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Age- and Sex-Associated Changes in mRNA Expression of Neurodegenerative Disorder-Related Molecules in the Hippocampus and Cerebellum of Rat Brain

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TITLE

AGE- AND SEX-ASSOCIATED CHANGES IN mRNA EXPRESSION OF NEURODEGENERATIVE DISORDER-RELATED MOLECULES IN THE HIPPOCAMPUS AND CEREBELLUM OF RAT BRAIN

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PART-I

Age- and Sex-associated Changes in mRNA Expression of Neurodegenerative Disorder-related Molecules in the Hippocampus of Rat Brains

Abstract

Age-associated oxidative stress is involved in neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, and sex-associated differences may also affect the risk for these neurodegenerative diseases. We compared the effects of aging and sex on the mRNA expression of five molecules that are closely related to oxidative stress, along with Alzheimer's and Parkinson's diseases in the hippocampus of both male and female Fischer 344xBrown Norway (F344BN) rats. The reverse transcription polymerase chain reaction was used to determine the mRNA expression level of superoxide dismutase 2 (SOD2), heme oxygenase 1 (HO1), amyloid precursor protein (APP), β-site APP-cleaving enzyme 1 (BACE1), and α-synuclein (ASN) in the hippocampus of 3 groups of male and female (young rats, aged rats, and very aged F344BN rats). No significant age- or sex-related changes were observed in the expression levels of SOD2, APP, or BACE1 mRNAs. The expression of HO1 mRNA in the very aged female rat hippocampus was significantly higher than that observed in the young female control and the aged females, when compared to male counterparts. No significant age-associated changes were observed in the expression of ASN mRNA; however, the expression of ASN was significantly higher in the hippocampus of male compared to female rats. Because the accumulation of iron in the brain plays a key role in Alzheimer's and Parkinson's diseases, we also investigated age- and sex-related expression of five mRNAs that are closely related to iron storage, transportation, and metabolism: ferritin heavy chain (FTH), ferritin light chain (FTL), transferrin receptor (TfR), divalent metal transporter 1 (DMT1), and iron-regulatory protein 1 (IRP1). No significant age-related changes were observed in the expression levels of any of these five molecules. The overall expression of FTH and IRP1 mRNAs was significantly lower in the

hippocampus of male rats when compared to females. This study paves the way for the further investigation of age- and sex-related changes in the protein expression and activities of these molecules, and will help clarify the mechanisms by which oxidative damage may affect neurodegenerative diseases.

CHAPTER 1

Introduction

According to the United States census, there were 31.2 million people aged 65 and older in 1990; this number increased to 35.0 million by 2000 [1]. The growth rate of the United States population in 2010-2020 is anticipated to be 8.3%; however, the growth rate of the portion of the population over 65 years old is projected to be 35.3% during this time. The aged now comprise the fastest growing segment of our population. Aging increases the risk of age-associated diseases such as neurodegenerative diseases, cardiovascular diseases, cancer, arthritis, osteoporosis, cataracts, type 2-diabetes, and hypertension [2, 3]. Aging is also accompanied by a decreased response to stress, and increased homeostatic imbalance.

The free-radical theory [4] proposes that reactive oxygen species (ROS), a cluster of highly-reactive oxygen-containing molecules, are responsible for the gradual damage to cell structure and function during aging and neurodegenerative diseases[5]. Cells normally have antioxidant systems to protect and repair themselves from oxidative damage. For example, superoxide dismutase (SOD) is an antioxidant enzyme that catalyzes the conversion of superoxide anions to hydrogen peroxide and molecular oxygen. The hydrogen peroxide is subsequently broken down by catalase or glutathione peroxidase to produce water. In this way, SOD acts as a defense against ROS damage. Cu/Zn superoxide dismutase (also called SOD1, a 32 kDa protein co-factored with copper and zinc) is localized primarily in the cytosol of the eukaryotic cells[6], while Mn superoxide dismutase (also called SOD2, a 23 kDa protein that binds manganese) is primarily present in the mitochondrial matrix[7].

Research data have demonstrated an increase in the SOD2 (but not SOD1) protein synthesis and activity in rodent brains during the course of normal aging [8, 9]. An increase in SOD2 expression has also been reported in the substantia nigra of Parkinson's disease and in the hippocampus of Alzheimer's disease subjects [10-12].

Heme oxygenase (HO) is another important antioxidant enzyme. As a microsomal enzyme, HO cleaves heme to produce biliverdin, inorganic iron, and carbon monoxide (CO). Biliverdin is subsequently converted to bilirubin by biliverdin reductase[13]. Bilirubin and CO demonstrate an intrinsic ability to exert potent antioxidant and anti-inflammatory processes. Heme oxygenase 1 (HO1) is a 32 kDa stress protein that catalyzes the rate-limiting step of the enzymatic degradation of heme in the brain and other tissues; its gene promoter region contains a heat shock element that is rapidly inducible when exposed to heme, sulfhydryl compounds, UV light, various pro-oxidants, or metal ions[14-16]. Deregulation of HO1 has been linked to several neurodegenerative disorders, including Alzheimer's disease [17-19]. Previous studies have linked the expression of HO1 with SOD2, which reveals an influence of these two genes on one another [7, 12].

Neurodegenerative disorders such as Alzheimer's and Parkinson's diseases are among the most prominent, but perplexing, diseases to understand and treat. Only recently have scientists begun to understand the molecular processes and pathways that are crucial in the development and progression of these diseases. Alzheimer's disease is currently a leading cause of death in people aged 65 and older, and is projected to affect 1 in 45 people worldwide by the year 2050[20, 21]. Patients with Alzheimer's disease experience

irreversible loss of neurons, which results in progressive memory decline, as well as inadequate thinking and behavioral abilities[22]. A dominant characteristic of Alzheimer's disease is the formation of extraneuronal plaques[23]. These plaques contain aggregated amyloid β peptide (Aβ), a 42-amino acid product that is produced by endoproteolysis of amyloid precursor protein (APP), a 100-140 kDa, large type 1 (α-helical) transmembrane glycoprotein[24]. The formation of Aβ from APP involves two steps; first β-secretase cleaves APP generating the N terminus of Aβ leaving a 99-amino acid C-terminal membrane-bound fragment. Next, γ-secretase cleaves the bound C-terminal domain to release the mature Aβ peptide [25, 26]. The accumulation of mature Aβ interrupts synaptic transmissions and alters synaptic plasticity.

β-secretase, commonly referred to as β-site APP-cleaving enzyme 1 (BACE1), is a 501-amino acid type 1 membrane protein with aspartic protease activity. BACE1 activity is optimized at low pH, and is predominantly localized in acidic intracellular areas, with its highest expression levels in the neurons of the brain[27].The BACE1 enzyme drives the rate-limiting step of APP cleavage[28, 29]. Recent studies have shown that in patients with Alzheimer's disease, levels of BACE1 increase with elevated levels of Aβ plaques[28, 30, 31]. This indicates that BACE1 may be initiating or accelerating the onset and progression of Alzheimer's disease [32, 33]. Other studies indicate the possibility that BACE1 may be a stress response protein. Impaired glucose metabolism is a characteristic of the Alzheimer's brain. The treatment of APP transgenic mice with drugs that disrupt energy metabolism causes levels of BACE1, and thus Aβ, to double[34]. BACE1 also actively participates in the processing of neuregulin 1, a ligand of a family of tyrosine receptor kinases[35]. Neuregulin

1 plays an important role in regulating synapse formation, synaptic plasticity, and the maintenance of synaptic connections. Research data have suggested that partial inhibition of BACE1 may benefit the central nervous system by reducing the accumulation of Aβ[36].

Another protein that may affect the onset and progression of Alzheimer's and Parkinson's diseases is α-synuclein (ASN). ASN is a 19 kDa, 140-amino acid, heat stable, presynaptic protein that is predominantly expressed in the brain. ASN is localized around the nucleus of mammalian brain cells in either a free or membrane-bound state. It is a precursor molecule to the non-Aβ-protein component of plaques found in Alzheimer's disease patients. ASN participates in the regulation of several enzymes and transporters, neurotransmitter release, and has also been identified as having potential roles in synaptic regulation and neuronal plasticity. Although ASN has been linked to cognitive impairment in Alzheimer's disease, it is most prominently associated with Parkinson's disease[37]. Parkinson's disease is the second most common human neurodegenerative disorder, affecting the motor system with symptoms that include trembling and stiffness of hands, arms, and legs, as well as poor balance and coordination [38, 39]. Elevated ASN protein expression has been reported in Parkinson's patients, and aggregated ASN is the main component of Lewy bodies, abnormal protein clusters found in the brains of Parkinson's patients[40]. ASN gene duplication and triplication, as well as genetic variability in the promoter and 3^1 untranslated region, are associated with familial Parkinson's disease [41-46].

Studies demonstrate that the accumulation of iron in the brain plays a key role in Alzheimer's and Parkinson's diseases, and it is known that excess free Fe^{2+} ions catalyze the

formation of free radicals that are toxic to neuronal cells[47-51]. Several types of molecules are involved in iron storage, transportation, and metabolism. A major intracellular iron storage protein is ferritin, which contains ferritin heavy chain (FTH, 21 kDa) and ferritin light chain (FTL, 19 kDa) [52, 53]. Transferrin receptor (TfR) is involved in iron transportation, while divalent metal transporter 1 (DMT1) is involved in iron metabolism by transporting Fe^{2+} from the intestinal lumen to the cytosol [54, 55]. Iron regulatory proteins (IRPs) post-transcriptionally control the expression of mRNAs bearing iron responsive elements (IREs) such as FTH, FTL, TfR, and DMT1[56].

In this study, we determined the mRNA expression level of five molecules (SOD2, HO1, APP, BACE1, and ASN) that are closely related to neurodegenerative diseases, as well as five molecules (FTH, FTL, TfR, DMT1, and IRP1) that are related to iron storage and transport. We used a rat model system specific for aging, and concentrated on examining hippocampus tissue. The purpose of this study was to test the hypothesis that normal aging may induce age-associated sex-associated changes in the mRNA expression of the molecules of interest.

CHAPTER 2

Experimental Methods

2.1. Animals

Animal care and use were performed in accordance with the Guide for the Care and Use of Laboratory Animals approved by the council of the American Physiological Society and by the Animal Use Review Board of Marshall University. Experimental procedures were conducted in strict accordance with the Public Health Service animal welfare policy. Male F344BN rats of 3 ages (6, 30, and 33 months old; 6 rats in each group), and female F344BN rats of 3 ages (6, 26, and 30 months old; 6 rats in each group) were obtained from the National Institute of Aging (Bethesda, MD). The rats were housed two per cage in a vivarium approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Housing conditions included a 12-hour light: 12-hour dark cycle, with the temperature maintained at 22 ± 2 °C. The rats were provided with food and water *ad libitum*, and allowed to recover from shipment for at least two weeks before experimentation began. During this recovery period, the rats were carefully observed and weighed weekly. None of the rats exhibited signs of failure to thrive such as precipitous weight loss, disinterest in the environment, and/or unexpected gait alterations.

2.2. Tissue Isolation

The rats were anesthetized by an intraperitoneal injection of a ketamine-xylazine (4:1) cocktail administered at 50 mg/kg, and supplemented as necessary for reflexive response. The brains were removed from the skull and rinsed in Krebs solution to remove the surface blood, after which they were dissected[57]. The hippocampus was quickly separated from the whole brain, blotted dry, and immediately frozen in liquid nitrogen and stored at -80 °C until further use[57].

2.3. RNA extraction and cDNA synthesis

The hippocampus of each male and female rat from each age group was homogenized, individually, on ice using TRI reagent (Ambion, Austin, TX) at a ratio of 1 mL of TRI reagent per 100 mg of tissue. The homogenate was incubated for 5 minutes on ice to allow nucleoprotein complexes to completely dissociate, after which it was centrifuged at 4 °C for approximately 15 minutes at 12,000 x g. The supernatant was separated from the pellet and transferred to a 2 mL RNAse-free tube. A 200-µl aliquot of chloroform (Sigma-Aldrich, Inc., St. Louis, MO) was added to the supernatant. The solution was mixed and incubated at room temperature for 10 minutes, followed by centrifugation at 4 °C for 15 minutes at 12,000 x g. The aqueous phase of the solution was collected and transferred to another tube. A 500-µl aliquot of isopropanol (Sigma-Aldrich, Inc., St. Louis, MO) was added to the aqueous phase, followed by thorough mixing. The resultant solution was incubated at room temperature for 10 minutes, followed by centrifugation at 4 °C for 10

minutes at 12,000 x g. The supernatant was then carefully removed without disturbing the pellet. The RNA pellet was washed free of salts by centrifugation in 75% ethanol at 7500 x g for 5 minutes. The ethanol wash was carefully removed without disturbing the pellet, and the residual ethanol was evaporated at room temperature by speed vacuuming for 1 minute. The RNA pellet was dissolved in 50 µl of 1xTE buffer (10 mM Tris at pH 8.0, 1 mM EDTA) and quantified at 260 nm using a NanoVue UV-Vis Spectrophotometer (GE Healthcare, Piscataway, NJ). The integrity of the RNA from each sample was determined through use of a RNA 6000 Nano Kit and an Agilent 2100 Bioanalyzer (both from Agilent Technologies, Santa Clara, CA), and subjected to reverse transcription in order to generate complementary DNA (cDNA) by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA).

2.4. Real-time polymerase chain reaction (RT-PCR)

The mRNA expression levels of SOD2, HO1, APP, BACE1, ASN, FTH, FTL, TfR, DMT1, and IRP1 in each hippocampus from each age group were determined by RT-PCR using the ABI 7000 Real-Time PCR system (Applied Biosystems Inc., Foster City, CA). The reactions were carried out in a 96-well optical reaction plate (Applied Biosystems Inc., Foster City, CA). Each 25-µl reaction was comprised of 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA), 0.5 µl of 10 µM forward primers, 0.5 µl of 10 µM reverse primer, 2 µl of cDNA, and 9.5 µl of nuclease-free water. An optical adhesive cover (Applied Biosystems Inc., Foster City, CA) was placed over the plate before it was spun at 300 rpm for 30 seconds. Cycling conditions for RT-PCR were as follows: 1

cycle of 2 minutes at 50 °C and 10 minutes at 95 °C; 40 cycles of 15 seconds at 95 °C; and a final 1 minute cycle at 60 °C. SYBR Green dye intensity for each RT-PCR reaction was analyzed using ABI prism 7000 SDS software (Applied Biosystems Inc., Foster City, CA), and the corresponding threshold cycle (Ct) value was determined by the relative quantification plate method.[58] All experiments were repeated three times, and β-actin was used as an internal control to offset the efficiency differences in RT-PCR. Primers used in this study were purchased from Integrated DNA technologies (San Diego, CA) with the sequences shown below.

Table 1.This table shows the list of Primers that we used in this study.

Title	**Primers used in this study**
SOD2 forward primer	5’-GGCCAAGGGAGATGTTACAA-3’
SOD2 reverse primer	5’-GCTTGATAGCCTCCAGCAAC-3’
HO1 forward primer	5’-TGCTCGCATGAACACTCTG-3’
HO1 reverse primer	5’-TCCTCTGTCAGCAGTGCCT-3’
APP forward primer	5’-GGCCCGAAAAACTCCTACTT-3’
APP reverse primer	5’-ATCGCAAAGAGAGCAGAAGC-3’
BACE1 forward primer	5’-GCTGCAGTCAAGTCCATCAA-3’
BACE1 reverse primer	5’-ATTGCTGAGGAAGGATGGTG-3’
ASN forward primer	5’-AGGGAGTCGTTCATGGAGTG-3’
ASN reverse primer	5’-CCCTCCACTGTCTTCTGAGC-3’
FTH forward primer	5’-CTGAATGCAATGGAGTGTGC-3’

FTH reverse primer	5’-TCTTGCGTAAGTTGGTCACG-3’
FTL forward primer	5’-GCACAGACCCTCACCTCTGT-3’
FTL reverse primer	5’-GGCGCTCAAAGAGATACTCG-3’
TfR forward primer	5’-GGGCTCTGGCTCTCACACCCT-3’
TfR reverse primer	5’-AGTCCACGTAGCCAGGGCCAA-3’
DMT1 forward primer	5’-CAGCAGCCCCCATGCTGACC-3’
DMT1 reverse primer	5’-CAGGATCCCCACCGCCCAGA-3’
IRP1 forward primer	5’-TTGCCGAGCCCTTGGACCCT-3’
IRP1 reverse primer	5’-TCACAGTTCCGAACGGCGGC-3’
β-actin forward primer	5’-CAACCTTCTTGCAGCTCCTC-3’
β-actin reverse primer	5’-TCTGACCCATACCCACCATC-3’

2.5. Data analysis

The expression of SOD2, HO1, BACE1, APP, ASN, FTH, FTL, TfR, DMT1, and IRP1 mRNAs were normalized to β-actin mRNA, and the $2^{-\Delta Ct}$ method[58] was used to calculate the relative mRNA expression level. Results are presented as the mean ± SEM (standard error of the mean). The SigmaStat 3.5 statistical program (SPSS Science Inc., Chicago, IL) was used to analyze the data; a two-way analysis of variance (ANOVA) was performed to determine any differences between mRNA expression levels from hippocampus tissues within same sex groups and between age-matched groups of the opposite sex. The significance level was set as $p < 0.05$ for each comparison.

CHAPTER 3

Results

3.1. Effect of aging on hippocampus mRNA levels of HO1, APP, SOD2, BACE1, or ASN

Based on a rat survivability curves obtained from the National Institutes of Health [59], 26-month-old female F344BN rats are an age match for 30-month-old male rats, and 30-month-old female F344BN rat's age-match for 33-month-old male rats. Six-month-old male or female rats were used as the controls. The expression of HO1 mRNA in the 30-month-old female rat hippocampus was significantly higher than in the 6-month and 26-month-old females. No significant age-related changes were observed in the expression levels of HO1 mRNA in the hippocampus of male rat brains when compared the 6-month-old male control group. No significant age-related changes were observed in the expression levels of SOD2, APP, BACE1, or ASN mRNAs in the hippocampus of either female or male rat brains when compared the 6-month-old control group (Figures1-5).

3.2. Effect of Sex on hippocampal mRNA of HO1, APP, SOD2, BACE1, ASN

The overall expression of HO1 mRNA in the hippocampus of both sexes was very low. The expression of HO1 mRNA in the 30-month-old female rat hippocampus was significantly higher than its age matched (33-month-old) male counterpart. No significant sex-related changes were observed in the expression levels of SOD2, APP, or BACE1 mRNAs when compared to the aged-matched group of the opposite sex.

