gene array experiments. Myelin analyses will be focused on the expression of myelin-associated proteins (Myelin Base protein, CNPase and Proteolipid protein) in white matter regions. Measurements of brain iron concentration, gene expression and myelin composition in two iron treatment groups (50 and 1000 ppm Fe) will be compared to the control groups (never ID) and H67D mice. We will include H67D mice because the H63D HFE or hemochromatosis gene variant is associated with iron overload and increased oxidative stress and it has been proposed as a genetic modifier for risk of neurodegenerative disorders. Therefore, comparing gene expression and myelin-analyses of the P8 treated group given severe iron intervention (1000 ppm) to H67D mice will further elucidate the potential for neurotoxicity if excessive iron is given to treat early ID.

We have made progress in two areas of the planned study. First, in the rat studies, it was not technically possible to get feed for the rats at 1000ppm levels and we had to revise protocol to feed 400ppm which is more physiologically realistic. Because of this change in protocol, we had a slower than expected start and decided to focus on the iron deficiency component of the proposal. No data were generated on the iron loading model. This was justified because of the novel receptor we discovered for iron uptake into oligodendrocytes which had not been investigated in models in which iron was being manipulated. Using an established

developmental iron deficient (ID) model known to have hypomyelination, we found that ferritin receptor protein expression was altered in the brain of ID rats. Tim2 protein levels were increased in whole brain homogenate of ID rats at day 36-48 ($p<0.05$; Figure 1) and day 100 ($p<0.01$; Figure 1). The ferritin receptor CXCR4 was decreased at day 36-48 in the brain of ID rats for both of the 60 kDa ($p<0.01$) and 50 kDa ($p<0.05$) isoforms (Figure 2). The 60 kDa isoform of CXCR4 is only detected at day 100, which is decreased in the brain of ID rats ($p<0.05$; Figure 2). Brain ID was confirmed by measuring TfR and ferritin in the brain, as these proteins are known to respond to reduced iron levels by increasing and decreasing respectively. As was expected, TfR protein was increased in the brain of ID rats at day 36-48 ($p<0.001$) and day 100 ($p<0.01$;) Hft protein was decreased in the brain of ID rats at day 36-48 ($p<0.001$) and day 100 ($p<0.001$). Additionally, Lft protein was decreased in the brain of ID rats at day 36-48 ($p<0.001$) and day 100 ($p<0.001$). Ferroportin (Fpn) was evaluated as well, as it has been reported to respond to iron levels similar to Hft and Lft and is critical for iron export from cells. In the brain of ID rats, Fpn protein was unchanged at day 36-48 ($p=0.123$) and was decreased at day 100 ($p<0.01$).

These data demonstrate that the novel receptor that we discovered that is present on oligodendrocytes for iron uptake will increase when iron availability to the brain is decreased. Moreover, the finding that CXCR4, the receptor for CXCL12, is decreased with ID is an exciting new observation. CXCR4 has been shown to bind to H-ferritin and it is also part of a protective, proliferative response.

Figure 1. Tim2 is increased at day 36-48 and is increased at day 100 in the brain of ID rats.

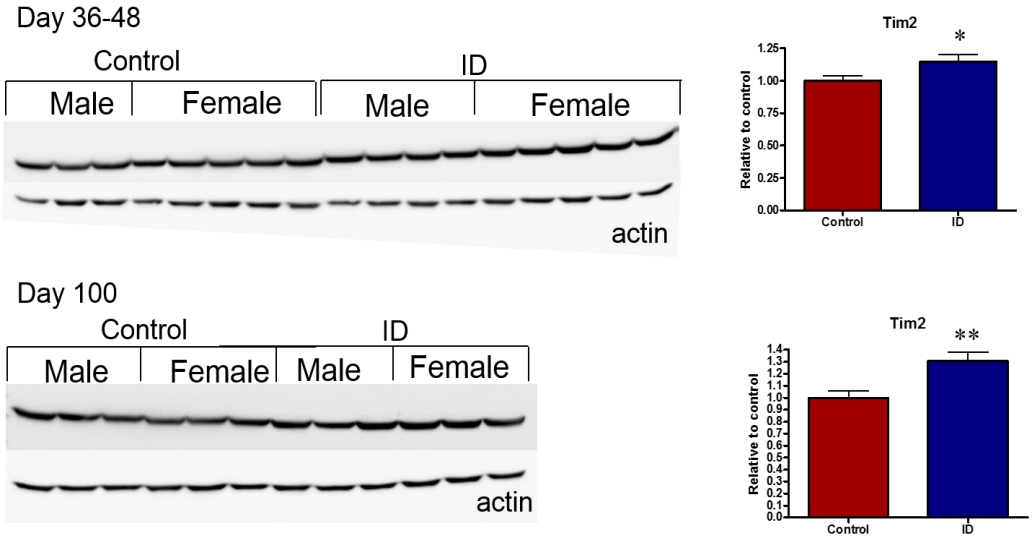
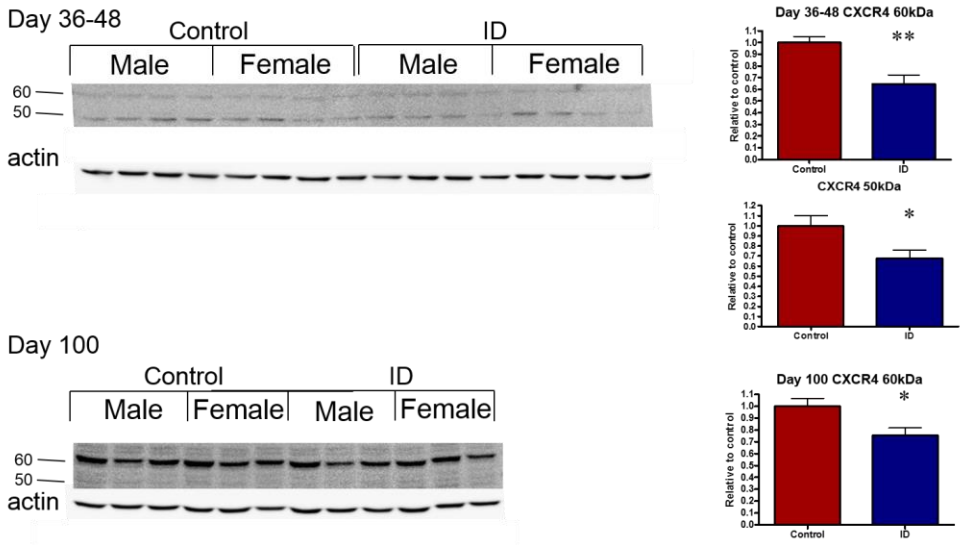


