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Regulation of Iron-Related Molecules In the Rat Hippocampus: Sex- and Age-Associated Differences

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Abstract. Iron accumulation, especially that of free oxidized ferrous iron, has been shown to induce tissue oxidative damage and contribute to brain aging and the development of neurodegenerative disease. Here we examine whether sex and advanced age affect the expression of iron-related molecules that participate in regulating free iron levels (heme oxygenase 1 (HO1), iron-regulatory protein 1 (IRP1), and ferritin heavy chain (FTH)) and whether changes in the expression of these molecules are associated with differences in the expression of alpha-synuclein (ASN) which is thought to be a critical regulator in the pathogenesis of neurodegeneration. Using a well-established aging animal model, we demonstrate that the expression of HO1, FTH, and IRP1 mRNAs is higher in the female hippocampus than that observed in male Fischer 344/NNiaHSD x Brown Norway/BiNia (F344BN) rats, regardless of age group. Consistent with these sex-associated alterations in iron-related regulators, the expression of ASN mRNA and protein in the female hippocampus was lower than that found in male rats. These results suggest a sex-dependent difference in regulating the expression of molecules involved in iron metabolism and neurodegeneration. A similar finding in humans, if present, may help to shed light on why sex may affect the incidence of neurodegenerative disorders.

Key words: Sex, Aging, Hippocampus, Heme oxygenase 1 (HO1), Ferritin, Iron-regulatory protein 1 (IRP1), Alpha-synuclein (ASN)

Introduction

It is well known that advanced age is associated with an elevated risk of developing Parkinson's disease and other neurodegenerative disorders [1, 2]. The mechanism(s) responsible for the pathological development of these disorders is currently unclear, but some have suggested that a diminished ability to respond to stress, elevations in tissue reactive oxygen species (ROS), and alterations in metal homeostasis may be involved [3-7]. Indeed, age-associated increases in tissue iron are associated with increased tissue ROS and neuronal damage, leading to the hypothesis that the accumulation of iron in the brain may play a key role in brain aging and in the development of age-related neurodegenerative disease [1, 8-10]. In addition to aging, it is also likely that sex plays a role, as males exhibit a significantly higher incidence of Parkinson's disease than females [2], although no significant differences in iron level have been reported between male and female brains [11]. Information regarding how sex may influence the development of age-associated neurodegenerative diseases is currently sparse.

Heme oxygenase (HO) is a ubiquitously expressed enzyme responsible for the degradation of heme [12]. The expression of HO1 is induced by cellular stress, such as that caused by exposure to UV light, elevated levels of pro-oxidants, or metal

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dyshomeostasis [13, 14]. Deregulation of HO1 has been linked to several neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease [15-17]. When released from heme, free iron can be sequestered by binding with ferritin, a globular protein that contains 24 subunits composed of two functionally-distinct proteins that are encoded at various ratios by two different genes: ferritin heavy chain (FTH, 21 kDa) and ferritin light chain (FTL, 19 kDa). FTH-rich ferritin predominates in cells and tissues that have high oxidative respiration because FTH functions to catalyze the oxidation of Fe^{2+} [18]. These ferritin molecules not only function in iron storage, but also help to prevent increased free-iron-mediated oxidative damage. Although not well understood, the regulation of iron metabolism is, at least partially, regulated by the iron-regulatory proteins (IRPs) [19]. IRP1 is the most abundant isoform that can bind to mRNAs bearing iron-responsive elements (IREs) and decrease expression of iron-related proteins such as FTH and FTL [19, 20].

Increased expression of alpha-synuclein (ASN) is thought to relate to the development of neurodegenerative diseases [21]. Elevated ASN mRNA and protein expression have been reported in Parkinson's patients [22-24]. The ASN molecules can aggregate and form abnormal protein clusters in the Lewy bodies, a structure commonly found in the brains of Parkinson's patients [22-25]. Interestingly, an IRE-like stem-loop structure has been found in the 5' untranslated region (5'UTR) of ASN mRNA [26, 27], which suggests the possibility of a link between iron dysregulation and the development of neurodegenerative diseases.