3.3. Sex Affects the Expression of ASN mRNA in the Hippocampus of F344BN Rats

Compared to 6-month-old control groups, no significant age-associated changes were observed in the expression of ASN mRNA in the hippocampus of either female or male rats. However, the overall expression of ASN mRNA was significantly higher in the hippocampus of male rats than in female rats (Figure 5).

3.4. Neither Age nor Sex Affects the Expression of FTL, TfR, or DMT1 mRNAs

We also investigated the expression of five mRNAs that are closely related to iron transport and storage within the cell: FTH, FTL, TfR, DMT1, and IRP1. Figure 6 illustrates the abundance of FTH (Figure 6A), FTL (Figure 6B), TfR (Figure 6C), DMT1 (Figure 6D), and IRP1 (Figure 6E) mRNA expression in the hippocampus of aged female (30-month-old) and male (33-month-old) F344BN rats as compared to the 6-month-old control groups. No significant age- or sex-related changes were observed in the expression levels of FTL, TfR, or DMT1 mRNAs.

3.5. Sex Affects the Expression of FTH and IRP1 mRNAs in the Hippocampus of F344BN Rats

Compared to sex-matched 6-month-old control groups, no significant age-associated changes were observed in the expression of FTH or IRP1 mRNAs in the hippocampus of

either female or male rats (Figures 6A, 6E). However, the overall expression of both FTH and IRP1 mRNAs was significantly lower in the hippocampus of male rats than in female rats (Figures 6A, 6E).

CHAPTER 4

Discussion

We first compared the effects of aging and sex on the mRNA expression of five molecules (SOD2, HO1, APP, BACE1, and ASN) in the hippocampus of both female and male F344BN rats. These molecules are closely related to oxidative stress, and to Alzheimer's and Parkinson's diseases. The results demonstrate that aging only significantly increases the expression of HO1 mRNA in the very aged, 30-month-old hippocampus of female F344BN rats (Figure 2).

Scapagnini's research group examined the expression levels of SOD2 and HO1 mRNAs in the hippocampus of male Wistar rats of various ages (6-, 12-, and 28-month-old). They observed a significant increase in the expression of HO1 mRNA, and a significant decrease in the expression of SOD2 mRNA, in the 28-month-old group when compared to their 6- and 12-month-old counterparts[7]. The proteins SOD2 and HO1 have been identified as being capable of protecting the brain from oxidative damage by radicals. Therefore, the reciprocal changes in the expression of HO1 and SOD2 mRNAs reported in Scapagnini's study may explain why the hippocampus is one of the areas of the brain that is most susceptible to oxidative damage. We did not observe statistically significant changes in the expression of either SOD2 or HO1 mRNA in the hippocampus of male rats, possibly because we used F344BN rats rather than Wistar rats.

In addition to hippocampus tissue, we also examined aged-related changes in the expression levels of SOD2 and HO1 mRNAs in the cerebellum of female and male F344BN rats (unpublished data). We did not observe statistically significant age- or sex-related changes in the expression of SOD2 mRNA. Our results demonstrate that a significant age-related increase in HO1 mRNA expression was, once again, only observed in female F344BN rats. We thus conclude that the brains of female F344BN rats may be better protected against oxidative damage than those of male F344BN rats.

A. Clark's research group studied age-associated expression of APP mRNA in the frontal cortex of male F344 rats. They found that the amount of APP mRNA was lower in the 13-month-old group, but not in the 29-month-old group, when each was compared to the 3-month-old control [60]. H. Chao and colleagues monitored age-associated expression of APP mRNA in the hippocampus of female and male Sprague-Dawley rats. They observed an decrease in APP mRNA expression in the hippocampus of female rats with age, but this phenomenon did not occur in the male rats[61]. In our case, the age- and sex-associated expression changes in APP mRNA were not statistically significant in the hippocampus of rat brains of either sex. The use of different strains of rats and/or different regions of the brains from the same type of rats may explain the differences in the observed expression levels of APP mRNA. To begin to test this hypothesis, we also examined aged-related changes in the expression levels of APP mRNA in the cerebellum of female and male F344BN rats (unpublished data). We did not observe statistically significant age-related changes in the expression of APP mRNA in the cerebellum of F344BN rats; however, the overall expression was significantly higher in the cerebellum of male rats than in female rats. Further studies

are needed to explore and interpret the differences in APP mRNA expression in various brain tissues.

To our knowledge, no research has yet addressed potential age-associated changes in the expression of BACE1 mRNA in rat brains during normal aging. Our data demonstrate that there were no significant age-associated changes in the expression of BACE1 mRNA in the hippocampus of either male or female rats, and no significant sex-associated changes in the expression of BACE1 mRNA between opposite-sex, age-matched groups.

S. Mak and coworkers investigated the expression of ASN mRNA in the hippocampus of male C57BL/6 mice of various ages (2-, 10-, and 20-month-old). They observed a significant decrease in the expression of ASN mRNA in the 10- and 20-month-old groups when compared to their 2-month-old counterparts[62]. J. Strosznajder's research group examined the expression of ASN mRNA in the hippocampus of 4- and 24-month-old male Wistar rats; no significant difference in the level of expression was observed[63]. In our study, no age-associated changes in ASN mRNA expression were observed in the hippocampus of either female or male rats; however, the overall expression of ASN mRNA was significantly higher in the hippocampus of male rats than in that of female rats (Figure 5). ASN is known to be involved in several neurodegenerative diseases, including Parkinson's disease, and researchers have reported a significantly higher incidence of Parkinson's disease in men than women. Our finding of sex-associated differences in ASN mRNA expression in the hippocampus of rats may shed light on the causes of the higher rate

of Parkinson's disease in males. Further studies are needed to examine sex-associated changes in ASN protein expression and activity.

Strosznajder's research group not only examined the effect of aging on ASN mRNA expression in the hippocampus of Wistar rats, but also in the cortex, striatum, and cerebellum of the Wistar rat brains. They observed no significant age-associated changes in ASN mRNA expression in the cortex or hippocampus, whereas they found significant age-associated decreases in the striatum and cerebellum[63]. We also examined age- and sex-associated changes in ASN mRNA expression in the cerebellum of female and male F344BN rats (unpublished data). The expression of ASN mRNA in the 33-month-old male rat cerebellum was significantly higher than in both the male control and the age-matched (30-month-old) female counterparts. The overall expression level of ASN mRNA in the cerebellum of both sexes was similar to that in female rat hippocampus, and much lower than in male rat hippocampus.

Researchers have reported that aging is associated with increases in iron accumulation. Therefore, we also examined the mRNA expression levels of five molecules (FTH, FTL, TfR, DMT1, and IRP1) related to iron transport and storage in the hippocampus of F344BN rat brains. As shown in Figure 6, we observed no significant age-associated changes in the expression of any of these 5 mRNAs, and no significant sex-associated changes in the expression of FTL, TfR, and DMT1 mRNAs. However, the overall expression of FTH and IRP1 mRNAs was significantly lower in the hippocampus of male rats than in female rats. It is well known that excess free iron catalyzes the formation of free radicals that

are toxic to neuronal cells. FTH is a major intracellular iron storage protein, and its expression is post-transcriptionally regulated by IRP1 via binding to the IRE in the 5' untranslated region of the FTH mRNA. When the intracellular free iron content is low, IRP1 binds to FTH IRE, thus inhibiting ribosome binding and the corresponding FTH mRNA translation. When the iron concentration is high, IRP1-IRE binding is inhibited, which allows the synthesis of additional FTH protein for iron storage [56, 64]. Additional research is necessary to determine whether the sex-associated differences in the expression of FTH and IRP1 mRNAs are related to a different rate of aging, and whether these differences have an effect on the incidence of neurodegenerative diseases in males and females.

Historically, animal-based studies are more commonly conducted using male, rather than female animals. Given that female subjects actually live longer and are more likely to experience age-associated diseases, more attention should focus on female subjects and on comparing sex-associated differences.

Acknowledgments

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Figure legends

Figure 1. The expression of HO1 mRNA in the hippocampus of female and male rats of various ages (F-6 refers to 6-month-old female rats; M-6 refers to 6-month-old male rats, etc.). N=6, and all values are expressed as the mean ± SEM. * indicates a significant difference from the 6-month-old control group of same sex ($p < 0.05$). # indicates a significant difference from the age-matched group of the opposite sex ($p < 0.05$). $ indicates a significant difference from the 26-month-old group of same sex ($p < 0.05$).

Figure 2. The expression of APP mRNA in the hippocampus of female and male rats of various ages (F-6 refers to 6-month-old female rats; M-6 refers to 6-month-old male rats, etc.). N=6, and all values are expressed as the mean ± SEM.

Figure 3. The expression of SOD2 mRNA in the hippocampus of female and male rats of various ages (F-6 refers to 6-month-old female rats; M-6 refers to 6-month-old male rats, etc.). N=6, and all values are expressed as the mean ± SEM.

Figure 4. The expression of BACE1 mRNA in the hippocampus of female and male rats of various ages (F-6 refers to 6-month-old female rats; M-6 refers to 6-month-old male rats, etc.). N=6, and all values are expressed as the mean ± SEM.

Figure 5. The expression of ASN mRNA in the hippocampus of female and male rats of various ages (F-6 refers to 6-month-old female rats; M-6 refers to 6-month-old male rats,

etc.). N=6, and all values are expressed as the mean ± SEM. # indicates a significant difference from the age-matched group of the opposite sex ($p < 0.05$).

Figure 6. The effect of aging on the expression levels of several iron transport and storage-related mRNAs in the hippocampus of female and male rats (F-6 refers to 6-month-old female rats; M-6 refers to 6-month-old male rats, etc.). (A) FTH mRNA, (B) FTL mRNA, (C) TfR mRNA, (D) DMT1 mRNA, and (E) IRP1 mRNA. N=6, and all values are expressed as the mean ± SEM. # indicates a significant difference from the age-matched group of the opposite sex ($p < 0.05$).