Figure 2. CXCR4 is decreased at day 36-48 and day 100 in the brain of ID rats.



In the second part of the study, we made significant progress in the development of the HFE mutant mouse line.

Generation of H67D Knock-in Mice

The H67D knock-in mice (mouse homologous to H63D in humans) were commercially generated (inGenious Targeting Laboratory, Inc, NY) as previously described by Tomatsu et al. Briefly, the HFE gene isolated from 129/SvJ mouse bacterial artificial chromosome library was subcloned into the pBS vector. The H67D point mutation (199C to –G) was introduced into exon 2 of HFE gene by site-directed mutagenesis, which destroyed a *Bsp*HI restriction site. The HFE gene fragment containing H67D mutation was added between the thymidine kinase (TK) and neo^r gene of a targeting vector (pPNT-loxP2 vector). The resulting targeting vector was linearized with *Not*I and introduced into the 129/Sv-derived embryonic stem (ES) cell line RW4 (Incyte Genomics Systems, St. Louis) by electroporation. ES clones that were resistant to both 200µg/ml G418 (BIBCO/BRL) and 2µM ganciclovir (Syntex Chemicals, Boulder, CO) were isolated and used for injection into C57BL/6J blastocysts. Chimeric males were bred to C57BL/6J females for germ-line transmission. The F1 heterozygous mice were bred to *Cre* mice to remove neo^r gene flanked by *lox*P sites. The resultant neo^r-excised heterozygotes were interbred to generate wild-type (+/+), heterozygous (+/H67D) and homozygous (H67D/H67D) H67D knock-in mice.

Mice were maintained under normal housing conditions. They were given *ad libitum* access to rodent chow pellets and water. Both males and females were included in all experiments. All procedures were conducted according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

Mice Genotyping

DNA was isolated from tail biopsies according to DNeasy blood and tissue kit (QIAGEN, CA). To amplify HFE gene including the H67D variant PCR analysis was conducted using a forward primer (5'AGGACTCACTCTCTGGCAGCAGGAGGTAACCA 3') and a reverse primer (5'TTTCTTTTACAAAGCTATATCCCCAGGGT3'). PCR conditions were: 94ºC for 15 mintues, 94ºC for 45 seconds, 58ºC for 45 seconds, 39 cycles of 72ºC for 90 seconds and 72ºC for 10 minutes. Amplified PCR fragments were digested with *Bsp*HI restriction enzyme for 2 hours at 37ºC to detect H67D point mutation. DNA fragments were separated by 2% agarose gel electrophoresis.

Measurement of Iron

Brain and Liver samples were harvested from 6- and 12-month-old wild-type (+/+), heterozygous (+/H67D) and homozygous (H67D/H67D) H67D knock-in mice. Samples were diluted 1:10 (wt:v) with 0.32 M sucrose and homogenized. Total brain and hepatic iron concentrations (µg/g of tissue; wet weight) were measured triplicate by graphite furnace atomic absorption spectrometry (model 5100AA, Perkin-Elmer, Norwalk, CT) according to standard protocol.

Immunblotting

Brain and liver tissues from 6- and 12-month-old wild-type (+/+), +/H67D and H67D/H67D mice (n = 4-6/genotype) were homogenized in homogenization buffer: 1xPBS, 0.5% NP-40 (IGEPAL; Sigma, St. Louis, MO) and protease inhibitor cocktail (1:100; Sigma, St. Louis, MO). The total protein concentration was determined with Pierce BCA protein assay kit (Thermo scientific, MA). Total brain or liver homogenates (20 µg) were separated by electrophoresis in Criterion polyacrylamide Tris-HCl gel (4-20%; Bio-Rad, Hercules, CA). Proteins were then transferred to nitrocellulose membranes and the membranes were blocked with 5% nonfat dry milk for one hour at room temperature. After overnight incubation at 4ºC with primary antibodies, membranes were then incubated with enhanced chemiluminescent (ECL) anti-host horseradish peroxidase-linked secondary antibodies (Amersham Bioscience, Piscataway, NJ) for an hour at room temperature. The signal was visualized by ECL detection (Perkin Elmer, Waltham, MA) and Multigauge software (V3.0; Fuji film system) was used to quantitate the intensity of the band. Following primary antibodies were used: HFE (1:500; Sigma, St. Louis, MO), H-ferritin (1:1000; Covence, Princeton, NJ), L-ferrtin (1:500; abcam, Cambridge, MA), transferrin receptor (1:500; ; Zymed Laboratories Inc., San Francisco, CA), divalent metal transporter-1 (1:1000; Convence, Princeton, NJ), transferrin (1:1000; MP biomedicals, Solon, OH), T cell immunoglobulin and mucin domain-containing protein-2 (Tim-2; 1:2000; abcam, Cambridge, MA), cystine/glutamate antiporter (xCT; 1:500; abcam, Cambridge, MA), hemeoxygenase-1 (HO-1; 1:500; Enzo Life Science, Farmingdale, NY), Nrf2 (1:1000; abcam, Cambridge, MA), GFAP (1:2000; Dako, Carpinteria, CA) and beta-actin (1:3000; Sigma, St. Louis, MO).

Histology

Six- and 12-month-old wild-type (+/+) and H67D/H67D mice (n = 4/genotype) were perfused transcardially with Ringer's solution followed by ice-cold 4% paraformaldehyde. The brains were paraffin-embedded and sectioned coronally at 6-µm-thick. The sections were

deparaffinized and then rehydrated through a series of ethanol. After antigen retrieval with sodium citrate (pH 6), endogenous peroxidase activity was blocked with hydrogen peroxide (3.7% in methanol) for 20 minutes at room temperature. The sections were then blocked for one hour with 2% milk and were incubated with primary antibodies overnight at 4ºC followed by an hour incubation at room temperature with biotinylated anti-host secondary antibody (1:200; vector laboratories, Burlingame, CA). Immunoreactivity was detected using the avidin biotin complex (ABC) and 3,3'-diaminobenzidine (DAB; vector laboratories, Burlingame, CA). The sections were analyzed with a bright-field microscopy by an investigator blinded for genotypes. The following primary antibodies were used for immunostaining: L-ferritin (1:250; abcam, Cambridge, MA), transferrin receptor (1:250; Zymed Laboratories Inc., San Francisco, CA), divalent metal transporter-1 (1:200; convence, Princeton, NJ) and transferrin (1:200; MP biomedicals, Solon, OH).

For immunofluorescence, after overnight incubation with rabbit anti-GFAP antibody (1:1000; Dako, Carpinteria, CA) or rabbit Iba-1 antibody (1:600; Wako, Richmond, VA), sections were probed with Alexa Flour 488 secondary antibody (Invitrogen, Grand Island, NY) for 1 hour in the dark at room temperature. After washes, slides were mounted and the sections were analyzed with fluorescence microscopy. For double immunofluorescence staining, because both L-ferritin and Iba-1 antibodies were made from the same host we fluorescently labeled L-ferritin using a DyLight 550 antibody labeling kit (Thermo fisher scientific, Waltham, MA) prior to the incubation with brain sections. The sections were first incubated overnight with rabbit Iba-1 antibody followed by overnight incubation with DyLight 550 labeled L-ferritin. The sections were then probed with Alexa Flour 488 secondary antibody for 1 hour in the dark at room temperature. After washes, the slides were mounted and the sections were analyzed with fluorescence microscopy.

Myelin isolation and Analysis of Myelin Protein

Brain samples from 6- and 12-month-old mice (5 to 6/genotype) were weighed and homogenized with 1.5 ml of 0.32M sucrose. After adding 2 ml of 0.85M sucrose samples were centrifuged for 30 minutes at 41000 rpm at 4ºC. The supernatant was discarded; the middle layer was collected and resuspended with 2.5 ml iron free water (Sigma, St. Louis, MO). The samples were centrifuged for 15 min at 41000 rpm at 4ºC. Supernatant was discarded and the pellet was resuspended with 3.0 ml iron free water. The samples were centrifuged 10 min at 17000 rpm at 4ºC and collected the pellet, which was crude myelin protein. The pellets were kept at -80ºC for 20 min and then were freeze-dry using a lyophilizer overnight. The pellets were then left at room temperature for 20 minutes and were weighed. The pellets were resuspended with RIPA buffer (Sigma, St. Louis, MO) and centrifuged 45 min at 51,000 rpm at 4 ºC. Supernatant was collected and protein concentration was determined with Pierce BCA protein assay kit (Thermo scientific, MA). Total protein of 10 µg was used for immunoblot analyses to determine the expression of myelin basic protein (MBP; 1:1000; abcam, Cambridge, MA), proteolipid protein (PLP; 1:1000; Millipore, Billerica, MA) and CNPase (1:500; abcam, Cambridge, MA).

Measurement of Oxidatively Modified Proteins

As a consequence of oxidative modification to proteins, carbonyl groups are introduced to the side chain of amino acids. These carbonyl groups hallmark the oxidative status of protein. An Oxyblot kit (Millipore, Billerica, MA) was used to measure protein carbonyl levels. Briefly, total

brain homogenates (20 µg) from 6- and 12-month-old wild-type, +/H67D and H67D/H67D mice (6/genotype) were reacted with 2,4-dinitrophenylhydrazone (DNP-hydrazone). Samples were then treated according to the manufacturer's protocol.