Figure 1: HO-1 mRNA expression in hippocampus of F344 BN Rat brain

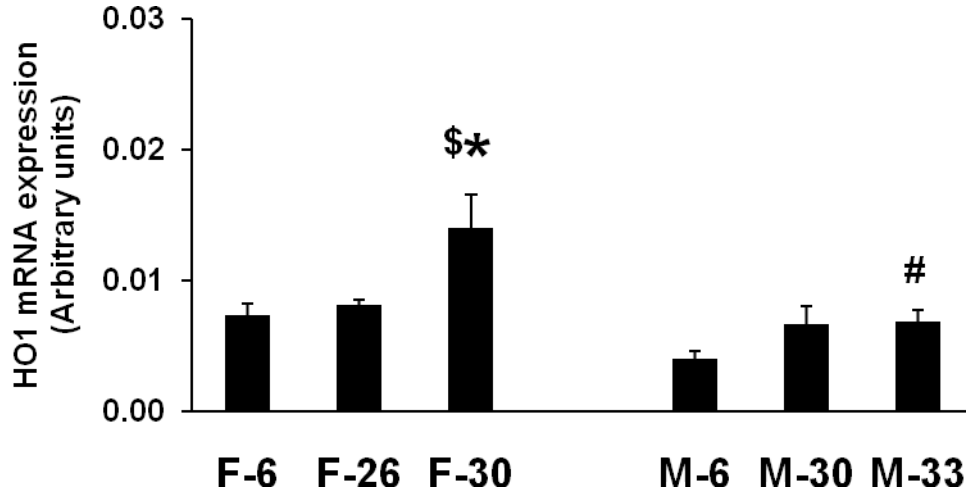


Figure 2: APP mRNA expression in hippocampus of F344 BN Rat brain

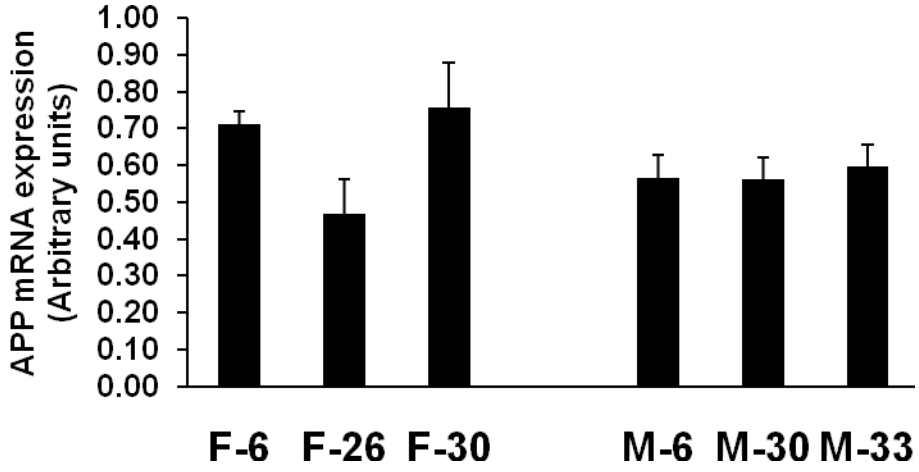


Figure 3: SOD-2 mRNA expression in hippocampus of F344 BN Rat brain

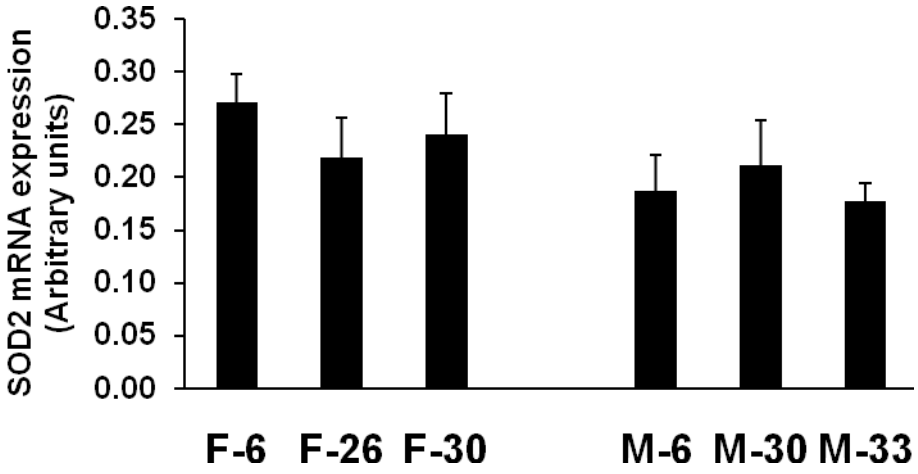


Figure 4: BACE1 mRNA expression in hippocampus of F344 BN Rat brain

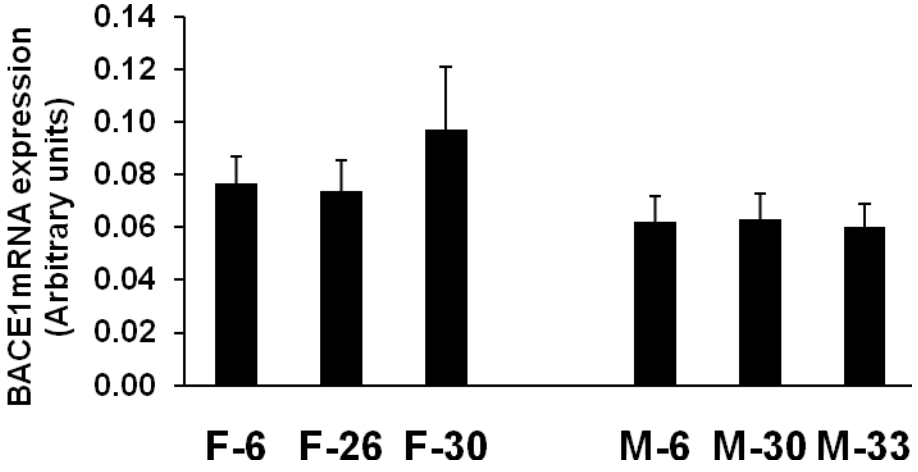


Figure 5 : ASN mRNA expression in hippocampus of F344 BN Rat brain

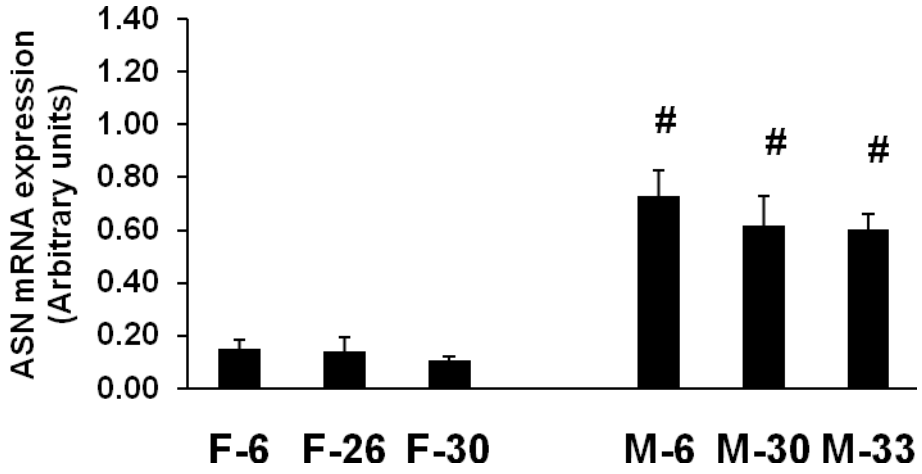


Figure 6A: FTH mRNA expression in hippocampus of F344 BN Rat brain

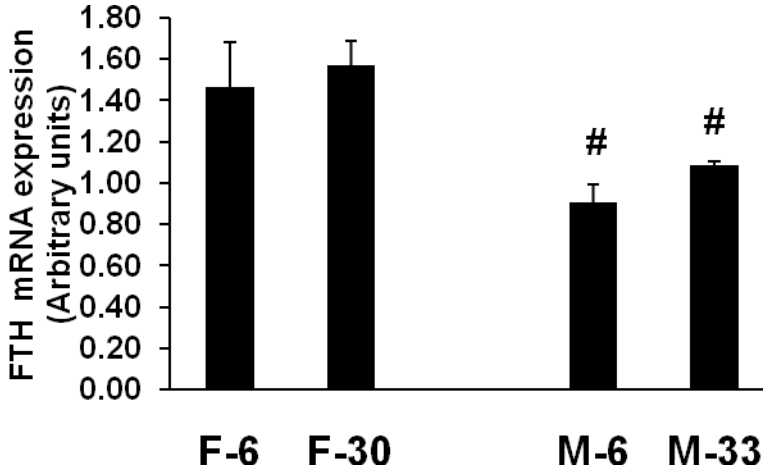


Figure 6B: FTL mRNA expression in hippocampus of F344 BN Rat brain

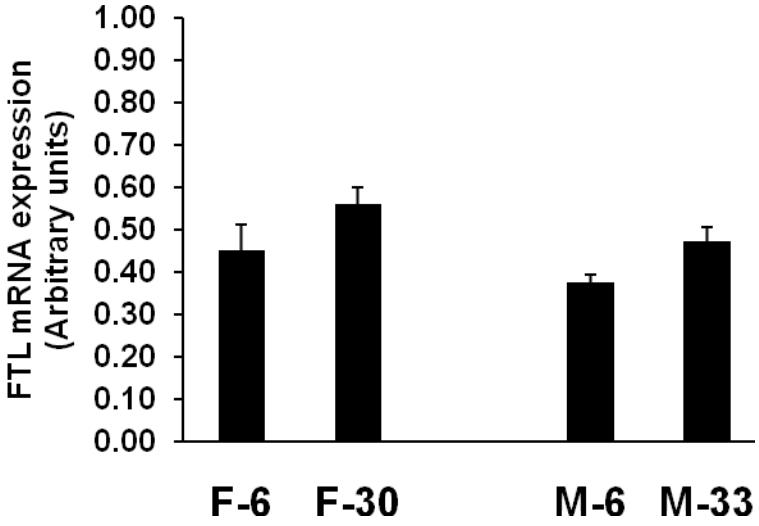


Figure 6C: TfR mRNA expression in hippocampus of F344 BN Rat brain

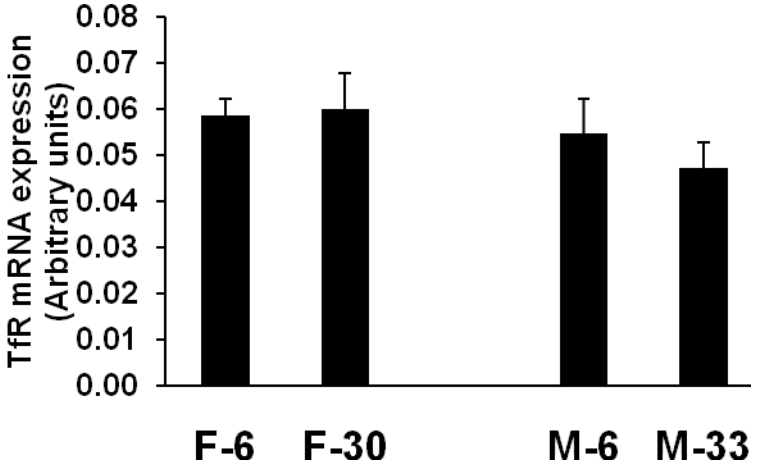


Figure 6D: DMT1 mRNA expression in hippocampus of F344 BN Rat brain

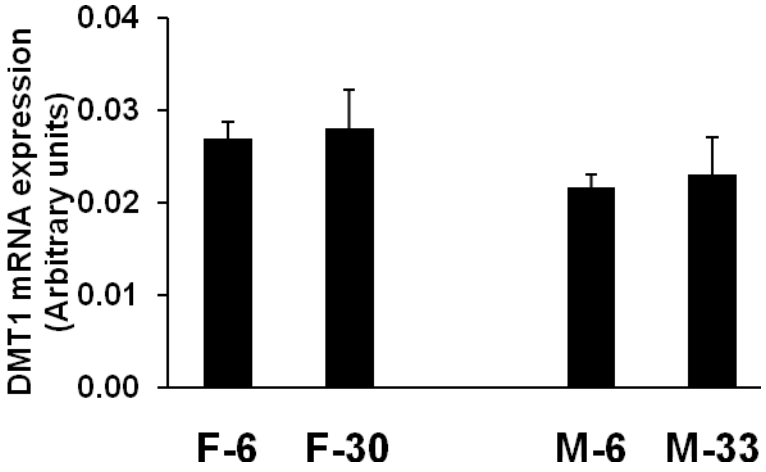
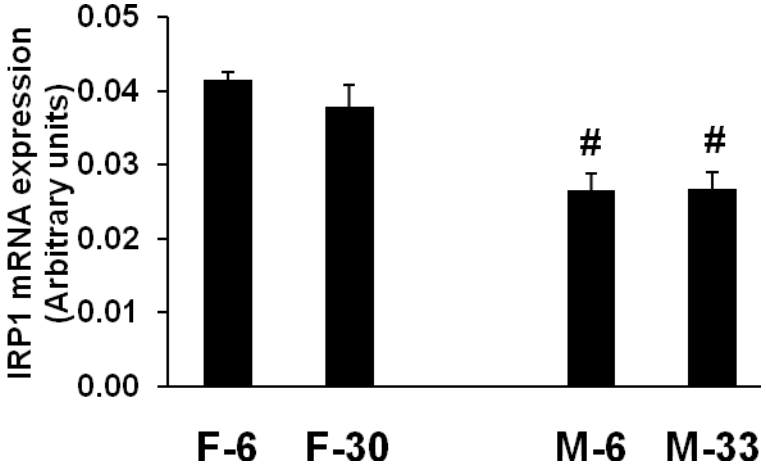


Figure 6E: IRP1 mRNA expression in hippocampus of F344 BN Rat brain



PART-II

Age- and Sex-associated Changes in the Expression of Neurodegenerative Disorder-related Molecules in the Cerebellum of Rat Brains

Abstract

Cerebellum tissue from both male and female F344BN rats (adult, aged, and very aged) was used to explore the effects of aging and sex on the expression of both the mRNA and associated protein of 5 molecules that are closely related to oxidative stress, and to Alzheimer's and Parkinson's diseases. The reverse transcription polymerase chain reaction and Western blot techniques were used to determine the mRNA and protein expression levels, respectively, of superoxide dismutase 2 (SOD2), heme oxygenase 1 (HO1), amyloid precursor protein (APP), β-site APP-cleaving enzyme 1 (BACE1), and α-synuclein (ASN). Results demonstrated 1) the expression of SOD2 protein in the very aged female cerebellum was significantly higher than in both the adult female control and the aged females; 2) the expression of SOD2 protein was significantly lower in the aged and very aged cerebellum of male rats when compared to the age-matched female groups; 3) the expression of HO1 mRNA in the aged and very aged female cerebellum was significantly higher than in the adult female control; 4) the expression of HO1 mRNA in the aged females was significantly higher than in the age-matched male counterparts, although the overall expression of HO1 mRNA in the cerebellum of both sexes was very low; 5) the expression of APP mRNA was significantly higher in the male rat cerebellum than in each group of age-matched female rats; 6) the expression of APP protein in the very aged female rat cerebellum was significantly higher than in the adult female control; 7) the expression of ASN mRNA in the very aged male rat cerebellum was significantly higher than in both the adult male control and the age-matched female counterparts; 8) the expression of ASN protein in the very aged female cerebellum was significantly higher than in both the adult female control and the aged females; and 9) the expression of ASN protein was significantly higher in the adult and aged

male rat cerebellum when compared to the respective age-matched female groups. We thus conclude that the female rat cerebellum may be better protected against age-associated oxidative damage than those of male F344BN rats. Sex-associated differences in the expression of ASN may relate to a different rate of aging, and may shed light on the causes of the higher rate of Parkinson's disease in males.

CHAPTER 1

Introduction

The increasing world population of people aged 65 and older has resulted in an increased incidence of age-associated neurodegenerative diseases such as Alzheimer's and Parkinson's diseases [1, 22, 39, 65], whose neurotoxicity has long been linked to an accumulation of oxidative stress[5, 66]. As a consequence, there has been an increase in research focused on the mechanism(s) of brain aging and age-related diseases, as well as investigations of whether different areas of the brain are affected differently by aging[2, 3, 7]. Studies have found that the human cortex undergoes more extensive age-associated changes than the cerebellum; this information led researchers to suggest that the difference in aging rates between brain regions may correspond to differences in function[67]. The more active a brain area is, the more free radicals form there. For example, the cortex is in charge of higher-level activities such as thought, and thus may experience more oxidative stress; whereas the cerebellum regulates basic processes such as balance, and thus may experience less oxidative stress. Historically, the cerebellum is a relatively neglected area in aging research; therefore, the investigation of age-associated changes in gene expression in the cerebellum may provide important information for the following reasons: 1) disturbances in body movement and cognitive function are among the most important health problems in the elderly, 2) the cerebellum is responsible for the coordination of muscle and motor skills, and 3) the cerebellum has recently been found to play a role in cognition and behavior[68].

In this study, we determined the level of expression of both the mRNA and associated protein for five molecules that are closely related to oxidative stress, and to Alzheimer's and Parkinson's diseases. Manganese superoxide dismutase (SOD2) is an antioxidant enzyme that catalyzes the conversion of superoxide anions to hydrogen peroxide and molecular oxygen [9, 69, 70]. Heme oxygenase 1 (HO1) is another important antioxidant enzyme, which catalyzes the rate-limiting step of the enzymatic degradation of heme in the brain and other tissues [12, 14, 17, 18]. Amyloid precursor protein (APP) is a precursor molecule to amyloid β (Aβ) peptides, a major component of the plaques found in Alzheimer's disease patients [23, 27]. Beta-site APP-cleaving enzyme 1 (BACE1) drives the rate-limiting step of APP cleavage to form Aβ [25, 29, 31, 33, 71]. Alpha-synuclein (ASN) is a precursor molecule to the non-Aβ component of plaques found in Alzheimer's disease patients, and is a main component of Lewy bodies, the abnormal protein clusters found in the brains of Parkinson's patients [38, 40, 42-45].

To evaluate the expression of these five neurodegenerative disorder-related molecules in cerebellum tissue, we chose to use a normal rat model system specific for aging rather than a transgenic model. Our reasons for using non-transgenic rodents include that previous studies addressing the expression of the molecules of interest have been focused on human postmortem brain tissues, human neuronal cell lines, and/or transgenic animal models of neurodegenerative diseases. Our aim for this study was to examine whether any expression changes occur during normal aging, and whether any expression differences exist between age-matched, opposite-sex animals. Specifically, as mentioned above, exploring gene expression in the cerebellum may provide important information toward understanding the

normal aging process, given that the cerebellum is not only involved in body movement, but also in cognition and behavior, both of which are major health issues encountered by the aged. Our results reveal some significant expression changes associated with aging and/or sex for some of the five molecules investigated from the cerebellum of F344BN rats.

CHAPTER 2

Experimental Methods

2.1. Animals

Animal care and use were performed in accordance with the Guide for the Care and Use of Laboratory Animals approved by the council of the American Physiological Society and by the Animal Use Review Board of Marshall University. Experimental procedures were conducted in strict accordance with the Public Health Service animal welfare policy. Male Fischer 344/NNiaHSd X Brown Norway/BiNia (F344BN) rats of 3 ages (27, 30, and 33 months old; 6 rats in each group), and female F344BN rats of 3 ages (20, 26, and 30 months old; 6 rats in each group) were obtained from the National Institute of Aging (Bethesda, MD). The rats were housed two per cage in a vivarium approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Housing conditions included a 12-hour light: 12-hour dark cycle, with the temperature maintained at 22 ± 2 °C. The rats were provided with food and water *ad libitum*, and allowed to recover from shipment for at least two weeks before experimentation began. During this recovery period, the rats were carefully observed and weighed weekly. None of the rats exhibited signs of failure to thrive such as precipitous weight loss, disinterest in the environment, and/or unexpected gait alterations.

2.2. Tissue Isolation

The rats were anesthetized by an intraperitoneal injection of a ketamine-xylazine (4:1) cocktail administered at 50 mg/kg, and supplemented as necessary for reflexive response. The brains were removed from the skull and rinsed in Krebs solution to remove the surface blood, after which they were dissected[57]. The cerebellum was quickly separated from the whole brain, blotted dry, and immediately frozen in liquid nitrogen and stored at -80 °C until further use [57].

2.3. RNA extraction and cDNA synthesis

The cerebellum of each male and female rat from each age group was homogenized, individually, on ice in a TRI reagent (Ambion, Austin, TX) at a ratio of 1 mL of TRI reagent per 100 mg of tissue. The homogenate was incubated for 5 minutes on ice to allow nucleoprotein complexes to completely dissociate, after which it was centrifuged at 4 °C for approximately 15 minutes at 12,000 x g. The supernatant was separated from the pellet and transferred to a 2 mL RNAse-free tube. A 200-µl aliquot of chloroform (Sigma-Aldrich, Inc., St. Louis, MO) was added to the supernatant. The solution was mixed and incubated at room temperature for 10 minutes, followed by centrifugation at 4 °C for 15 minutes at 12,000 x g. The aqueous phase of the solution was collected and transferred to another tube. A 500-µl aliquot of isopropanol (Sigma-Aldrich, Inc., St. Louis, MO) was added to the aqueous phase, followed by thorough mixing. The resultant solution was incubated at room

temperature for 10 minutes, followed by centrifugation at 4 °C for 10 minutes at 12,000 x g. The supernatant was then carefully removed without disturbing the pellet. The RNA pellet was washed free of salts by centrifugation in 75% ethanol at 7500 x g for 5 minutes. The ethanol wash was carefully removed without disturbing the pellet, and the residual ethanol was evaporated at room temperature by speed vacuuming for 1 minute. The RNA pellet was dissolved in 50 µl of 1xTE buffer (10 mM Tris at pH 8.0, 1 mM EDTA) and quantified at 260 nm using a NanoVue UV-Vis Spectrophotometer (GE Healthcare, Piscataway, NJ). The integrity of the RNA from each sample was determined through use of a RNA 6000 Nano Kit and an Agilent 2100 Bioanalyzer (both from Agilent Technologies, Santa Clara, CA), and subjected to reverse transcription in order to generate complementary DNA (cDNA) by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA).

2.4. Real-time polymerase chain reaction (RT-PCR)

The mRNA expression levels of SOD2, HO1, APP, BACE1, and ASN in each cerebellum from each age group were determined by RT-PCR using the ABI 7000 Real-Time PCR system (Applied Biosystems Inc., Foster City, CA). The reactions were carried out in a 96-well optical reaction plate (Applied Biosystems Inc., Foster City, CA). Each 25-µl reaction was comprised of 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA), 0.5 µl of 10 µM forward primers, 0.5 µl of 10 µM reverse primer, 2 µl of cDNA, and 9.5 µl of nuclease-free water. An optical adhesive cover (Applied Biosystems Inc., Foster City, CA) was placed over the plate before it was spun at 300 rpm for

30 seconds. Cycling conditions for RT-PCR were as follows: 1 cycle of 2 minutes at 50 °C and 10 minutes at 95 °C; 40 cycles of 15 seconds at 95 °C; and a final 1 minute cycle at 60 °C. SYBR Green dye intensity for each RT-PCR reaction was analyzed using ABI prism 7000 SDS software (Applied Biosystems Inc., Foster City, CA), and the corresponding threshold cycle (Ct) value was determined by the relative quantification plate method.[58] All experiments were repeated three times, and β-actin was used as an internal control to offset the efficiency differences in RT-PCR. The expression of SOD2, HO1, BACE1, APP, ASN, FTH, FTL, TfR, DMT1, and IRP1 mRNAs were normalized to β-actin mRNA, and the $2^{-\Delta Ct}$ method[58] was used to calculate the relative mRNA expression level. Primers used in this study were purchased from Integrated DNA technologies (San Diego, CA) with the sequences shown in table2.

Table 2. This table shows the list of Primers that we used in this study.

Title	**Primers used in this study**
SOD2 forward primer	5'-GGCCAAGGGAGATGTTACAA-3'
SOD2 reverse primer	5'-GCTTGATAGCCTCCAGCAAC-3'
HO1 forward primer	5'-TGCTCGCATGAACACTCTG-3'
HO1 reverse primer	5'-TCCTCTGTCAGCAGTGCCT-3'
APP forward primer	5'-GGCCCGAAAAACTCCTACTT-3'
APP reverse primer	5'-ATCGCAAAGAGAGCAGAAGC-3'
BACE1 forward primer	5'-GCTGCAGTCAAGTCCATCAA-3'
BACE1 reverse primer	5'-ATTGCTGAGGAAGGATGGTG-3'

ASN forward primer	5’-AGGGAGTCGTTCATGGAGTG-3’
ASN reverse primer	5’-CCCTCCACTGTCTTCTGAGC-3’
β-actin forward primer	5’-CAACCTTCTTGCAGCTCCTC-3’
β-actin reverse primer	5’-TCTGACCCATACCCACCATC-3’

2.5. Immunoblotting

Primary antibodies against SOD2, HO1, BACE1, and ASN were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Primary antibodies against APP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary antibody, and NIH-3T3 control cell extracts were obtained from Cell Signaling Technology (Danvers, MA). Precast 10% and 15% SDS-PAGE gels were purchased from Lonza Rockland, Inc. (Rockland, ME), while the Enhanced Chemiluminescence (ECL) Western Blot Detection Reagents, Hyperfilm, and Hybond nitrocellulose membranes were obtained from Amersham Biosciences (Piscataway, NJ). Tissue protein extraction reagent (TPER) was obtained from Pierce (Rockford, IL). Dual Color Molecular Weight Markers were purchased from Bio-Rad (Hercules, CA).

The cerebellum of each rat from each age group was homogenized individually, on ice, twice for 30 seconds in TPER (1 mL/100 mg tissue) supplemented with protease and phosphatase inhibitors (both from Sigma-Aldrich, Inc., St. Louis, MO). The homogenized sample was sonicated for approximately 90 seconds, followed by a 10-minute incubation on

ice. The sample was then centrifuged at 4 °C for 15 minutes at 10,000 x g. The supernatant was separated from the pellet and stored at -80 °C until further use.

The protein concentration in the supernatant was determined in duplicate using the Pierce 660 nm protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard. The sample was then diluted to 2.0 µg/µl in a SDS-loading buffer, and boiled for 5 minutes. A 20-µl aliquot (i.e., approximately 40 µg of protein) of this solution was loaded on to a 10% SDS-PAGE gel. Cerebellum samples from different age groups of female and male rats were run on the same gel to allow direct comparison.

After the gels were run, the protein was transferred onto nitrocellulose membranes as previously described by Towbin *et al*.[72] After transfer, in order to block non-specific binding, the membranes were incubated at room temperature for 1 hour in Tris-buffered saline with 0.05% Tween 20 (TBS-T) that also contained 5% milk. The membranes were then washed with TBS-T and incubated with the appropriate primary antibody overnight at 4°C. After incubation, the membranes were exposed to HRP-conjugated anti-rabbit IgG secondary antibody at room temperature for 1 hour. Protein bands were visualized with ECL (Amersham Biosciences, Piscataway, NJ), and the exposure time was adjusted to keep the integrated optical densities (IODs) within a linear and non-saturated range. Band signal intensity was quantified by densitometry using Imaging software (Alpha Ease FC) and normalized to GAPDH to verify equal protein transfer to membranes. Molecular weight markers (Bio-Rad, Hercules, CA) were used as molecular mass standards, and NIH-3T3 cell lysates (Cell Signaling Technology, Danvers, MA) were included as positive controls.