Statistical Analyses

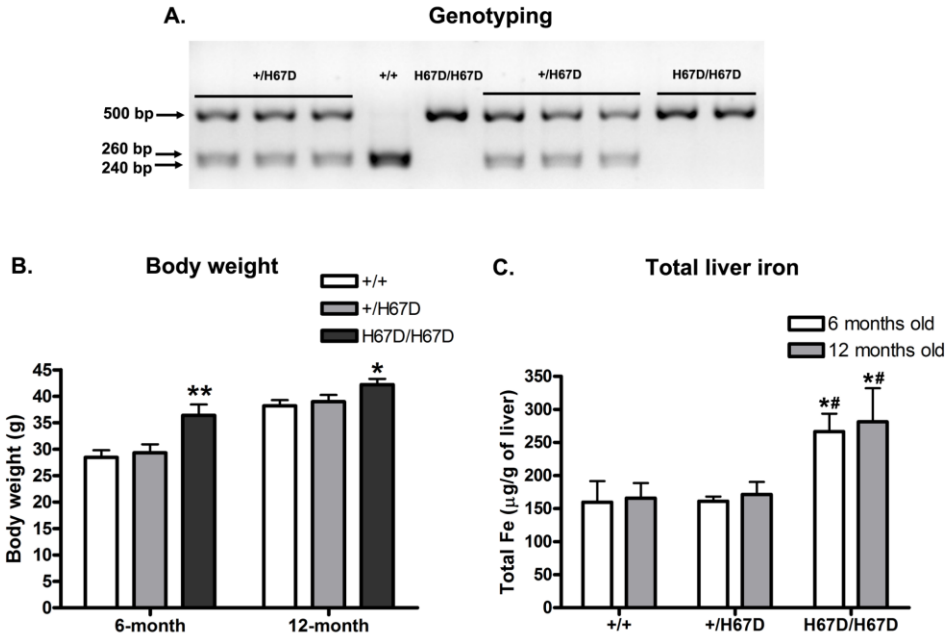
Data were expressed as mean ± standard error. An analysis of variance (one-way ANOVA; GraphPad Prism 4) followed by a Tukey multiple comparison or Dunnett test was used to compare between experimental groups. For iron measurement, two-way ANOVA was performed to analyze the interaction of age and genotype with iron concentration. Bonferroni posttest was used to compare between experimental parameters. A value of $p < 0.05$ was considered significant for all experiments.

Results

Generation of H67D mice

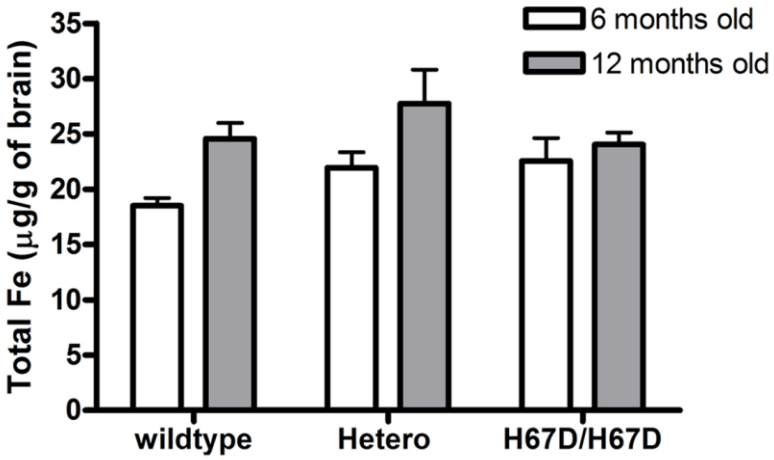
A H67D knock-in mouse line (mouse homologue of the human H63D) was generated by site-directed mutagenesis to murine HFE gene. The resultant heterozygous mice were bred to generate wild-type, heterozygous and homozygous H67D knock-in mice. Mice carrying the H67D variant were indistinguishable at birth from their littermate controls. Homozygous H67D mice develop normally and are reproductively viable. Genotyping of mice was performed by PCR analysis of DNA obtained from tail biospies and subsequent digestion with *Bsp*HI restriction enzyme. Body weights of mice were taken at 6- and 12-months of age. At both ages, H67D/H67D mice had significantly higher body weight than wild-type mice while the heterozygous H67D (+/H67D) mice had similar body weight as the wild-type.

To confirm whether the allelic variant is functional we determined hepatic iron levels in 6- and 12-month-old H67D mice. Compared to the wild-type mice, 6- and 12-month-old H67D/H67D mice had a 67% (266.4 ± 27.3 vs. 159.6 ± 31.9 µg/g of liver) and 70% (281.35 ± 50.7 vs. 165.7 ± 22.9 µg/g of liver) increase in hepatic iron concentration while +/H67D mice had similar hepatic iron concentration as wild-type mice at both ages. Six and 12-month-old H67D/H67D mice also had changes in iron management protein expressions in the liver. Consistent with increased iron concentration iron storage proteins, H-ferritin and L-ferritin were significantly increased while iron transport protein transferrin receptor (TfR) was significantly decreased in H67D/H67D mice compared to wild-type mice at both ages. Together these results indicated that the allelic variant is functional in H67D mice.



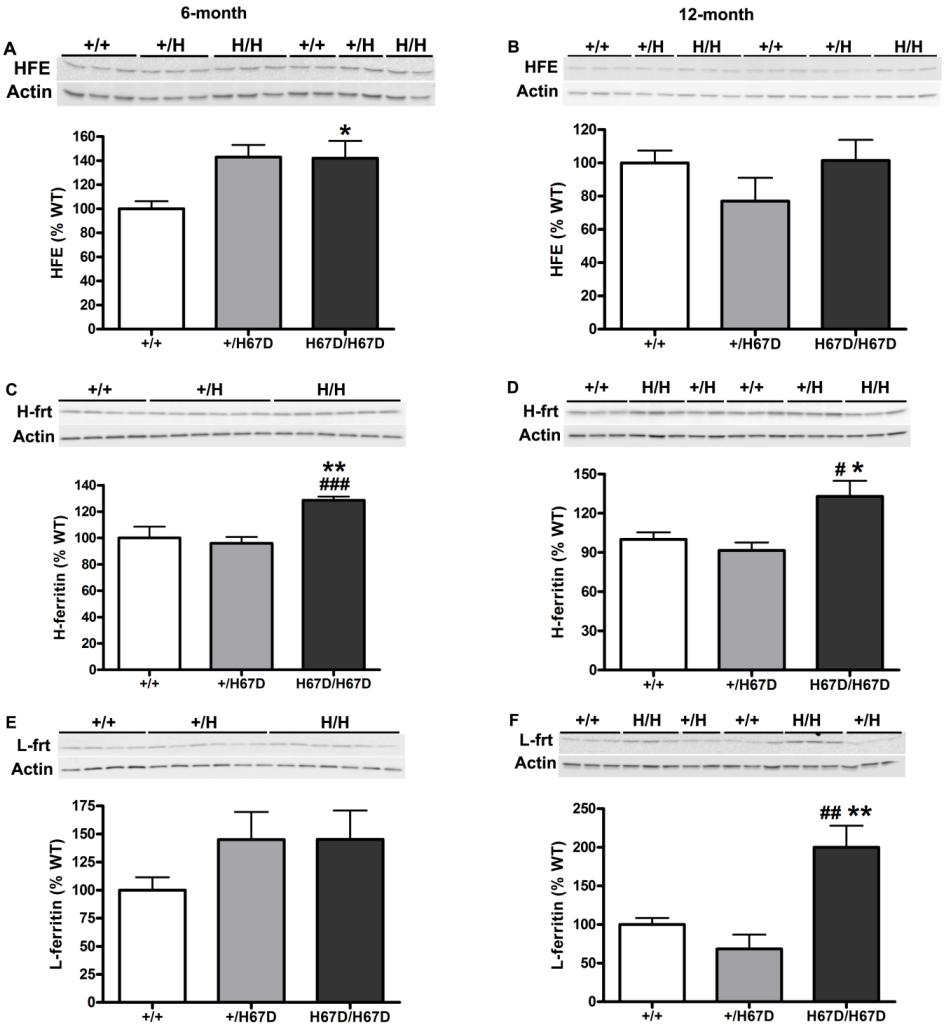
Brain iron concentration in H67D mice

Total brain iron was measured in 6- and 12-month-old mice by atomic absorption spectrometry. At 6-months of age, total brain iron level was 22% higher in H67D/H67D (22.57 ± 2.0 µg/g of brain) and 18.6% higher in +/H67D mice (21.95 ± 1.4 µg/g of brain) compared to wild-type mice (18.5 ± 0.7 µg/g of brain) although these differences did not reach statistical significance ($p = 0.26$). At 12-months of age, total brain iron concentration in H67D/H67D mice (24.04 ± 1.1 µg/g) and +/H67D mice (27.76 ± 3.0 µg/g) was not different compared to wild-type mice (24.58 ± 1.4 µg/g). There was a significant interaction for age and iron concentrations indicating that regardless of genotypes brain iron concentration increased with age ($p = 0.01$). Compared to 6-month-old mice, by 12-months, brain iron concentration increased by 33% in the wild-type, 26.5% in the +/H67D but only 6.5% in H67D/H67D mice.



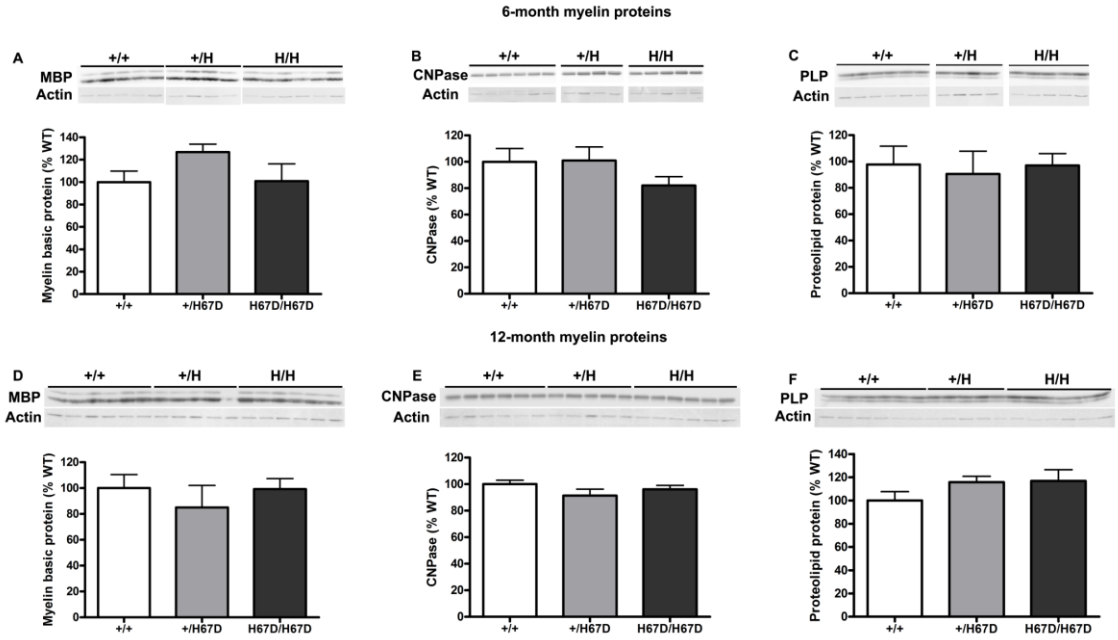
Alteration in expression of proteins involved in iron homeostasis in H67D mice