2.5. Data analysis

Results are presented as the mean ± SEM (standard error of the mean). The SigmaStat 3.5 statistical program (SPSS Science Inc., Chicago, IL) was used to analyze both the mRNA and protein data; a two-way analysis of variance (ANOVA) was performed to determine any differences between expression levels from cerebellum tissues within same sex groups and between age-matched groups of the opposite sex. The significance level was set as $p < 0.05$ for each comparison.

CHAPTER 3

Results

Figures 7-11 illustrate the levels of mRNA and protein expression of SOD2, HO1, APP, BACE1, and ASN in the cerebellum of female and male F344BN rats of various ages. Based on a rat survivability curve obtained from the National Institutes of Health [59] , aged, 26-month-old female F344BN rats age-match 30-month-old male rats, and very aged, 30-month-old female F344BN rats age-match 33-month-old male rats. Adult, 20-month-old female rats age-match 27-month-old male rats, and were used as the controls in this study.

3.1. Neither Age nor Sex Affects the Expression of SOD2 mRNA in the Cerebellum of F344BN Rats

No significant age-related changes were observed in the expression level of SOD2 mRNA in the cerebellum of either female or male rat brains when compared to the sex-matched control group. In addition, no significant sex-related changes were observed in the expression level of SOD2 mRNA when compared to the age-matched group of the opposite sex (Figure 7A).

3.2. Aging Increases the Expression of SOD2 Protein in the Cerebellum of Female F344BN Rats

Figure 7B indicates that the expression of SOD2 protein in the 30-month-old female rat cerebellum was significantly higher than in both the 20-month-old female control and the 26-month-old females. No significant age-related changes were observed in the expression levels of SOD2 protein in the cerebellum of either 30- or 33-month-old male rat brains when compared to the 27-month-old sex-matched control group.

3.4. With Aging, Sex Affects the Expression of SOD2 Protein in the Cerebellum of F344BN Rats

No significant sex-related change was observed in the expression level of SOD2 protein in the cerebellum of adult (27-month-old) male rat brains when compared to the 20-month-old age-matched female group (Figure 7B). However, Figure 7B also illustrates that the expression of SOD2 protein was significantly lower in the aged (30-month-old) and very aged (33-month-old) cerebellum of male rats when compared to the 26- and 30-month-old age-matched female groups, respectively.

3.5. Aging Increases the Expression of HO1 mRNA in the Cerebellum of Female F344BN Rats

Figure 8A indicates that the expression of HO1 mRNA in the 26- and 30-month-old female rat cerebellum was significantly higher than in the 20-month-old female control. In

addition, the expression of HO1 mRNA in the 26-month-old females was significantly higher than in the age-matched (30-month-old) male counterparts, although the overall expression of HO1 mRNA in the cerebellum of both sexes was very low. No significant age-related changes were observed in the expression levels of HO1 mRNA in the cerebellum of either 30- or 33-month-old male rat brains when compared to the 27-month-old male control group.

3.6. Neither Age nor Sex Affects the Expression of HO1 Protein in the Cerebellum of F344BN Rats

Figure 8B illustrates that neither age nor sex affects the expression of HO1 protein significantly. The overall expression of HO1 protein was very low in the cerebellum of F344BN rats of both sexes.

3.7. Sex Affects the Expression of APP mRNA in the Cerebellum of F344BN Rats

Compared to sex-matched control groups, no significant age-associated changes were observed in the expression of APP mRNA in the cerebellum of either female or male rats (Figure 9A). However, the overall expression of APP mRNA was significantly higher in the cerebellum of male rats than in each group of age-matched female rats (Figure 9A).

3.8. Aging Increases the Expression of APP Protein in the Cerebellum of Female F344BN Rats

Figure 9B indicates that the expression of APP protein in the 30-month-old female rat cerebellum was significantly higher than in the 20-month-old female control. No significant age-related changes were observed in the expression levels of APP protein in the cerebellum of either 30- or 33-month-old male rat brains when compared to the 27-month-old sex-matched control group.

3.9. Neither Age nor Sex Affects the Expression of BACE1 mRNA or Its Protein in the Cerebellum of F344BN Rats

No significant age-related changes were observed in the expression levels of BACE1 mRNA or its protein in the cerebellum of either female or male rat brains when compared to the sex-matched control group. In addition, no significant sex-related changes were observed in the expression levels of BACE1 mRNA or its protein when compared to the aged-matched group of the opposite sex. The overall expression of both BACE1 mRNA and its protein was low in the cerebellum of both sexes (Figure 10A, B).

3.10. Aging Increases the Expression of ASN mRNA in the Cerebellum of Male F344BN Rats

Figure 11A illustrates that the expression of ASN mRNA in the 33-month-old male rat cerebellum was significantly higher than in both the 27-month-old male control and the age-matched (30-month-old) female counterparts. No significant age-associated changes were observed in the expression of ASN mRNA in the cerebellum of female rats (Figure 11A).

3.11. Aging Increases the Expression of ASN Protein in the Cerebellum of Female F344BN Rats

Figure 11B indicates that the expression of ASN protein in the 30-month-old female rat cerebellum was significantly higher than in both the 20-month-old female control and the 26-month-old females. No significant age-related changes were observed in the expression levels of ASN protein in the cerebellum of either 30- or 33-month-old male rat brains when compared to the 27-month-old sex-matched control group (Figure 11B).

3.12. Sex Affects the Expression of ASN Protein in the Cerebellum of F344BN Rats

Figure 11B further indicates that the expression of ASN protein was significantly higher in the adult (27-month-old) and aged (30-month-old) cerebellum of male rats when compared to the 20- and 26-month-old age-matched female groups, respectively. No

significant sex-related change was observed in the expression level of ASN protein in the cerebellum of very aged (33-month-old) male rat brains when compared the 30-month-old age-matched female group (Figure 11B).

CHAPTER 4

Discussion

The purpose of this study was to determine the existence of potential age- or sex-related expression changes in SOD2, HO1, APP, BACE1, or ASN mRNAs or their correlated proteins in the cerebellum of female and male F344BN rats. SOD2 and HO1 are both proteins that act as antioxidants, and their expression levels were expected to increase with age in order to counteract the associated increase in oxidative stress. Although no statistically significant changes were observed in the expression of SOD2 mRNA with aging, we found an age-associated increase in the expression of HO1 mRNA in the cerebellum of female rats (Figures 7A, 8A). G. Scapagnini's research group examined the expression levels of SOD2 and HO1 mRNA in the cerebellum of male Wistar rats of various ages (6-, 12-, and 28-month-old). They observed a significant increase in the expression of both SOD2 and HO1 mRNA in the 28-month-old group when compared to their 6- and 12-month-old counterparts[7]. We did not observe statistically significant changes in the expression of either SOD2 or HO1 mRNA in the cerebellum of male rats, possibly because we used F344BN rats rather than Wistar rats or because we used different age groups (27-, 30-, and 33-month-old).

As expected, the expression of SOD2 protein in the very aged, 30-month-old female rat cerebellum was significantly higher than in both the 20-month-old female control and the 26-month-old females (Figure 7B). However, this age-associated increase was not observed in the male rat cerebellum. Moreover, the expression of SOD2 protein was

significantly lower in the aged (30-month-old) and very aged (33-month-old) cerebellum of male rats when compared to the 26- and 30-month-old age-matched female groups, respectively (Figure 7B). We thus conclude that the brains of female F344BN rats may be better protected against age-associated oxidative damage than those of male F344BN rats.

Our data demonstrate that the overall expression of HO1 protein in the cerebellum of rats of both sexes was lower than the expression of SOD2 protein (see Western blot images in Figures 7B and 8B). In contrast with changes that occurred in SOD2 protein levels, there were no significant age- or sex-related changes in HO1 protein expression in the rat cerebellum. Although we did not find any published research concerning age- or sex-related changes in the expression of HO1 protein in rodent brains, M. Bergeron and coworkers investigated developmental changes, rather than age-associated changes, in the expression of HO1 protein in the cerebral cortex and hippocampus of Sprague-Dawley rats at 7, 14, and 21 days after birth, and adulthood. They observed a sustained reduction in HO1 expression during brain development [73].

We observed no statistically significant age-related changes in the expression of APP mRNA in the cerebellum of rats of either sex; however, the overall expression was significantly higher in the cerebellum of male rats than in female rats (Figure 9A). We further examined the expression of APP mRNA in the hippocampus of female and male F344BN rats; no significant age- or sex-related changes were observed (unpublished data). A.Clark's research group studied age-associated expression of APP mRNA in the frontal cortex of male F344 rats. They found that the amount of APP mRNA was lower in the 13-month-old group,

but not in the 29-month-old group, when each was compared to the 3-month-old control [60]. H.Chao and colleagues monitored age-associated expression of APP mRNA in the hippocampus of female and male Sprague-Dawley rats. They observed a decrease in APP mRNA expression in the hippocampus of female rats with age, but this phenomenon did not occur in the male rats[61]. The use of different strains of rats and/or different regions of the brain from the same type of rats may explain the differences in the observed expression levels of APP mRNA. Further studies are needed to explore and interpret the differences in APP mRNA expression in various brain tissues.

APP is the main precursor molecule in the formation of Aβ plaques, which amass with the increased oxidative stress associated with aging. Because the overall expression of APP mRNA in the male cerebellum was significantly higher than in female rats, we anticipated a corresponding elevated protein expression in the male cerebellum relative to those of females. However, our data showed no significant sex-associated changes in the expression of APP protein. An age-associated increase was observed in the 30-month-old female cerebellum when compared the 20-month-old female control, but this pattern was not observed in the male cerebellum (Figure 9B). To our knowledge, no research has yet addressed potential age-associated changes in the expression of APP protein in the rodent cerebellum. Jeong and coworkers explored age-related changes in the expression of APP protein in the hippocampus of senescence-accelerated prone mouse 10 (SAMP10) and senescence-accelerated resistant mouse 1(SAMR1).They observed an age-associated increase in the expression of APP protein in the SAMP10 hippocampus, while also finding an age-associated decrease in the expression of APP protein in the SAMR1 hippocampus [74].

We observed no significant age-or sex-related changes in the expression levels of BACE1 mRNA or protein in the cerebellum of F344BN rats. The overall expression of both BACE1 mRNA and its protein was low in the cerebellum of rats of both sexes (Figure 10). V. Bigl's research group compared the levels of BACE1 protein in brain homogenates of N2-generation mice of two ages (6- and 16-month-old); they also did not detect any age-associated changes [75].

Figure 11A shows that the expression of ASN mRNA in the very aged, 33-month-old male rat cerebellum was significantly higher than in both the adult (27-month-old) male control and the age-matched (30-month-old) female counterparts. We also examined age- and sex-associated changes in ASN mRNA expression in the hippocampus of female and male F344BN rats (unpublished data). No significant age-associated changes were observed in the expression of ASN mRNA in the hippocampus of either female or male rats. However, the overall expression of ASN mRNA was significantly higher in the hippocampus of male rats than in female rats. J. Strosznajder's research group examined the effect of aging on ASN mRNA expression in the hippocampus, cortex, striatum, and cerebellum of the male Wistar rats of two ages (4- and 24-month-old). They observed no significant age-associated changes in ASN mRNA expression in the cortex or hippocampus, whereas they found significant age-associated decreases in the striatum and cerebellum [63]. These contradictory results may be due to the usage of different strain of rats and/or different age groups.

Figure 11B indicates that the expression of ASN protein in the 30-month-old female rat cerebellum was significantly higher than in both the 20-month-old female control and the 26-month-old females; in addition, the expression of ASN protein was significantly higher in the cerebellum of adult (27-month-old) and aged (30-month-old) male rats when compared to the 20- and 26-month-old age-matched female groups. ASN is known to be involved in several neurodegenerative diseases, especially Parkinson's disease, and researchers have reported a significantly higher incidence of Parkinson's disease in men than women. Our finding of sex-associated differences in ASN mRNA expression may shed light on the causes of the higher rate of Parkinson's disease in males.

Other researchers have investigated the expression of ASN protein in different brain regions using different animal models. Strosznajder's research group measured the levels of ASN protein in the brain cortex of male Wistar rats of two ages (4- and 24-month-old). They found that the immunoactivity of ASN in synaptic plasma membranes from the brain cortex of 24-month-old male rats was 39% lower than in the 4-month-old control group [63]. S. Mak and coworkers observed age-dependent decreases of ASN protein in the substantia nigra and hippocampus of male C57BL/6 mice[62]. Additional research is necessary to uncover the significance of these various results, and to determine whether the sex-associated differences in the expression of ASN are related to a different rate of aging, and whether these differences have an effect on the incidence of neurodegenerative diseases in males and females.

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FIGURE LEGENDS

Figure 7. The expression of (A) SOD2 mRNA, and (B) SOD2 protein in the cerebellum of female and male rats of various ages (F-20 refers to 20-month-old female rats; M-27 refers to 27-month-old male rats, etc.). N=6, and all values are expressed as the mean ± SEM. * indicates a significant difference from the 20-month-old control group of the same sex ($p < 0.05$). $ indicates a significant difference from the 26-month-old group of the same sex ($p < 0.05$). # indicates a significant difference from the age-matched group of the opposite sex ($p < 0.05$). One set of Western blot images for SOD2 protein from female and male cerebellum samples are shown below the graph. GAPDH was used as an internal control.

Figure 8. The expression of (A) HO1 mRNA, and (B) HO1 protein in the cerebellum of female and male rats of various ages (F-20 refers to 20-month-old female rats; M-27 refers to 27-month-old male rats, etc.). N=6, and all values are expressed as the mean ± SEM. * indicates a significant difference from the 20-month-old control group of the same sex ($p < 0.05$). # indicates a significant difference from the age-matched group of the opposite sex ($p < 0.05$). One set of Western blot images for HO1 protein from female and male cerebellum samples are shown below the graph. GAPDH was used as an internal control.

Figure 9. The expression of (A) APP mRNA, and (B) APP protein in the cerebellum of female and male rats of various ages (F-20 refers to 20-month-old female rats; M-27 refers to 27-month-old male rats, etc.). N=6, and all values are expressed as the mean ± SEM. * indicates a significant difference from the 20-month-old control group of the same sex ($p <$

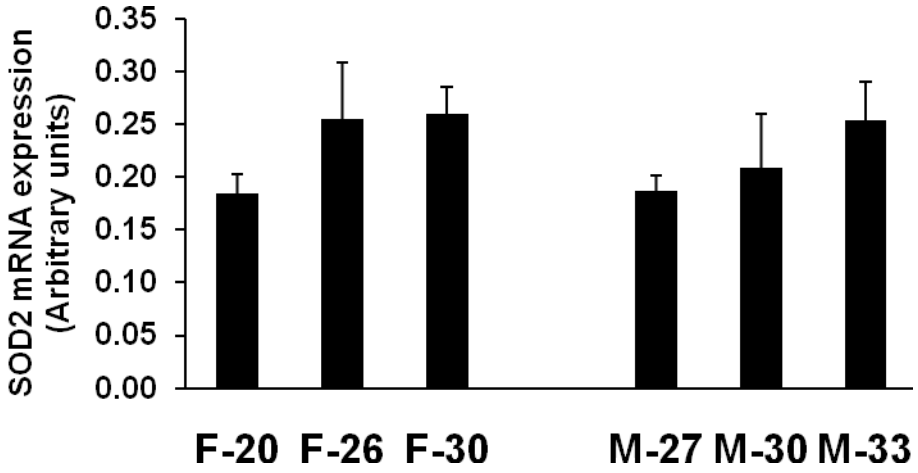
0.05). # indicates a significant difference from the age-matched group of the opposite sex ($p < 0.05$). One set of Western blot images for APP protein from female and male cerebellum samples are shown below the graph. GAPDH was used as an internal control.

Figure 10. The expression of (A) BACE1 mRNA, and (B) BACE1 protein in the cerebellum of female and male rats of various ages (F-20 refers to 20-month-old female rats; M-27 refers to 27-month-old male rats, etc.). N=6, and all values are expressed as the mean ± SEM. One set of Western blot images for BACE1 protein from female and male cerebellum samples are shown below the graph. GAPDH was used as an internal control.

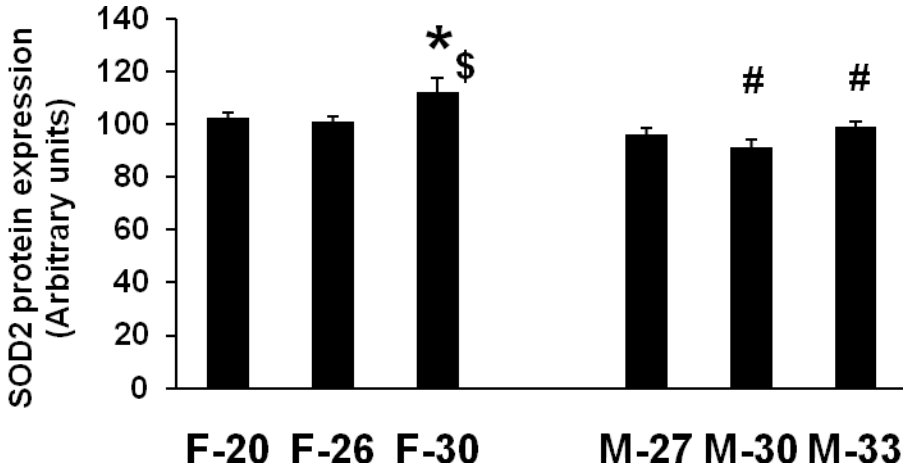
Figure 11. The expression of (A) ASN mRNA, and (B) ASN protein in the cerebellum of female and male rats of various ages (F-20 refers to 20-month-old female rats; M-27 refers to 27-month-old male rats, etc.). N=6, and all values are expressed as the mean ± SEM. * indicates a significant difference from the 20-month-old control group of the same sex ($p < 0.05$). $ indicates a significant difference from the 26-month-old group of the same sex ($p < 0.05$). # indicates a significant difference from the age-matched group of the opposite sex ($p < 0.05$). One set of Western blot images for ASN protein from female and male cerebellum samples are shown below the graph. GAPDH was used as an internal control.