Although total iron levels are important, it is the response of the regulatory proteins that are perhaps most important because they maintain homeostasis. Thus, we evaluated the expressions of proteins involved in brain iron homeostasis: HFE, H-ferritin, L-ferritin, transferrin receptor (TfR), transferrin (Tf), divalent metal transporter-1 (DMT-1) and T cell immunoglobulin and mucin domain-containing protein-2 (Tim-2). Western blot analyses of brain homogenates from 6- and 12-month-old mice revealed differences in the levels of iron management proteins in H67D/H67D mice compared to the wild-type. At 6-months H67D/H67D mice had significant increases in HFE and H-ferritin and a 40% decrease in DMT-1 which did not reach statistical significance ($p = 0.07$). L-ferritin, Tf, Tim-2 levels and TfR (data not shown) in H67D/H67D mice were not different from wild-type mice. At 12 months of age, H67D/H67D mice had increases in H- and L-ferritin levels, and decreases in Tf and Tim-2 levels compared to wild-type mice. The relative concentrations of HFE, DMT- and TfR (not shown) in H67D/H67D mice were not different from wild-type mice at this age. None of the iron management proteins levels in +/H67D mice were different from wild-type at any ages.



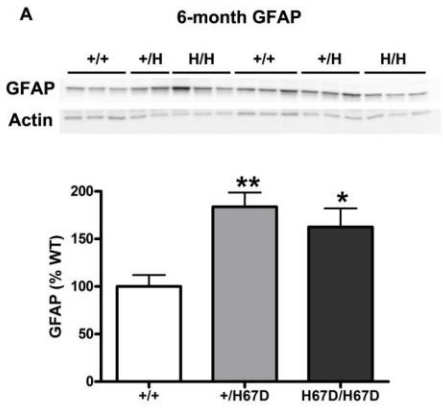
Myelin proteins were not changed in H67D mice

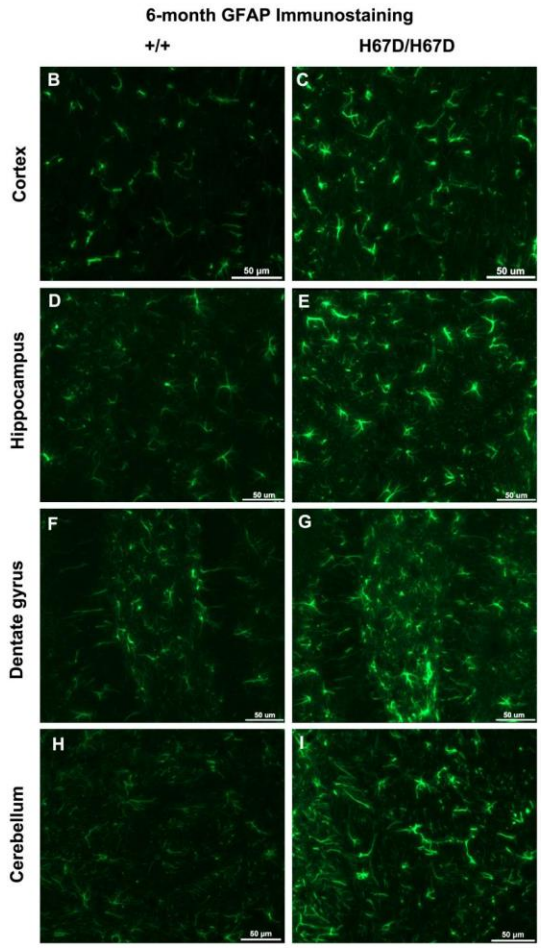
Crude myelin protein was extracted by sucrose gradient and ultracentrifugation and three major myelin proteins in myelin extract were analyzed in 6- and 12-month-old mice by immunoblotting. Myelin basic protein (MBP), proteolipid protein (PLP) and CNPase protein were not changed significantly between any of the groups at either age.



H67D HFE is associated with astrogliosis

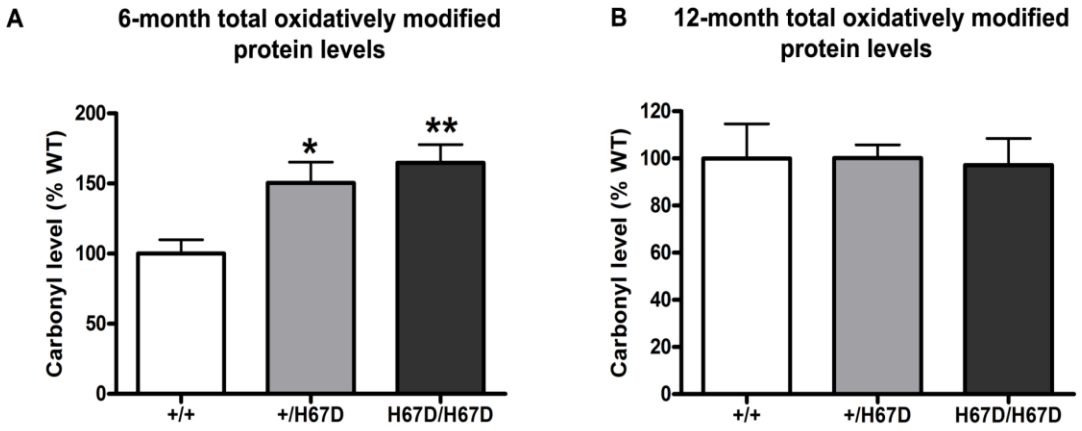
To determine whether H67D HFE is associated with increased gliosis, we evaluated GFAP staining in H67D mice at both ages. GFAP staining was increased in all observed brain regions; the cortex, hippocampus and the cerebellum in 6-month-old H67D/H67D, compared to wild-type mice, which is consistent with the significant increase in total GFAP level in H67D knock-in mice determined by immunoblotting. GFAP staining and total GFAP level in 12-month-old H67D mice was not changed significantly from wild-type mice (data not shown).