Figure 7

A: SOD-2 mRNA expression in cerebellum of F344 BN Rat brain



B: SOD-2 protein expression in cerebellum of F344 BN Rat brain



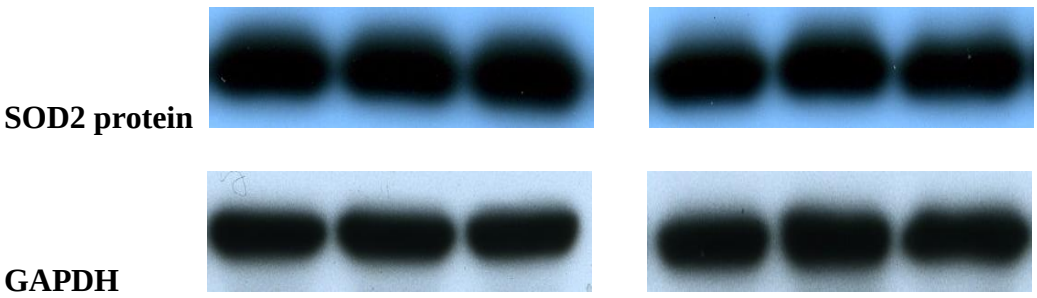
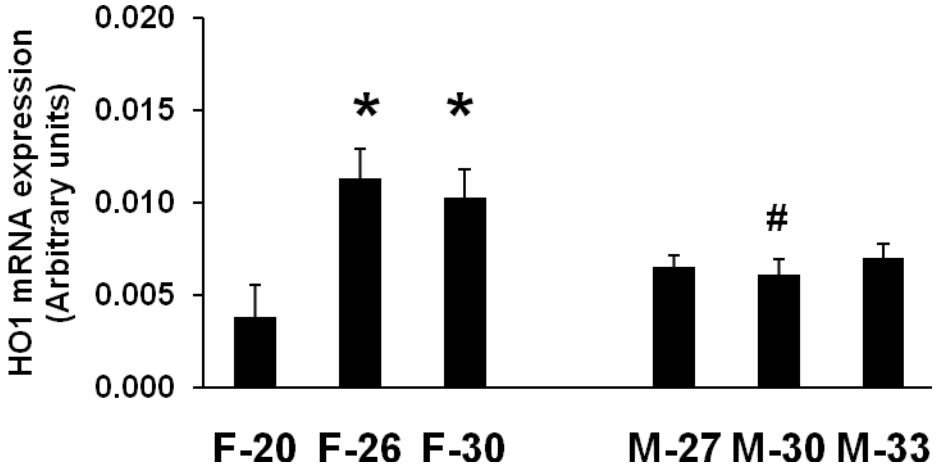
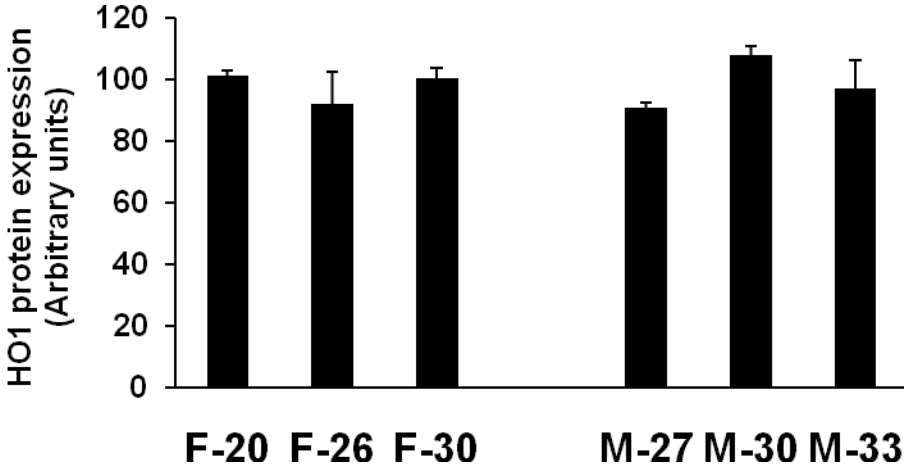


Figure 8

A: HO-1 mRNA expression in cerebellum of F344 BN Rat brain



B: HO-1 protein expression in cerebellum of F344 BN Rat brain



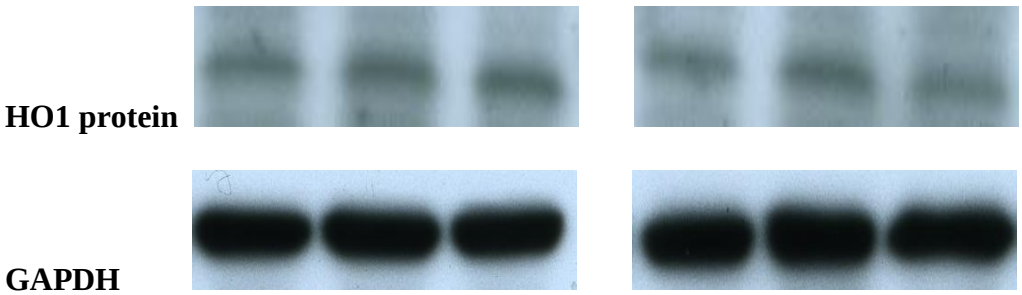
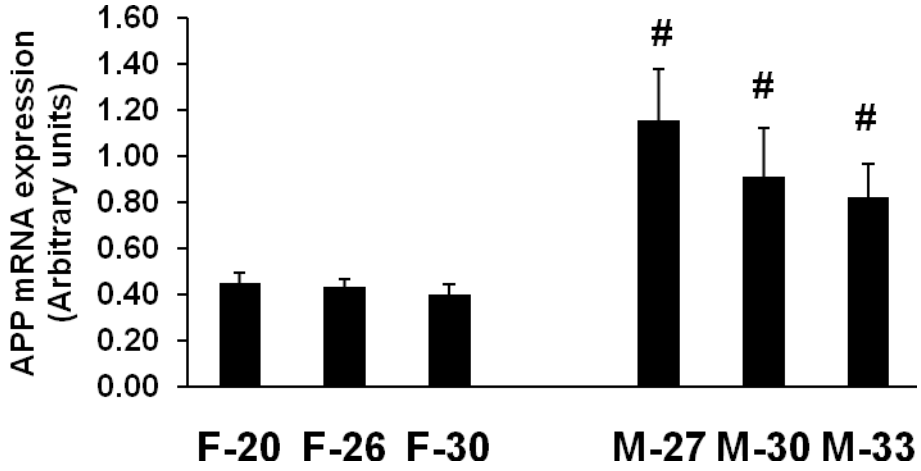
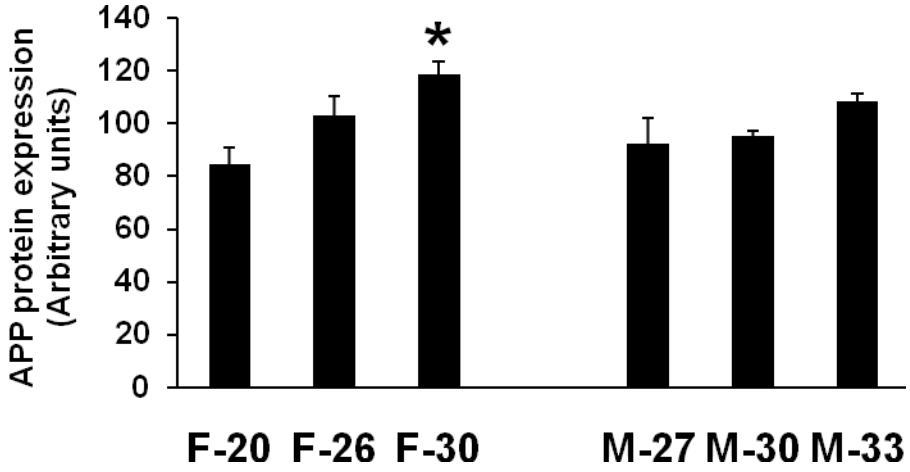


Figure 9

A: APP mRNA expression in cerebellum of F344 BN Rat brain



B: APP protein expression in cerebellum of F344 BN Rat brain



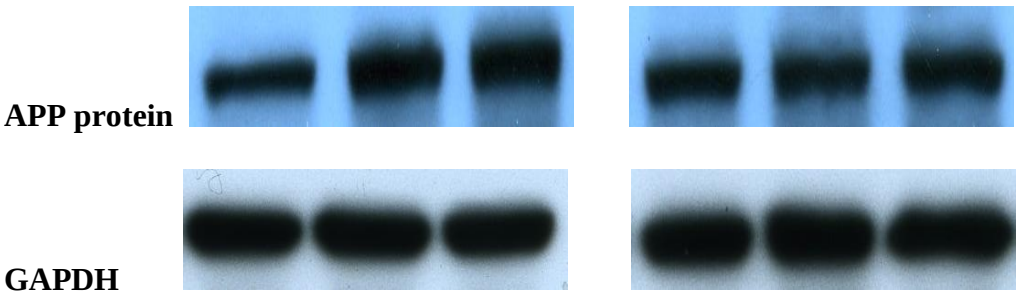
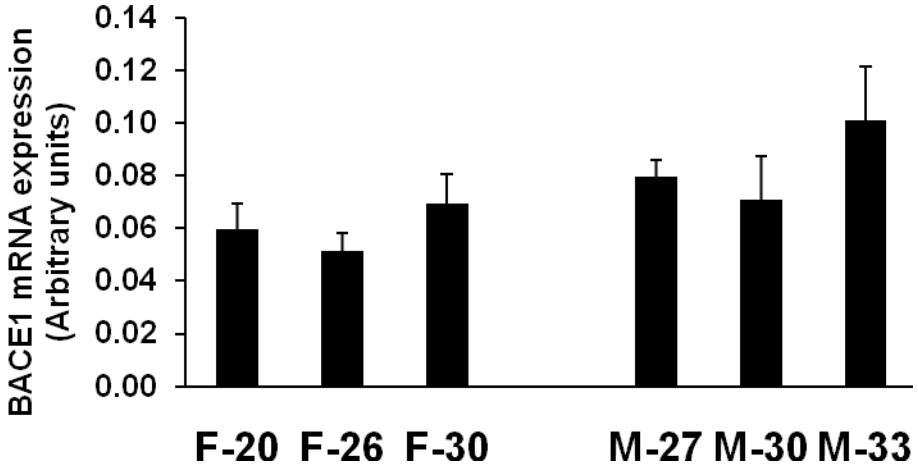
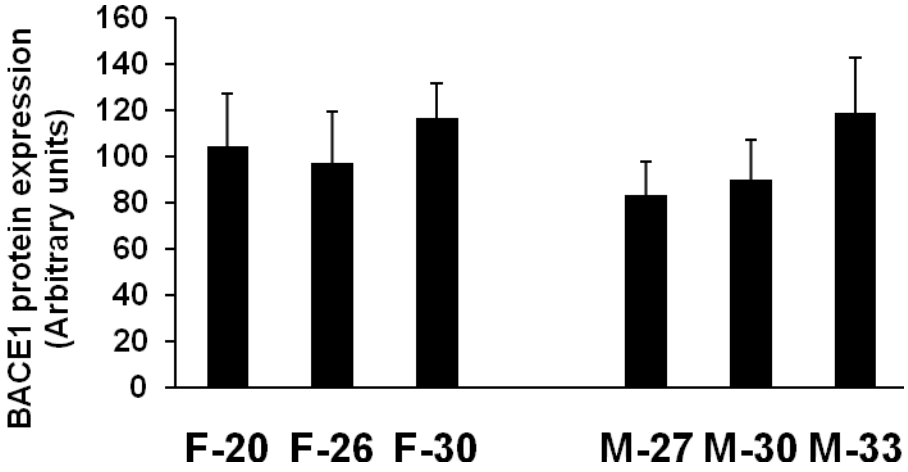


Figure 10

A: BACE1 mRNA expression in cerebellum of F344 BN Rat brain



B: BACE1 protein expression in cerebellum of F344 BN Rat brain



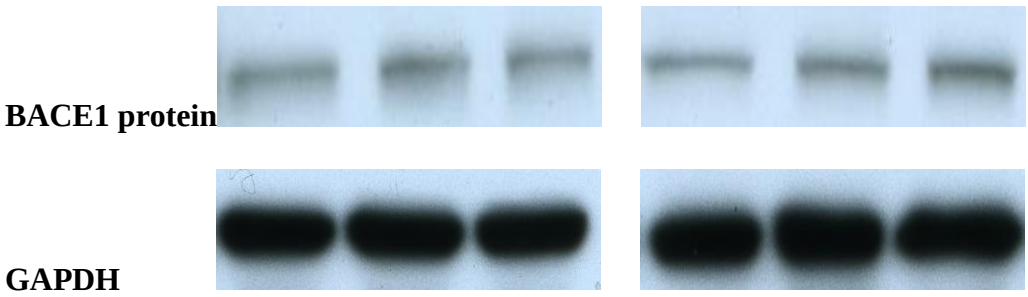
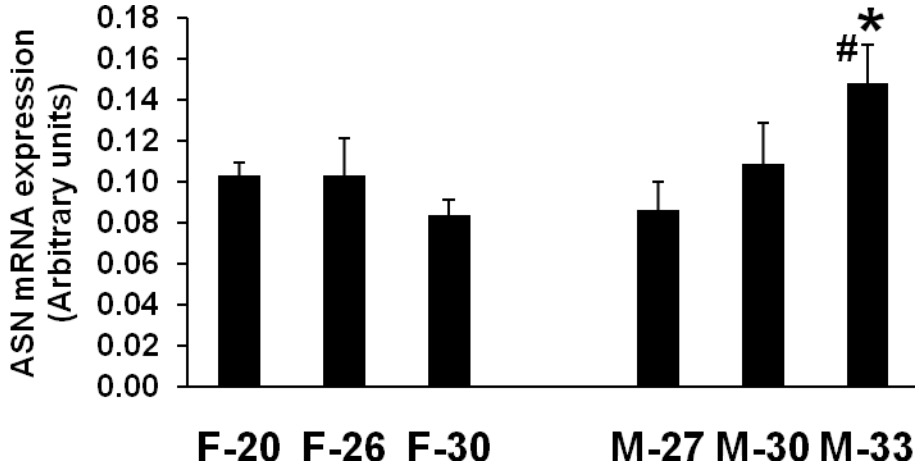
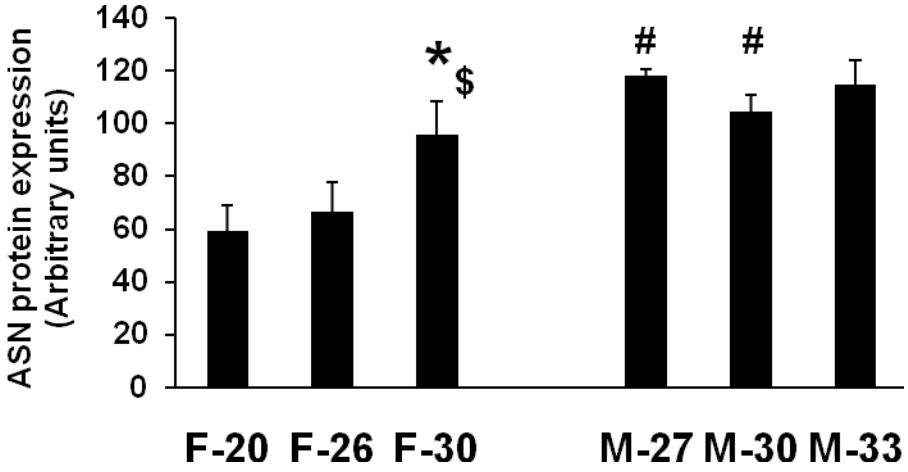


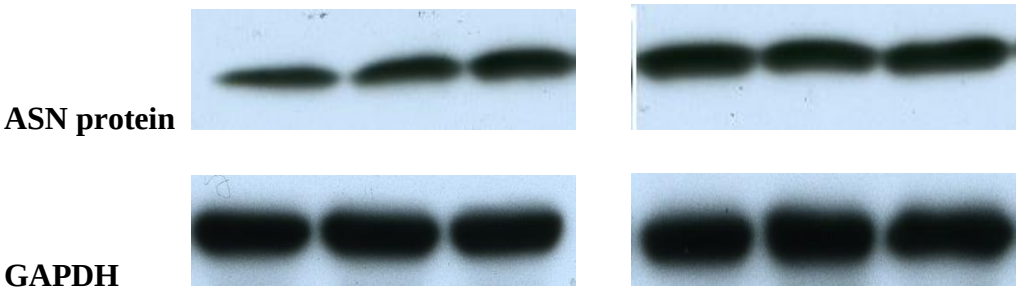
Figure 11

A: ASN mRNA expression in cerebellum of F344 BN Rat brain



B: ASN protein expression in cerebellum of F344 BN Rat brain





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APPENDIX

This section includes western blot film properties reports, raw data tables, QRT-PCR data and statistics of various molecules in Hippocampus and Cerebellum used for this study.

Laboratory of Molecular Physiology

Western Blot Film Record

Print Form

Experimenter: Srinivasarao Thulluri

Date: 2010/03/27

Project: Aging and sex associated changes

Report Number:

Tissue/ cell line/ etc.: Rat brain (cerebellum)

Gel type: 15%

Electrophoresis Voltage: 124

Transfer Voltage: 25

Protien Load per Well: 40 ug

Duration: 01 :30 hrs

Duration: 70 minutes

Primary Antibody

Name (Do Not Abbreviate): Alpha synuclein (ASN)

Dilution: 1:300

Medium: 5% BSA in TBST

Incubation time: Overnight

Secondary Antibody

Name: Anti mouse

Dilution: 1:1000

Medium: 5% Milk in TBST

Incubation time: 1 :00hr

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Positive control	20 m Female	26 m Female	30 m Female	27 m Male	30 m Male	33 m Male	20 m Female	26 m Female	30 m Female	Soleus	BL+MW

Place Scan of Film Here

Exposure time

3'

Molecular Wieght

19 Kda

☒ Used For Analysis

Location of Scan: \\Blough-raid1\shared-data-server\Post docs, staff and students folders\Masters Graduate Students\THULLURI\Gende

Location of Excel Data: \\Blough-raid1\shared-data-server\Post docs, staff and students folders\Masters Graduate Students\THULLURI\Gender

Notes

email Report

Signature

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http://www.science.marshall.edu/blough

Raw data

This section represents the raw data tables produced from spot densitometry of the Alpha Synuclein(ASN) immunoblot films.