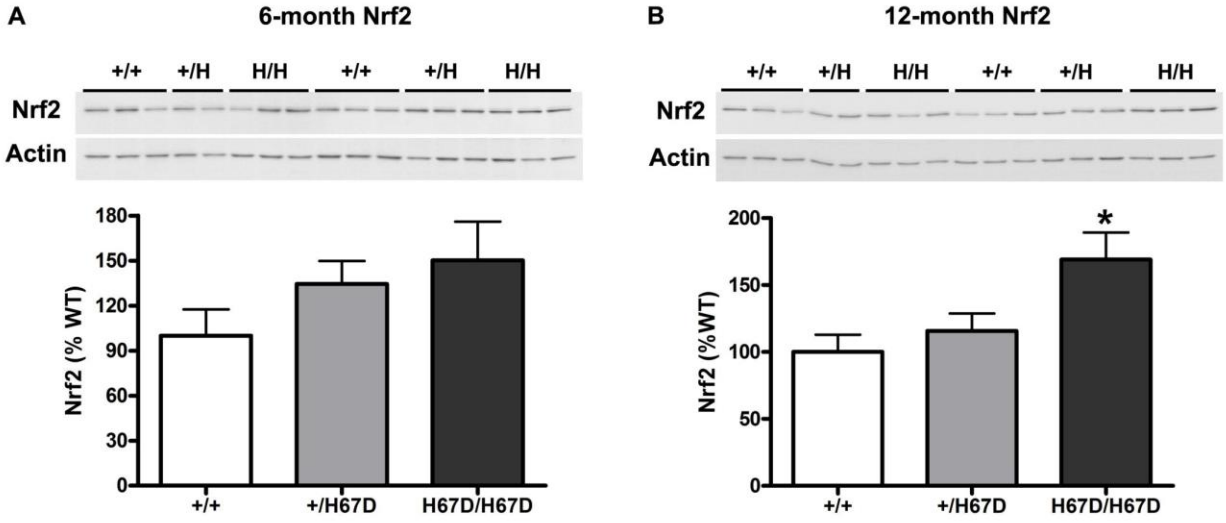


<u>H67D HFE is associated with oxidative stress</u>

Because H63D HFE is associated with higher baseline stress in our cell models, we determined the level of oxidatively modified proteins (carbonyl level), and expression of xCT antiporter, which is essential for maintaining intracellular glutathione level, and hemeoxygenase-1 (HO-1) as indices of oxidative stress in H67D mice. The level of oxidatively modified proteins increased in both H67D/H67D (64%) and +/H67D (50%) mice at <u>6-months</u> of age compared to the wild-type. Moreover, xCT expression was 86% higher and HO-1 expression was 27% higher in the brains of 6-month-old H67D/H67D mice compared to the wild-type mice. However, there was no difference in the oxidatively modified proteins level, and xCT and HO-1 expression between three groups at 12-months of age.



To examine whether an increase oxidative stress observed in 6-month-old H67D mice, is associated with an impaired cellular defense system against redox stress we measured the expression of nuclear factor E2-related factor 2 (Nrf2) that controls endogenous anti-oxidant genes transcription. The expression of Nrf2 in 6-month-old H67D/H67D and +/H67D mice was not different from wild-type mice but was 69% higher ($p < 0.05$) in 12-month-old H67D/H67D mice compared to the wild-type.



18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04),

the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
None				☐Submitted ☐ Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____X____ No_____

If yes, please describe your plans:

We plan to continue to develop the mouse model. We have already had a paper accepted with this model studying changes in the brain at 18 months.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable."

Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No___

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes__ No___

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes___ No___ ____X___

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/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
James R. Connor, Ph.D.	Distinguished Professor
eRA COMMONS USER NAME James_R_Connor	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Thomas More College, Ft. Mitchell, KY	B.A.	05/75	Psychology
Wright State University, Dayton, OH	M.S.	05/78	Physiology
University of California, Berkeley, CA	Ph.D.	06/81	Anatomy
Boston University Medical Center, Boston, MA	Postdoc	06/81-07/83	Neuroscience

A. Positions and Honors

Professional Positions:

1983-1987 Research Biologist, Veteran's Administration Medical Center, Washington, DC
1983-1987 Assistant Research Professor, Department of Physiology, The George Washington University School of Medicine
1987-1990 Assistant Professor, Department of Anatomy Pennsylvania State University College of Medicine (PSUCOM) Neuroscience & Anatomy, (PSUCOM)
1990-1996 Associate Professor, Department of Neuroscience & Anatomy, (PSUCOM)
1996- Professor, Department of Neuroscience & Anatomy, (PSUCOM)
1999-2002 Vice Chair Department of Neuroscience & Anatomy, (PSUCOM)
2002-2003 Interim Chair Department of Neuroscience & Anatomy, (PSUCOM)
2004- Professor Neurosurgery, (PSUCOM)
2006 Co-Director, Neuro-oncology Experimental Therapeutic Group (PSU Cancer Institute)
2007 University Distinguished Professor (PSUCOM)

Grant Review Experience:

NIH, NIEHS, Veterans Administration, Department of Defense USDA, NSF, MRC, Wellcome Trust, Israel Academy of Science, Alzheimer's Association, Hong Kong Research Council, American Federation for Aging Research, American Heart Association

Honors and Awards:

Samuel Hinkle Society Outstanding Young Investigator Award, Pennsylvania State University College of Medicine (1990);
Mentored two graduate students awarded Marion Kies Award by the American Society for Neurochemistry for outstanding doctoral thesis (1999, 2010);
Elected Chair of the East Coast Iron Club (2000-2002);
Dean's Lecturer Pennsylvania State University College of Medicine/M.S. Hershey Medical Center (2003);
Distinguished Alumni Award, Thomas More College (2007);

Named Distinguished Professor Penn State University (2007)
Honorary Professor, Department of Neurology, Tianjin First Center Hospital, Tianjin (2008-)

Editorial Boards:
Journal of Neuroscience Research (1994-); GLIA (2001-); Fluids and Barriers of the CNS (2004-); Current Neurovascular Research (2004-); CNS Drug Discovery (2005-); Recent Patents on Biotechnology (2008-); Journal of Neurochemistry (Handling Editor, 2011-); PlosOne (2012-).

B. Selected Peer-reviewed Publications

1. Wang X, Lee S, Simmons Z, Boyer P, Scott K, Liu W, **Connor J**. Increased incidence of the Hfe mutation in amyotrophic lateral sclerosis and related cellular consequences. *Journal of Neurological Sciences* 227(1) 27-33, 2004.
2. Lee SY, Patton SM, Henderson RJ, **Connor JR.** Consequences of expressing mutants of the hemochromatosis gene (JFE) into a human neuronal cell line lacking endogenous HFE. *The FASEB Journal* 21(2):564-576, 2007.
3. Mitchell RM, Freeman WM, Randazzo WT, Stephens HE, Beard JL, Simmons Z, **Connor JR**. A CSF biomarker panel for identification of patients with Amyotrophic Lateral Sclerosis. *Neurology* 72:14-19, 2009.
4. Mitchell RM, Lee SY, Simmons Z, **Connor JR**: HFE polymorphisms affect cellular glutamate regulation. *Neurobiology of Aging* 6(6):1-12, 2009.
5. Mitchell RM, Lee SY, Randazoo WT, Simmons Z, **Connor JR**: Influence of HFE variants and cellular iron regulation on monocyte chemoattractant protein-1. *J Neuroinflamm* 6:6, 1-12 Feb, 2009. PMCID: 2656486
6. Hall EC, Lee SY, Simmons Z, Neely EB, Nandar W, **Connor JR**: Prolyl-peptidyl isomerase, Pin1, phosphorylation is compromised in association with the expression of the HFE polymorphic allele, H63D. *Biochimica et Biophysica Acta* 1802:389-395, 2010.
7. Mitchell RM, Beard JL, Stephens HE, Simmons Z, Connor JR. Plasma Biomarkers Associated with ALS and Their Relationship to Iron Homeostasis. *Muscle and Nerve* 42:95-103, 2010.
8. Bartzokis G, Lu PH, Tishler TA, Peters DG, Kosenko A, Barrall KA, Finn JP, Villablanca P, Laub G, Altshuler LL, Geschwind DH, Mintz J, Neely E, **Connor JR**. Prevalent Iron Metabolism Gene Variants Associated with Increased Brain Ferritin Iron in Healthy Older Men. *Journal of Alzheimer's Disease* Apr;20(1):333-341, 2010. PMID: 20164577
9. Mairuae N, Hall EC II, Cheepsunthorn P, Lee SY, Connor JR: The H63D HFE Gene Variant promostes Activation of the Intrinsic Apoptotic Pathway via Mitochondria Dysfunction Following β–Amyloid Peptide Exposure. *Journal of Neuroscience Research* 1;88(14):3079-3089, 2010.
10. Bartzokis G, Po HL, Tingus K, Peters D, Amar CP, Tishler TA, Finn JP, Villablanca P, Altshuler LL, Mintz J, Neely E, **Connor JR**. Gender and Iron Genes May Modify Associations Between Brain Iron and Memory in Health Aging. *Neuropsychopharmacology*. June 36:1375-1384, 2011.
11. **Connor JR**, Ponnuru P, Wang X, Patton S, Allen R, Earley C. (2010) Profile of altered brain iron acquisition in restless legs syndrome. *Brain*. April 134(4):959-968, 2011.
12. Nandar W, **Connor JR**. HFE Gene Variants Affect Iron in the Brain. *The Journal of Nutrition*. April, 141:729S-739S, 2011.
13. Liu Y, Lee SY, Neely E, Nandar W, Moyo M, Simmons Z, **Connor JR**. Mutant HFE H63D Protein Is Associated with Prolonged Endoplasmic Reticulum Stress and Increased Neuronal Vulnerability. *Journal of Biological Chemistry*. 286:13161-13170, 2011.

14. Hall EC II, Lee SY, Mariuae N, Simmons Z, **Connor JR**: Expression of the HFE allelic variant H63D in SH-SY5Y cells affects tau phosphorylation at serine residues. *Neurobiology of Aging*. 32:1409-1419, 2011.
15. Su X, Lee S, Mitchell R, Stephens H, Simmons Z, **Connor, JR**. H63D HFE polymorphisms are associated with increased disease duration and decreased muscle SOD1 expression in amyotrophic lateral sclerosis patients. *Muscle and Nerve*. Accepted for publication 2012.