	F-20	F-26	F-30	M-27	M-30	M-33
	39.47	49.45	76.15	119.52	87.77	103.58
	75.68	90.44	122.30	115.25	108.65	126.67
	70.84	75.07	106.12	117.36	103.44	117.36
	51.54	50.83	77.69	125.94	123.22	135.75
				111.68	99.09	89.23
N	4	4	4	5	5	5
Mean	59.4	66.4	95.6	117.9	104.4	114.5
STDEV	16.9	19.9	22.5	5.3	13.0	18.5
SEM	9.7	11.5	13.0	2.7	6.5	9.2

Statistics

Two Way Analysis of Variance Friday, June 25, 2010, 11:54:12 AM
Data source: Data 1 in Notebook 1
General Linear Model
Dependent Variable: ASN PROTEIN
Normality Test: Passed (P = 0.772)
Equal Variance Test: Passed (P = 0.093)

Source of Variation	DF	SS	MS	F	P
GENDER	1	9881.897	9881.897	36.301	<0.001
AGE	2	1963.851	981.926	3.607	0.045
GENDER x AGE	2	1744.755	872.378	3.205	0.061
Residual	21	5716.676	272.223		
Total	26	19035.053	732.117		

The difference in the mean values among the different levels of GENDER is greater than would be expected by chance after allowing for effects of differences in AGE. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of AGE is greater than would be expected by chance after allowing for effects of differences in GENDER. There is a statistically significant difference (P = 0.045). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of GENDER does not depend on what level of AGE is present. There is not a statistically significant interaction between GENDER and AGE. (P = 0.061)

Power of performed test with alpha = 0.0500: for GENDER : 1.000
Power of performed test with alpha = 0.0500: for AGE : 0.453
Power of performed test with alpha = 0.0500: for GENDER x AGE : 0.389
Least square means for GENDER :

Group	Mean	SEM
F 73.799	4.763	
M112.299	4.260	

Least square means for AGE :

Group	Mean	SEM
20.000	88.666	5.534
26.000	85.440	5.534
30.000	105.042	5.534

Least square means for GENDER x AGE :

GroupMean	SEM	
F x 20.000	59.382	8.250
F x 26.000	66.448	8.250
F x 30.000	95.567	8.250
M x 20.000	117.949	7.379
M x 26.000	104.432	7.379
M x 30.000	114.517	7.379

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05
Comparisons for factor: **GENDER**

ComparisonDiff of Means	t	Unadjusted P	Critical Level	Significant?
M vs. F 38.500	6.025	0.00000559	0.050	Yes

Comparisons for factor: **AGE**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
30.000 vs. 26.000	19.602	2.505	0.0206	0.017	No
30.000 vs. 20.000	16.376	2.092	0.0487	0.025	No
20.000 vs. 26.000	3.226	0.412	0.684	0.050	No

Comparisons for factor: **AGE within F**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
30.000 vs. 20.000	36.184	3.102	0.005	0.017	Yes
30.000 vs. 26.000	29.118	2.496	0.021	0.025	Yes
26.000 vs. 20.000	7.066	0.606	0.551	0.050	No

Comparisons for factor: **AGE within M**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
20.000 vs. 26.000	13.517	1.295	0.209	0.017	No
30.000 vs. 26.000	10.085	0.966	0.345	0.025	No
20.000 vs. 30.000	3.432	0.329	0.745	0.050	No

Comparisons for factor: **GENDER within 20**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
M vs. F	58.567	5.292	0.000	0.050	Yes

Comparisons for factor: **GENDER within 26**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
M vs. F	37.984	3.432	0.003	0.050	Yes

Comparisons for factor: **GENDER within 30**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
M vs. F	18.950	1.712	0.102	0.050	N

Laboratory of Molecular Physiology
Western Blot Film Record

Print Form

Experimenter	Srinivasarao Thulluri		
Date	2010/03/27	Project	Aging and sex associated changes
Report Number		Tissue/ cell line/ etc.	Rat brain (cerebellum)
Gel type	15%	Electrophoresis Voltage	124
Protien Load per Well	40 ug	Duration	01 : 30 hrs
Transfer Voltage	25		
Duration	70 minutes		

Primary Antibody

Name (Do Not Abbreviate) superoxide dismutase-2 (SOD-2)

Dilution 1:2000 Medium 5% BSA in TBST

Incubation time Overnight

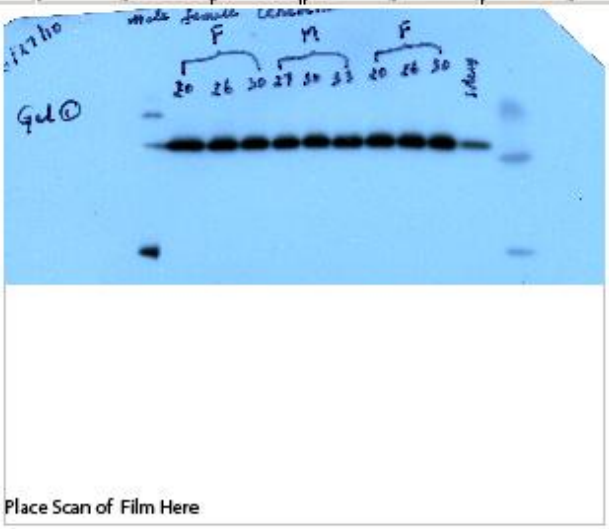
Secondary Antibody

Name Anti Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00hr

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Positive control	20 m Female	26 m Female	30 m Female	27 m Male	30 m Male	33 m Male	20 m Female	26 m Female	30 m Female	Soleus	BL + MW



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Exposure time

1'

Molecular Wieght

22 Kda

☒ Used For Analysis

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Location of Excel Data \\Blough-raid1\shared-data-server\Post docs, staff and students folders\Masters Graduate Students\THULLURI\Gender

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Raw data

This section represents the raw data tables produced from spot densitometry of the Superoxide dismutase (SOD-2) immunoblot films.

	F-20	F-26	F-30	M-27	M-30	M-33
	105.96	106.20	104.76	93.09	87.97	93.84
	103.73	98.26	111.71	101.40	93.06	105.29
	101.75	99.99	125.33	94.71	100.23	95.59
	99.55	100.23	107.27	91.73	88.25	100.81
				100.45	88.29	99.04
N	4	4	4	5	5	5
Mean	102.75	101.17	112.27	96.27	91.56	98.91
STDEV	2.74	3.47	9.17	4.39	5.29	4.50
SEM	1.58	2.00	5.29	2.19	2.64	2.25

Statistics

SOD2 CERE PROTEIN
Two Way Analysis of Variance Friday, June 25, 2010, 11:51:07 AM
Data source: Data 1 in Notebook 1
General Linear Model
Dependent Variable: SOD2
Normality Test: Passed (P = 0.230)
Equal Variance Test: Passed (P = 0.663)

Source of Variation	DF	SS	MS	F	P
GENDER	1	641.854	641.854	23.211	<0.001
AGE	2	391.136	195.568	7.072	0.004
GENDER x AGE	2	52.750	26.375	0.954	0.401
Residual	21	580.713	27.653		
Total	26	1649.823	63.455		

The difference in the mean values among the different levels of GENDER is greater than would be expected by chance after allowing for effects of differences in AGE. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of AGE is greater than would be expected by chance after allowing for effects of differences in GENDER. There is a statistically significant difference (P = 0.004). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of GENDER does not depend on what level of AGE is present. There is not a statistically significant interaction between GENDER and AGE. (P = 0.401)

Power of performed test with alpha = 0.0500: for GENDER : 0.997
Power of performed test with alpha = 0.0500: for AGE : 0.843
Power of performed test with alpha = 0.0500: for GENDER x AGE : 0.0500
Least square means for GENDER :

Group	Mean	SEM
F105.395	1.518	
M95.583	1.358	

Least square means for AGE :

Group	Mean	SEM
20.000	99.510	1.764
26.000	96.365	1.764
30.000	105.592	1.764

Least square means for GENDER x AGE :

GroupMean	SEM	
F x 20.000	102.747	2.629
F x 26.000	101.169	2.629
F x 30.000	112.269	2.629
M x 20.000	96.274	2.352
M x 26.000	91.560	2.352
M x 30.000	98.914	2.352

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05
Comparisons for factor: **GENDER**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
F vs. M	9.812	4.818	0.0000923	0.050	Yes

Comparisons for factor: **AGE**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
30.000 vs. 26.000	9.227	3.699	0.00133	0.017	Yes
30.000 vs. 20.000	6.081	2.438	0.0237	0.025	Yes
20.000 vs. 26.000	3.145	1.261	0.221	0.050	No

Comparisons for factor: **AGE within F**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
30.000 vs. 26.000	11.099	2.985	0.007	0.017	Yes
30.000 vs. 20.000	9.522	2.561	0.018	0.025	Yes
20.000 vs. 26.000	1.577	0.424	0.676	0.050	No

Comparisons for factor: **AGE within M**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
30.000 vs. 26.000	7.354	2.211	0.038	0.017	No
20.000 vs. 26.000	4.714	1.417	0.171	0.025	No
30.000 vs. 20.000	2.641	0.794	0.436	0.050	No

Comparisons for factor: **GENDER within 20**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
F vs. M	6.473	1.835	0.081	0.050	No

Comparisons for factor: **GENDER within 26**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
F vs. M	9.609	2.724	0.013	0.050	Yes

Comparisons for factor: **GENDER within 30**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
F vs. M	13.354	3.786	0.001	0.050	Yes

Laboratory of Molecular Physiology
Western Blot Film Record

Print Form

Experimenter: Srinivasarao Thulluri

Date: 2010/03/27

Project: Aging and sex associated changes

Report Number:

Tissue/ cell line/ etc.: Rat brain (cerebellum)

Gel type: 15%

Electrophoresis Voltage: 124

Transfer Voltage: 25

Protien Load per Well: 40 ug

Duration: 01 : 30 hrs

Duration: 70 minutes

Primary Antibody

Name (Do Not Abbreviate): Amyloid precurser protein(APP)

Dilution: 1:2000

Medium: 5% BSA in TBST

Incubation time: Overnight

Secondary Antibody

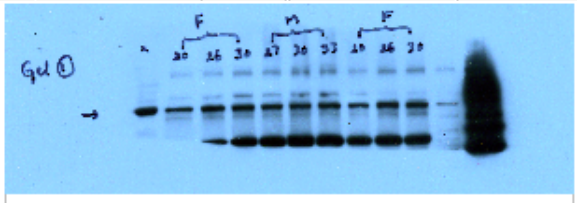
Name: Anti Rabbit

Dilution: 1:200

Medium: 5% Milk in TBST

Incubation time: 1 : 00hr

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Positive control	20 m Female	26 m Female	30 m Female	27 m Male	30 m Male	33 m Male	20 m Female	26 m Female	30 m Female	Soleus	BL+MW



Place Scan of Film Here

Exposure time

3'

Molecular Wieght

100 Kda

☒ Used For Analysis

Location of Scan: \\Blough-raid1\shared-data-server\Post docs, staff and students folders\Masters Graduate Students\THULLURI\Gende

Location of Excel Data: \\Blough-raid1\shared-data-server\Post docs, staff and students folders\Masters Graduate Students\THULLURI\Gender

Notes

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Signature

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Raw data

This section represents the raw data tables produced from spot densitometry of the Amyloid precursor protein(APP) immunoblot films.

	F-20	F-26	F-30	M-27	M-30	M-33
	95.10	114.54	105.95	89.76	92.39	107.07
	86.63	106.91	124.06	79.52	98.95	116.06
	69.19	104.98	119.74	92.69	89.78	102.32
	87.05	84.82	124.79	75.13	98.15	111.76
				124.79	97.16	104.69
N	4	4	4	5	5	5
Mean	84.50	102.81	118.63	92.38	95.29	108.38
STDEV	10.92	12.68	8.74	19.50	4.00	5.53
SEM	6.31	7.32	5.05	9.75	2.00	2.77

Statistics

Two Way Analysis of Variance Friday, June 25, 2010, 11:53:00 AM
Data source: Data 1 in Notebook 1
General Linear Model
Dependent Variable: APP PROTEIN
Normality Test: Passed (P = 0.131)
Equal Variance Test: Passed (P = 0.414)

Source of Variation	DF	SS	MS	F	P
GENDER	1	72.584	72.584	0.549	0.467
AGE	2	2815.423	1407.711	10.647	<0.001
GENDER x AGE	2	425.110	212.555	1.608	0.224
Residual	21	2776.421	132.211		
Total	26	5910.810	227.339		

The difference in the mean values among the different levels of GENDER is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in AGE. There is not a statistically significant difference (P = 0.467).

The difference in the mean values among the different levels of AGE is greater than would be expected by chance after allowing for effects of differences in GENDER. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of GENDER does not depend on what level of AGE is present. There is not a statistically significant interaction between GENDER and AGE. (P = 0.224)

Power of performed test with alpha = 0.0500: for GENDER : 0.0500
Power of performed test with alpha = 0.0500: for AGE : 0.971
Power of performed test with alpha = 0.0500: for GENDER x AGE : 0.133
Least square means for GENDER :

Group	Mean	SEM
F101.981	3.319	
M98.681	2.969	

Least square means for AGE :

Group	Mean
20.000	88.437
26.000	99.049
30.000	113.507

Std Err of LS Mean = 3.857
Least square means for GENDER x AGE :

GroupMean		SEM
F x 20.000	84.495	5.749
F x 26.000	102.813	5.749
F x 30.000	118.635	5.749
M x 20.000	92.378	5.142
M x 26.000	95.285	5.142
M x 30.000	108.380	5.142

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05
Comparisons for factor: **GENDER**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
F vs. M	3.300	0.741	0.467	0.050	No

Comparisons for factor: **AGE**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
30.000 vs. 20.000	25.071	4.597	0.000156	0.017	Yes
30.000 vs. 26.000	14.458	2.651	0.0149	0.025	Yes
26.000 vs. 20.000	10.612	1.946	0.0652	0.050	No

Comparisons for factor: **AGE within F**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
30.000 vs. 20.000	34.140	4.199	0.000	0.017	Yes
26.000 vs. 20.000	18.318	2.253	0.035	0.025	No
30.000 vs. 26.000	15.822	1.946	0.065	0.050	No

Comparisons for factor: **AGE within M**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
30.000 vs. 20.000	16.002	2.200	0.039	0.017	No
30.000 vs. 26.000	13.095	1.801	0.086	0.025	No
26.000 vs. 20.000	2.907	0.400	0.693	0.050	No

Comparisons for factor: **GENDER within 20**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
M vs. F	7.883	1.022	0.318	0.050	No

Comparisons for factor: **GENDER within 26**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
F vs. M	7.527	0.976	0.340	0.050	No

Comparisons for factor: **GENDER within 30**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
F vs. M	10.255	1.329	0.198	0.050	No

Laboratory of Molecular Physiology

Western Blot Film Record

Print Form

Experimenter	Srinivasarao Thulluri		
Date	2010/03/27	Project	Aging and sex associated changes
Report Number		Tissue/ cell line/ etc.	Rat brain (cerebellum)

Gel type	15%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	01 : 30 hrs	Duration	70 minutes

Primary Antibody

Name (Do Not Abbreviate) Beta site APP cleaving enzyme (BACE1)

Dilution 1:2000 Medium 5% BSA in TBST

Incubation time Overnight

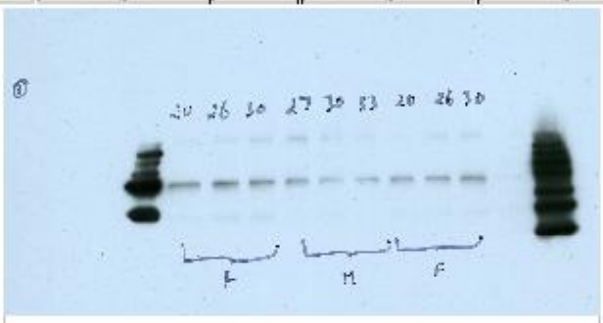
Secondary Antibody

Name Anti mouse

Dilution 1:300 Medium 5% Milk in TBST

Incubation time 1 : 00hr

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Positive control	20 m Female	26 m Female	30 m Female	27 m Male	30 m Male	33 m Male	20 m Female	26 m Female	30 m Female	Soleus	BL+MW



Place Scan of Film Here

Exposure time

3'

Molecular Wieght

70 Kda

☒ Used For Analysis

Location of Scan \\\Blough-raid1\shared-data-server\Post docs, staff and students folders\Masters Graduate Students\THULLURI\Gende

Location of Excel Data \\Blough-raid1\shared-data-server\Post docs, staff and students folders\Masters Graduate Students\THULLURI\Gender

Notes

email Report

Signature

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http://www.science.marshall.edu/blough

Raw data

This section represents the raw data tables produced from spot densitometry of the Beta site APP cleaving enzyme (BACE1) immunoblot films.

	F-20	F-26	F-30	M-27	M-30	M-33
	70.35	49.58	79.17	120.48	134.80	165.09
	157.79	134.91	137.59	39.94	100.35	158.40
	78.51	83.41	127.47	89.12	50.77	59.41
	110.36	120.77	122.38	81.39	103.52	132.61
				86.12	60.32	78.91
N	4	4	4	5	5	5
Mean	104.25	97.17	116.65	83.41	89.95	118.88
STDEV	39.65	38.45	25.77	28.76	34.34	47.48
SEM	22.89	22.20	14.88	14.38	17.17	23.74

Statistics

Two Way Analysis of Variance Friday, June 25, 2010, 11:52:26 AM
Data source: Data 1 in Notebook 1
General Linear Model
Dependent Variable: BACE1 PROTEIN
Normality Test: Passed (P = 0.361)
Equal Variance Test: Passed (P = 0.442)

Source of Variation	DF	SS	MS	F	P
GENDER	1	494.223	494.223	0.368	0.550
AGE	2	3434.338	1717.169	1.279	0.299
GENDER x AGE	2	598.078	299.039	0.223	0.802
Residual	21	28186.801	1342.229		
Total	26	33022.959	1270.114		

The difference in the mean values among the different levels of GENDER is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in AGE. There is not a statistically significant difference (P = 0.550).

The difference in the mean values among the different levels of AGE is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in GENDER. There is not a statistically significant difference (P = 0.299).

The effect of different levels of GENDER does not depend on what level of AGE is present. There is not a statistically significant interaction between GENDER and AGE. (P = 0.802)

Power of performed test with alpha = 0.0500: for GENDER : 0.0500
Power of performed test with alpha = 0.0500: for AGE : 0.0861
Power of performed test with alpha = 0.0500: for GENDER x AGE : 0.0500
Least square means for GENDER :

Group	Mean	SEM
F106.025	10.576	
M97.415	9.459	

Least square means for AGE :

Group	Mean
20.000	93.831
26.000	93.560
30.000	117.768

Std Err of LS Mean = 12.288
Least square means for GENDER x AGE :

GroupMean	SEM	
F x 20.000	104.254	18.318
F x 26.000	97.168	18.318
F x 30.000	116.653	18.318
M x 20.000	83.409	16.384
M x 26.000	89.951	16.384
M x 30.000	118.884	16.384

Laboratory of Molecular Physiology

Western Blot Film Record

Print Form

Experimenter: Srinivasarao Thulluri

Date: 2010/03/27

Project: Aging and sex associated changes

Report Number:

Tissue/ cell line/ etc.: Rat brain (cerebellum)

Gel type: 15%

Electrophoresis Voltage: 124

Transfer Voltage: 25

Protien Load per Well: 40 ug

Duration: 01 : 30 hrs

Duration: 70 minutes

Primary Antibody

Name (Do Not Abbreviate): Haemoxigenase1(HO1)

Dilution: 1:500

Medium: 5% BSA in TBST

Incubation time: Overnight

Secondary Antibody

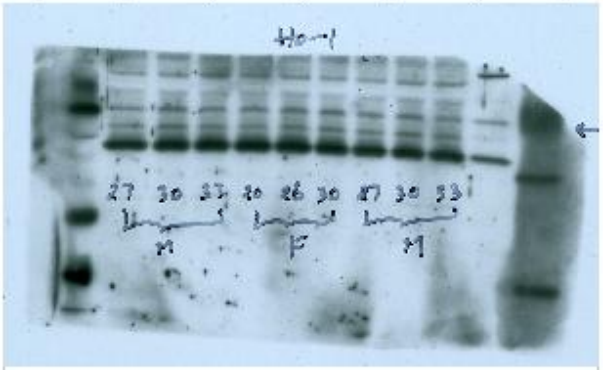
Name: Anti Rabbit

Dilution: 1:1000

Medium: 5% Milk in TBST

Incubation time: 1 : 00hr

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Positive control	20 m Female	26 m Female	30 m Female	27 m Male	30 m Male	33 m Male	20 m Female	26 m Female	30 m Female	Soleus	BL + MW



Place Scan of Film Here

Exposure time

3'

Molecular Wieght

32 Kda

☒ Used For Analysis

Location of Scan: \\\Blough-raid1\shared-data-server\Post docs, staff and students folders\Masters Graduate Students\THULLURI\Gende

Location of Excel Data: \\Blough-raid1\shared-data-server\Post docs, staff and students folders\Masters Graduate Students\THULLURI\Gender

Notes

email Report

Signature

Laboratory of Molecular Physiology, Marshall University, Science Bldg. Suite 311, 1 John Marshall Drive, Huntington, WV, 25755 * Phone: 304-696-3267 Fax: 304-696-7136
http://www.science.marshall.edu/blough

Raw data

This section represents the raw data tables produced from spot densitometry of the Haemoxigenase(HO-1) immunoblot films.

	F-20	F-26	F-30	M-27	M-30	M-33
	102.36	106.00	92.69	89.58	104.95	89.18
	104.25	77.53	104.96	89.45	105.38	110.93
	100.49	76.13	106.08	95.26	104.56	72.21
	97.24	108.89	97.48	86.97	118.97	97.13
				93.24	105.28	116.83
N	4	4	4	5	5	5
Mean	101.08	92.14	100.30	90.90	107.83	97.25
STDEV	2.99	17.72	6.35	3.31	6.24	17.76
SEM	1.72	10.23	3.67	1.65	3.12	8.88

Statistics

Two Way Analysis of Variance Friday, June 25, 2010, 11:55:11 AM
Data source: Data 1 in Notebook 1
General Linear Model
Dependent Variable: HO1 PROTEIN

Normality Test: Failed (P < 0.050)
Equal Variance Test: Passed (P = 0.355)

Source of Variation	DF	SS	MS	F	P
GENDER	1	476.188	476.188	0.305	0.586
AGE	2	4135.520	2067.760	1.326	0.287
GENDER x AGE	2	3334.124	1667.062	1.069	0.361
Residual	21	32746.480	1559.356		
Total	26	40254.623	1548.255		

The difference in the mean values among the different levels of GENDER is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in AGE. There is not a statistically significant difference (P = 0.586).

The difference in the mean values among the different levels of AGE is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in GENDER. There is not a statistically significant difference (P = 0.287).

The effect of different levels of GENDER does not depend on what level of AGE is present. There is not a statistically significant interaction between GENDER and AGE. (P = 0.361)

Power of performed test with alpha = 0.0500: for GENDER : 0.0500
Power of performed test with alpha = 0.0500: for AGE : 0.0926
Power of performed test with alpha = 0.0500: for GENDER x AGE : 0.0580

Least square means for GENDER :

Group	Mean	SEM
F115.680	11.399	
M107.228	10.196	

Least square means for AGE :

Group	Mean	SEM
20.000	99.210	13.245
26.000	106.614	13.245
30.000	128.539	13.245

Least square means for GENDER x AGE :

GroupMean	SEM	
F x 20.000	105.222	19.744
F x 26.000	96.339	19.744
F x 30.000	145.479	19.744
M x 20.000	93.198	17.660
M x 26.000	116.888	17.660
M x 30.000	111.599	17.660

Raw data

This section represents the raw data tables produced from qRTPCR (2^-dct values) of the ASN mRNA expression in cerebellum.

F-20	F-26	F-30		M-27	M-30	M-33
	0.10	0.11	0.10	0.09	0.14	0.18
	0.09	0.07	0.08	0.11	0.05	0.13
	0.11	0.12	0.10	0.07	0.13	0.17
	0.11	0.07	0.09	0.11	0.13	0.12
		0.15	0.06	0.05	0.09	
N	4	5	5	5	5	4
Mean	0.10	0.10	0.08	0.09	0.11	0.15
STDEV	0.01	0.04	0.01	0.03	0.04	0.03
SEM	0.01	0.02	0.01	0.01	0.02	0.02

Statistics

Two Way Analysis of Variance Saturday, July 03, 2010, 11:05:25 AM

Data source: Data 1 in Notebook 1
General Linear Model
Dependent Variable: ASN
Normality Test: Passed (P = 0.720)
Equal Variance Test: Passed (P = 0.129)

Source of Variation	DF	SS	MS	F	P
GENDER	1	0.00217	0.00217	2.551	0.125
AGE	2	0.00206	0.00103	1.208	0.318
GENDER x AGE	2	0.00792	0.00396	4.645	0.021
Residual	22	0.0188	0.000852		
Total	27	0.0304	0.00113		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.
The effect of different levels of GENDER depends on what level of AGE is present. There is a statistically significant interaction between GENDER and AGE. (P = 0.021)
Power of performed test with alpha = 0.0500: for GENDER : 0.209
Power of performed test with alpha = 0.0500: for AGE : 0.0765
Power of performed test with alpha = 0.0500: for GENDER x AGE : 0.607
Least square means for GENDER :

Group	Mean
F	0.0968
M	0.115

Std Err of LS Mean = 0.00785
Least square means for AGE :

Group	Mean	SEM
20.000	0.0946	0.00979
26.000	0.106	0.00923
30.000	0.116	0.00979

Least square means for GENDER x AGE :

Group	Mean	SEM
F x 20.000	0.103	0.0146
F x 26.000	0.103	0.0131
F x 30.000	0.0839	0.0131
M x 20.000	0.0861	0.0131
M x 26.000	0.109	0.0131
M x 30.000	0.148	0.0146

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05
Comparisons for factor: **GENDER**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
M vs. F	0.0177	1.597	0.125	0.050	No

Comparisons for factor: **AGE**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
30.000 vs. 20.000	0.0215	1.553	0.135	0.017	No
26.000 vs. 20.000	0.0115	0.853	0.403	0.025	No
30.000 vs. 26.000	0.0100	0.745	0.464	0.050	No

Comparisons for factor: **AGE within F**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significa**
26.000 vs. 30.000	0.0193	1.048	0.306	0.017	No
20.000 vs. 30.000	0.0193	0.986	0.335	0.025	No
26.000 vs. 20.000	0.0000385	0.00196	0.998	0.050	No

Comparisons for factor: **AGE within M**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significa**
30.000 vs. 20.000	0.0623	3.182	0.004	0.017	Yes
30.000 vs. 26.000	0.0394	2.011	0.057	0.025	No
26.000 vs. 20.000	0.0229	1.242	0.227	0.050	No

Comparisons for factor: **GENDER within 20**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
F vs. M	0.0171	0.874	0.392	0.050	No

Comparisons for factor: **GENDER within 26**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significa**
M vs. F	0.00577	0.313	0.758	0.050	No

Comparisons for factor: **GENDER within 30**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
M vs. F	0.0645	3.294	0.003	0.050	Yes

Raw data

This section represents the raw data tables produced from qRTPCR (2^-dct values) of the SOD-2 mRNA expression in Cerebellum.

	F-20	F-26	F-30	M-27	M-30	M-33
	0.18	0.26	0.24	0.22	0.15	0.34
	0.20	0.27	0.35	0.18	0.12	0.19
	0.16	0.41	0.26	0.18	0.31	0.26
	0.24	0.12	0.24	0.17	0.25	0.23
	0.13	0.22	0.22			
	0.14					
N	6	5	5	4	4	4
Mean	0.17	0.26	0.26	0.19	0.21	0.25
STDEV	0.04	0.11	0.05	0.02	0.09	0.06
SEM	0.02	0.05	0.03	0.01	0.05	0.04

Statistics

Two Way Analysis of Variance Wednesday, June 23, 2010, 9:30:48 AM

Data source: 2^-DCT CERE in Notebook 1
General Linear Model
Dependent Variable: SOD-2

Normality Test:	Passed	(P = 0.227)
Equal Variance Test:	Passed	(P = 0.136)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Gender	1	0.00130	0.00130	0.282	0.601
AGE	2	0.0279	0.0140	3.032	0.069
Gender x AGE	2	0.00428	0.00214	0.465	0.634
Residual	22	0.101	0.00460		
Total	27	0.137	0.00508		

The difference in the mean values among the different levels of Gender is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in AGE. There is not a statistically significant difference (P = 0.601).
The difference in the mean values among the different levels of AGE is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Gender. There is not a statistically significant difference (P = 0.069).
The effect of different levels of Gender does not depend on what level of AGE is present. There is not a statistically significant interaction between Gender and AGE. (P = 0.634)

Power of performed test with alpha = 0.0500: for Gender : 0.0500
Power of performed test with alpha = 0.0500: for AGE : 0.363
Power of performed test with alpha = 0.0500: for Gender x AGE : 0.0500
Least square means for Gender :

Group	**Mean**	**SEM**
F	0.230	0.0170
M	0.217	0.0196

Least square means for AGE :

Group	**Mean**	**SEM**
20.000	0.181	0.0219
26.000	0.232	0.0228
30.000	0.257	0.0228

Least square means for Gender x AGE :

Group	**Mean**	**SEM**
F x 20.000	0.175	0.0277
F x 26.000	0.256	0.0303
F x 30.000	0.261	0.0303
M x 20.000	0.188	0.0339
M x 26.000	0.209	0.0339
M x 30.000	0.254	0.0339

Raw data

This section represents the raw data tables produced from qRTPCR (2^-dct values) of the HO-1 mRNA expression in Cerebellum.

	F-20	F-26	F-30	M-27	M-30	M-33
	0.0041	0.0161	0.0112	0.0054	0.0064	0.0057
	0.0013	0.0118	0.0150	0.0065	0.0084	0.0074
	0.0019	0.0084	0.0074	0.0078	0.0063	0.0087
	0.0079	0.0116	0.0100	0.0077	0.0056	0.0062
		0.0088	0.0079	0.0050	0.0037	
N	4	5	5	5	5	4
Mean	0.0038	0.0113	0.0103	0.0065	0.0061	0.0070
STDEV	0.0030	0.0031	0.0031	0.0013	0.0017	0.0013
SEM	0.0017	0.0015	0.0015	0.0006	0.0008	0.0008

Statistics

Two Way Analysis of Variance Wednesday, June 23, 2010, 9:48:43 AM
Data source: 2^-DCT HO-1 in Notebook 2
General Linear Model
Dependent Variable: HO1
Normality Test: Passed (P = 0.180)
Equal Variance Test: Passed (P = 0.350)

Source of Variation	DF	SS	MS	F	P
Gender	1	0.0000264	0.0000264	4.620	0.043
AGE	2	0.0000759	0.0000379	6.628	0.006
Gender x AGE	2	0.0000787	0.0000394	6.880	0.005
Residual	22	0.000126	0.00000572		
Total	27	0.000309	0.0000114		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.
The effect of different levels of Gender depends on what level of AGE is present. There is a statistically significant interaction between Gender and AGE. (P = 0.005)
Power of performed test with alpha = 0.0500: for Gender : 0.433
Power of performed test with alpha = 0.0500: for AGE : 0.814
Power of performed test with alpha = 0.0500: for Gender x AGE : 0.833
Least square means for Gender :

Group	Mean
F	0.00848
M	0.00652

Std Err of LS Mean = 0.000643
Least square means for AGE :

Group	Mean	SEM
20.000	0.00514	0.000802
26.000	0.00871	0.000756
30.000	0.00865	0.000802

Least square means for Gender x AGE :

Group	Mean	SEM
F x 20.000	0.00380	0.00120
F x 26.000	0.0113	0.00107
F x 30.000	0.0103	0.00107
M x 20.000	0.00648	0.00107
M x 26.000	0.00608	0.00107
M x 30.000	0.00701	0.00120

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :
Comparisons for factor: **Gender**

Comparison	Diff of Means	p	q	P	P<0.050
F vs. M	0.00195	2	3.040	0.043	Yes

Comparisons for factor: **AGE**

Comparison	Diff of Means	p	q	P	P<0.050
26.000 vs. 20.000	0.00357	3	4.579	0.010	Yes
26.000 vs. 30.000	0.0000597	2	0.0766	0.957	No
30.000 vs. 20.000	0.00351	2	4.375	0.005	Yes

Comparisons for factor: **AGE within F**

Comparison	Diff of Means	p	q	P	P<0.05
26.000 vs. 20.000	0.00754	3	6.642	<0.001	Yes
26.000 vs. 30.000	0.00104	2	0.976	0.497	No
30.000 vs. 20.000	0.00649	2	5.722	<0.001	Yes

Comparisons for factor: **AGE within M**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
30.000 vs. 26.000	0.000925	3	0.815	0.834	No
30.000 vs. 20.000	0.000529	2	0.466	0.745	Do Not Test
20.000 vs. 26.000	0.000396	2	0.371	0.796	Do Not Test

Comparisons for factor: **Gender within 20**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
M vs. F	0.00268	2	2.360	0.109	No

Comparisons for factor: **Gender within 26**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
F vs. M	0.00526	2	4.912	0.002	Yes

Comparisons for factor: **Gender within 30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
F vs. M	0.00329	2	2.896	0.053	No

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Raw data

This section represents the raw data tables produced from qRTPCR (2^-dct values) of the BACE1 mRNA expression in Cerebellum.

	F-20	F-26	F-30	M-27	M-30	M-33
	0.06	0.07	0.06	0.07	0.05	0.10
	0.06	0.04	0.12	0.07	0.03	0.07
	0.08	0.05	0.04	0.08	0.07	0.13
	0.04	0.04	0.06	0.10	0.12	
		0.06	0.07	0.08	0.08	
			0.07			
N	4	5	6	5	5	3
Mean	0.06	0.05	0.07	0.08	0.07	0.10
STDEV	0.02	0.01	0.02	0.01	0.03	0.03
SEM	0.01	0.01	0.01	0.01	0.02	0.02

Statistics

Two Way Analysis of Variance Wednesday, June 23, 2010, 11:11:38 AM

Data source: BACE1 2^-DCT CERE in Notebook 1
General Linear Model
Dependent Variable: BACE1

Normality Test:	Passed	(P = 0.059)
Equal Variance Test:	Passed	(P = 0.366)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Gender	1	0.00373	0.00373	7.369	0.013
AGE	2	0.00261	0.00131	2.583	0.098
Gender x AGE	2	0.000202	0.000101	0.200	0.820
Residual	22	0.0111	0.000506		
Total	27	0.0166	0.000617		

The difference in the mean values among the different levels of Gender is greater than would be expected by chance after allowing for effects of differences in AGE. There is a statistically significant difference (P = 0.013). To isolate which group(s) differ from the others use a multiple comparison procedure.
The difference in the mean values among the different levels of AGE is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Gender. There is not a statistically significant difference (P = 0.098).
The effect of different levels of Gender does not depend on what level of AGE is present. There is not a statistically significant interaction between Gender and AGE. (P = 0.820)
Power of performed test with alpha = 0.0500: for Gender : 0.678
Power of performed test with alpha = 0.0500: for AGE : 0.289
Power of performed test with alpha = 0.0500: for Gender x AGE : 0.0500

Least square means for Gender :

Group	**Mean**	**SEM**
F	0.0603	0.00589
M	0.0840	0.00642

Least square means for AGE :

Group	**Mean**	**SEM**
20.000	0.0698	0.00755
26.000	0.0613	0.00711
30.000	0.0854	0.00795

Least square means for Gender x AGE :

Group	**Mean**	**SEM**
F x 20.000	0.0599	0.0112
F x 26.000	0.0516	0.0101
F x 30.000	0.0696	0.00918
M x 20.000	0.0797	0.0101
M x 26.000	0.0711	0.0101
M x 30.000	0.101	0.0130

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Gender**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
M vs. F	0.0237	2	3.839	0.013	Yes

Comparisons for factor: **AGE**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
30.000 vs. 26.000	0.0241	3	3.196	0.083	No
30.000 vs. 20.000	0.0157	2	2.020	0.167	Do Not Test
20.000 vs. 26.000	0.00845	2	1.152	0.424	Do Not Test

Comparisons for factor: **AGE within F**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
30.000 vs. 26.000	0.0180	3	1.873	0.397	No
30.000 vs. 20.000	0.00975	2	0.950	0.509	Do Not Test
20.000 vs. 26.000	0.00829	2	0.777	0.588	Do Not Test

Comparisons for factor: **AGE within M**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
30.000 vs. 26.000	0.0302	3	2.598	0.181	No
30.000 vs. 20.000	0.0216	2	1.857	0.203	Do Not Test
20.000 vs. 26.000	0.00861	2	0.855	0.552	Do Not Test

Comparisons for factor: **Gender within 20**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
M vs. F	0.0198	2	1.857	0.203	No

Comparisons for factor: **Gender within 26**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
M vs. F	0.0195	2	1.938	0.184	No

Comparisons for factor: **Gender within 30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
M vs. F	0.0316	2	2.813	0.059	No

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Raw data

This section represents the raw data tables produced from qRTPCR (2^-dct values) of the APP mRNA expression in Cerebellum.

	F-20	F-26	F-30	M-27	M-30	M-33
	0.41	0.42	0.46	1.24	0.88	1.09
	0.49	0.49	0.39	0.88	0.42	0.80
	0.53	0.46	0.29	1.83	1.01	0.90
	0.37	0.33	0.45	1.19	1.56	0.50
		0.46		0.65	0.68	
N	4	5	4	5	5	4
Mean	0.45	0.43	0.40	1.15	0.91	0.82
STDEV	0.07	0.06	0.08	0.44	0.42	0.25
SEM	0.04	0.03	0.05	0.22	0.21	0.14

Statistics

Two Way Analysis of Variance Wednesday, June 23, 2010, 11:17:48 AM
Data source: APP 2^-DCT CERE in Notebook 2
General Linear Model
Dependent Variable: APP
Normality Test: Failed (P < 0.050)
Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Gender	1	1.915	1.915	23.115	<0.001
AGE	2	0.165	0.0824	0.995	0.386
Gender x AGE	2	0.0970	0.0485	0.586	0.566
Residual	21	1.740	0.0828		
Total	26	4.026	0.155		

The difference in the mean values among the different levels of Gender is greater than would be expected by chance after allowing for effects of differences in AGE. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.
The difference in the mean values among the different levels of AGE is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Gender. There is not a statistically significant difference (P = 0.386).
The effect of different levels of Gender does not depend on what level of AGE is present. There is not a statistically significant interaction between Gender and AGE. (P = 0.566)
Power of performed test with alpha = 0.0500: for Gender : 0.997
Power of performed test with alpha = 0.0500: for AGE : 0.0500
Power of performed test with alpha = 0.0500: for Gender x AGE : 0.0500
Least square means for Gender :

Group	Mean	SEM
F	0.426	0.0803
M	0.962	0.0773

Least square means for AGE :

Group	Mean	SEM
20.000	0.802	0.0965
26.000	0.671	0.0910
30.000	0.610	0.102

Least square means for Gender x AGE :

Group	Mean	SEM
F x 20.000	0.449	0.144
F x 26.000	0.432	0.129
F x 30.000	0.398	0.144
M x 20.000	1.155	0.129
M x 26.000	0.909	0.129
M x 30.000	0.823	0.144

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Gender**

Comparison	Diff of Means	p	q	P	P<0.050
M vs. F	0.536	2	6.799	<0.001	Yes

Comparisons for factor: **AGE**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
20.000 vs. 30.000	0.192	3	1.932	0.376	No
20.000 vs. 26.000	0.131	2	1.398	0.334	Do Not Test
26.000 vs. 30.000	0.0604	2	0.626	0.663	Do Not Test

Comparisons for factor: **AGE within F**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
20.000 vs. 30.000	0.0515	3	0.358	0.965	No
20.000 vs. 26.000	0.0169	2	0.124	0.931	Do Not Test
26.000 vs. 30.000	0.0346	2	0.253	0.860	Do Not Test

Comparisons for factor: **AGE within M**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
20.000 vs. 30.000	0.332	3	2.430	0.222	No
20.000 vs. 26.000	0.245	2	1.907	0.192	Do Not Test
26.000 vs. 30.000	0.0863	2	0.632	0.660	Do Not Test

Comparisons for factor: **Gender within 20**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
M vs. F	0.706	2	5.168	0.002	Yes

Comparisons for factor: **Gender within 26**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
M vs. F	0.477	2	3.706	0.016	Yes

Comparisons for factor: **Gender within 30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
M vs. F	0.425	2	2.955	0.049	Yes

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Raw data

This section represents the raw data tables produced from qRTPCR (2^-dct values) of the ASN mRNA expression in Hippocampus.

	F-6	F-26	F-30	M-6	M-30	M-33
	0.141	0.074	0.038	0.613	0.536	0.613
	0.062	0.062	0.097	0.401	0.860	0.547
	0.071	0.112	0.089	0.694	0.663	0.558
	0.227	0.380	0.112	0.872	0.415	0.663
	0.186	0.084	0.159	1.052	1.968	0.410
	0.209	0.121	0.130	0.729		0.812
N	6	6	6	6	5	6
Mean	0.137	0.142	0.099	0.727	0.888	0.558
STDEV	0.072	0.134	0.044	0.249	0.626	0.095
SEM	0.032	0.060	0.019	0.111	0.313	0.043

Statistics

Two Way Analysis of Variance Wednesday, June 23, 2010, 3:34:02 PM
Data source: Data 1 in Notebook 1
General Linear Model
Dependent Variable: ASN

Normality Test: Failed (P < 0.050)
Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
GENDER	1	3.219	3.219	46.451	<0.001
AGE	2	0.149	0.0747	1.077	0.354
GENDER x AGE	2	0.0944	0.0472	0.681	0.514
Residual	29	2.010	0.0693		
Total	34	5.380	0.158		

The difference in the mean values among the different levels of GENDER is greater than would be expected by chance after allowing for effects of differences in AGE. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.
The difference in the mean values among the different levels of AGE is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in GENDER. There is not a statistically significant difference (P = 0.354).
The effect of different levels of GENDER does not depend on what level of AGE is present. There is not a statistically significant interaction between GENDER and AGE. (P = 0.514)
Power of performed test with alpha = 0.0500: for GENDER : 1.000
Power of performed test with alpha = 0.0500: for AGE : 0.0593
Power of performed test with alpha = 0.0500: for GENDER x AGE : 0.0500
Least square means for GENDER :

Group	Mean	SEM
F	0.131	0.0620
M	0.739	0.0641

Least square means for AGE :

Group	Mean	SEM
6.000	0.438	0.0760
26.000	0.514	0.0797
30.000	0.352	0.0760

Least square means for GENDER x AGE :

Group	Mean	SEM
F x 6.000	0.149	0.107
F x 26.000	0.139	0.107
F x 30.000	0.104	0.107
M x 6.000	0.727	0.107
M x 26.000	0.888	0.118
M x 30.000	0.601	0.107

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :
Comparisons for factor: **GENDER**

Comparison	Diff of Means	p	q	P	P<0.050
M vs. F	0.608	2	9.639	<0.001	Yes

Comparisons for factor: **AGE**

Comparison	Diff of Means	p	q	P	P<0.050
26.000 vs. 30.000	0.161	3	2.071	0.322	No
26.000 vs. 6.000	0.0755	2	0.970	0.498	Do Not Test
6.000 vs. 30.000	0.0858	2	1.129	0.431	Do Not Test

Comparisons for factor: **AGE within F**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
6.000 vs. 30.000	0.0453	3	0.421	0.952	No
6.000 vs. 26.000	0.0104	2	0.0965	0.946	Do Not Test
26.000 vs. 30.000	0.0349	2	0.325	0.820	Do Not Test

Comparisons for factor: **AGE within M**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
26.000 vs. 30.000	0.288	3	2.553	0.186	No
26.000 vs. 6.000	0.161	2	1.432	0.320	Do Not Test
6.000 vs. 30.000	0.126	2	1.175	0.413	Do Not Test

Comparisons for factor: **GENDER within 6**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
M vs. F	0.578	2	5.375	<0.001	Yes

Comparisons for factor: **GENDER within 26**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
M vs. F	0.749	2	6.649	<0.001	Yes

Comparisons for factor: **GENDER within 30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
M vs. F	0.497	2	4.621	0.003	Yes

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Raw data

This section represents the raw data tables produced from qRTPCR (2^-dct values) of the SOD2 mRNA expression in Hippocampus.

	F-6	F-26	F-30	M-6	M-30	M-33
	0.37	0.15	0.12	0.26	0.12	0.23
	0.21	0.19	0.21	0.07	0.20	0.19
	0.26	0.26	0.17	0.17	0.16	0.15
	0.24	0.11	0.27	0.22	0.25	0.18
	0.24	0.27	0.35	0.26	0.33	0.14
	0.30	0.34	0.32	0.14		
N	6	6	6	6	5	5
Mean	0.27	0.20	0.22	0.20	0.21	0.18
STDEV	0.06	0.07	0.09	0.08	0.08	0.03
SEM	0.03	0.03	0.04	0.04	0.04	0.02

Statistics

Two Way Analysis of Variance Wednesday, June 23, 2010, 3:03:26 PM
Data source: HIPPO RNA in Notebook 1
General Linear Model
Dependent Variable: SOD2
Normality Test: Passed (P = 0.232)
Equal Variance Test: Passed (P = 0.415)

Source of Variation	DF	SS	MS	F	P
Gender	1	0.0177	0.0177	3.149	0.086
AGE	2	0.00125	0.000623	0.111	0.895
Gender x AGE	2	0.00850	0.00425	0.756	0.479
Residual	29	0.163	0.00562		
Total	34	0.191	0.00563		

The difference in the mean values among the different levels of Gender is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in AGE. There is not a statistically significant difference (P = 0.086).
The difference in the mean values among the different levels of AGE is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Gender. There is not a statistically significant difference (P = 0.895).
The effect of different levels of Gender does not depend on what level of AGE is present. There is not a statistically significant interaction between Gender and AGE. (P = 0.479)

Power of performed test with alpha = 0.0500: for Gender : 0.279
Power of performed test with alpha = 0.0500: for AGE : 0.0500
Power of performed test with alpha = 0.0500: for Gender x AGE : 0.0500
Least square means for Gender :

Group	Mean	SEM
F	0.243	0.0177
M	0.198	0.0182

Least square means for AGE :

Group	Mean	SEM
6.000	0.229	0.0216
26.000	0.215	0.0227
30.000	0.218	0.0216

Least square means for Gender x AGE :

Group	Mean	SEM
F x 6.000	0.271	0.0306
F x 26.000	0.219	0.0306
F x 30.000	0.240	0.0306
M x 6.000	0.187	0.0306
M x 26.000	0.212	0.0335
M x 30.000	0.196	0.0306

Raw data

This section represents the raw data tables produced from qRTPCR (2^-dct values) of the HO-1 mRNA expression in Hippocampus.

	F-6	F-26	F-30	M-6	M-30	M-33
	0.0110	0.0079	0.0061	0.0039	0.0041	0.0074
	0.0066	0.0079	0.0136	0.0022	0.0070	0.0102
	0.0076	0.0085	0.0083	0.0040	0.0071	0.0068
	0.0060	0.0070	0.0173	0.0043	0.0055	0.0053
	0.0054	0.0091	0.0197	0.0062	0.0042	0.0068
	0.0072	0.0086	0.0189	0.0038	0.0123	0.0048
N	6	6	6	6	6	6
Mean	0.0073	0.0081	0.0130	0.0041	0.0056	0.0073
STDEV	0.0022	0.0008	0.0058	0.0014	0.0015	0.0018
SEM	0.0010	0.0004	0.0026	0.0006	0.0007	0.0008

Statistics

Two Way Analysis of Variance Wednesday, June 23, 2010, 3:25:45 PM
Data source: Data 1 in Notebook 1
Balanced Design
Dependent Variable: HO1
Normality Test: Failed (P < 0.050)
Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
GENDER	1	0.000141	0.000141	16.453	<0.001
AGE	2	0.000139	0.0000696	8.147	0.001
GENDER x AGE	2	0.0000495	0.0000247	2.894	0.071
Residual	30	0.000256	0.00000855		
Total	35	0.000586	0.0000167		

The difference in the mean values among the different levels of GENDER is greater than would be expected by chance after allowing for effects of differences in AGE. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.
The difference in the mean values among the different levels of AGE is greater than would be expected by chance after allowing for effects of differences in GENDER. There is a statistically significant difference (P = 0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.
The effect of different levels of GENDER does not depend on what level of AGE is present. There is not a statistically significant interaction between GENDER and AGE. (P = 0.071)

Power of performed test with alpha = 0.0500: for GENDER : 0.979
Power of performed test with alpha = 0.0500: for AGE : 0.917
Power of performed test with alpha = 0.0500: for GENDER x AGE : 0.351
Least square means for GENDER :

Group	Mean
F	0.00983
M	0.00588

Std Err of LS Mean = 0.000689
Least square means for AGE :

Group	Mean
6.000	0.00569
26.000	0.00742
30.000	0.0105

Std Err of LS Mean = 0.000844
Least square means for GENDER x AGE :

Group	Mean
F x 6.000	0.00732
F x 26.000	0.00817
F x 30.000	0.0140
M x 6.000	0.00407
M x 26.000	0.00667
M x 30.000	0.00690

Std Err of LS Mean = 0.00119
All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **GENDER**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
F vs. M	0.00395	4.056	0.000327	0.050	Yes

Comparisons for factor: **AGE**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
30.000 vs. 6.000	0.00476	3.987	0.000396	0.017	Yes
30.000 vs. 26.000	0.00303	2.542	0.0164	0.025	Yes
26.000 vs. 6.000	0.00172	1.445	0.159	0.050	No

Comparisons for factor: **AGE within F**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
30.000 vs. 6.000	0.00668	3.960	0.000	0.017	Yes
30.000 vs. 26.000	0.00584	3.461	0.002	0.025	Yes
26.000 vs. 6.000	0.000842	0.499	0.621	0.050	No

Comparisons for factor: **AGE within M**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
30.000 vs. 6.000	0.00283	1.678	0.104	0.017	No
26.000 vs. 6.000	0.00261	1.544	0.133	0.025	No
30.000 vs. 26.000	0.000226	0.134	0.894	0.050	No

Comparisons for factor: **GENDER within 6**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
F vs. M	0.00326	1.930	0.063	0.050	No

Comparisons for factor: **GENDER within 26**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
F vs. M	0.00149	0.885	0.383	0.050	No

Comparisons for factor: **GENDER within 30**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
F vs. M	0.00711	4.211	0.000	0.050	Yes

Raw data

This section represents the raw data tables produced from qRTPCR (2^-dct values) of the BACE1 mRNA expression in Hippocampus.

	F-6	F-26	F-30	M-6	M-30	M-33
	0.12	0.05	0.03	0.07	0.05	0.09
	0.07	0.05	0.08	0.03	0.07	0.08
	0.07	0.08	0.05	0.06	0.05	0.06
	0.08	0.06	0.10	0.08	0.06	0.05
	0.06	0.09	0.16	0.08	0.09	0.05
	0.06	0.12	0.16	0.04		0.04
N	6	6	6	6	5	6
Mean	0.08	0.07	0.08	0.07	0.06	0.06
STDEV	0.02	0.02	0.05	0.02	0.02	0.02
SEM	0.01	0.01	0.02	0.01	0.01	0.01

Statistics

Two Way Analysis of Variance Wednesday, June 23, 2010, 3:15:23 PM
Data source: Data 1 in Notebook 1
General Linear Model
Dependent Variable: BACE1
Normality Test: Passed (P = 0.431)
Equal Variance Test: Passed (P = 0.053)

Source of Variation	DF	SS	MS	F	P
GENDER	1	0.00367	0.00367	4.143	0.051
AGE	2	0.000744	0.000372	0.419	0.662
GENDER x AGE	2	0.00117	0.000584	0.658	0.525
Residual	29	0.0257	0.000887		
Total	34	0.0314	0.000923		

The difference in the mean values among the different levels of GENDER is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in AGE. There is not a statistically significant difference (P = 0.051).
The difference in the mean values among the different levels of AGE is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in GENDER. There is not a statistically significant difference (P = 0.662).
The effect of different levels of GENDER does not depend on what level of AGE is present. There is not a statistically significant interaction between GENDER and AGE. (P = 0.525)
Power of performed test with alpha = 0.0500: for GENDER : 0.389
Power of performed test with alpha = 0.0500: for AGE : 0.0500
Power of performed test with alpha = 0.0500: for GENDER x AGE : 0.0500
Least square means for GENDER :

Group	Mean	SEM
F	0.0825	0.00702
M	0.0620	0.00725

Least square means for AGE :

Group	Mean	SEM
6.000	0.0694	0.00860
26.000	0.0686	0.00902
30.000	0.0787	0.00860

Least square means for GENDER x AGE :

Group	Mean	SEM
F x 6.000	0.0765	0.0122
F x 26.000	0.0739	0.0122
F x 30.000	0.0971	0.0122
M x 6.000	0.0622	0.0122
M x 26.000	0.0633	0.0133
M x 30.000	0.0604	0.0122

Raw data

This section represents the raw data tables produced from qRTPCR (2^-dct values) of the APP mRNA expression in Hippocampus.

	F-6	F-26	F-30	M-6	M-30	M-33
	0.723	0.370	0.426	0.404	0.427	0.719
	0.656	0.573	1.222	0.643	0.678	0.711
	0.785	0.745	0.583	0.554	0.539	0.507
	0.669	0.633	0.695	0.723	0.599	0.466
	0.627	0.222	0.842	0.509		0.583
	0.811	0.270	0.780			
N	6	6	6	5	4	5
Mean	0.69	0.51	0.75	0.57	0.56	0.60
STDEV	0.06	0.21	0.30	0.12	0.11	0.12
SEM	0.03	0.09	0.14	0.06	0.06	0.06

Statistics

Two Way Analysis of Variance Wednesday, June 23, 2010, 3:20:20 PM
Data source: Data 1 in Notebook 1
General Linear Model
Dependent Variable: APP
Normality Test: Passed (P = 0.405)
Equal Variance Test: Passed (P = 0.309)

Source of Variation	DF	SS	MS	F	P
GENDER	1	0.0124	0.0124	0.350	0.559
AGE	2	0.0738	0.0369	1.044	0.366
GENDER x AGE	2	0.213	0.106	3.011	0.066
Residual	27	0.955	0.0354		
Total	32	1.278	0.0399		

The difference in the mean values among the different levels of GENDER is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in AGE. There is not a statistically significant difference (P = 0.559).
The difference in the mean values among the different levels of AGE is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in GENDER. There is not a statistically significant difference (P = 0.366).
The effect of different levels of GENDER does not depend on what level of AGE is present. There is not a statistically significant interaction between GENDER and AGE. (P = 0.066)
Power of performed test with alpha = 0.0500: for GENDER : 0.0500
Power of performed test with alpha = 0.0500: for AGE : 0.0548
Power of performed test with alpha = 0.0500: for GENDER x AGE : 0.368
Least square means for GENDER :

Group	Mean	SEM
F	0.646	0.0443
M	0.607	0.0486

Least square means for AGE :

Group	Mean
6.000	0.639
26.000	0.563
30.000	0.678

Std Err of LS Mean = 0.0569

Least square means for GENDER x AGE :

Group	Mean	SEM
F x 6.000	0.712	0.0768
F x 26.000	0.469	0.0768
F x 30.000	0.758	0.0768
M x 6.000	0.567	0.0841
M x 26.000	0.658	0.0841
M x 30.000	0.597	0.0841