Here we hypothesize that sex and advanced age affect the expression of iron-related molecules, which in turn alter the expression of alpha-synuclein. It has been suggested that the hippocampus is more vulnerable to oxidative damage, and that dopamine denervation in Parkinson's disease is associated with ASN deposition in the hippocampus [28]. Therefore, we chose to examine how sex and advanced age affect iron-related molecules and expression of ASN in the hippocampus of Fischer 344/NNiaHSD x Brown Norway/BiNia (F344BN) rats, a well-established aging animal model [29]. Our findings provide evidence for sex-dependent differences in the expression of molecules involved in iron metabolism and neurodegeneration in the rat hippocampus.

Materials and Methods

Animals. Animal care and use were approved by the Marshall University Institutional Animal Care and Use Committee (IACUC approval number #346), and the "Principles of Laboratory Animal Care" (NIH publications No. 86-23, revised 1985) were followed. Male (6- and 33-month-old) and female (6- and 30-month-old) F344BN rats (n = 6 in each of the 4 groups) were obtained from the National Institute of Aging (Bethesda, MD). Animals were housed 2 per cage in a vivarium approved by the American Association of Laboratory Animal Care. Housing conditions included a 12-hour light/dark cycle, with the temperature maintained at 22 ± 2°C as previously outlined [5]. Food and water were provided *ad libitum*, and animals were allowed to recover from shipment for at least 2 weeks before experimentation. Based on rat survival curves obtained from the National Institute of Aging, the age of a 30-month-old female F344BN is approximately equivalent to that of a 33-month-old male rat. Six-month-old male and female rats (n = 6 per group) were used as the adult controls.

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 as detailed elsewhere [5]. Brains were removed and rinsed in Krebs solution to remove any superficial blood. The hippocampus was quickly separated from the whole brain, blotted dry, and immediately frozen in liquid nitrogen [30]. Tissue samples were stored at -80°C until further use.

RNA extraction and real-time quantitative PCR (RT-qPCR). The hippocampus tissue was homogenized in TRI reagent (Ambion, Austin, TX) at a ratio of 1 mL of TRI reagent per 100 mg of tissue, as outlined by the manufacturer. After a 5 minute incubation, the homogenates were centrifuged (12,000 x g for 15 min at 4°C). The supernate

Table 1. PCR primers used to quantify the levels of HO1, FTH, IRP1, ASN, β-actin, and GAPDH transcripts

HO1 forward primer 5'-TGCTCGCATGAACACTCTG-3'
HO1 reverse primer 5'-TCCTCTGTCAGCAGTGCCT-3'
FTH forward primer 5'-CTGAATGCAATGGAGTGTGC-3'
FTH reverse primer 5'-TCTTGCGTAAGTTGGTCACG-3'
IRP1 forward primer 5'-TTGCCGAGCCCTTGGACCCT-3'
IRP1 reverse primer 5'-TCACAGTTCCGAACGGCGGC-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'
GAPDH forward primer 5'-GGCTCTCTGCTCCTCCCTGTTCT-3'
GAPDH reverse primer 5'-GCCAAATCCGTTCACACCGACCTT-3'

was collected and mixed with 200 μL chloroform (Sigma-Aldrich, Inc., St. Louis, MO). After a 10 minute incubation at room temperature, the aqueous phase of the mixed solution was collected via centrifugation (12,000 x g for 15 min at 4°C) and then mixed with 500 μL isopropanol (Sigma-Aldrich Inc., St. Louis, MO). RNA pellets were precipitated (12,000 x g for 10 min at 4°C), washed with 75% ethanol, and then dried under vacuum (Eppendorf Vacufuge Model 5301 Vacuum Concentrator, Westbury, NY). After dissolving the RNA pellet with 50 μL of nuclease-free water, RNA was quantified at 260 nm using a NanoVue UV-Vis Spectrophotometer (GE Healthcare, Piscataway, NJ). RNA integrity was confirmed using a RNA 6000 Nano Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA samples were stored at -80°C until further use.

Reverse transcription coupled with RT-qPCR was used to quantify the relative abundance of HO1, FTH, IRP1, and ASN mRNAs in hippocampus samples. Complimentary DNA (cDNA) libraries were synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA) as per the manufacturer's instructions. RT-qPCR was set up using the Power SYBR Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA) as previously outlined [31, 32]. PCR reactions were run on an ABI 7000 Real-Time PCR system (Applied Biosystems Inc., Foster City, CA) using the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute [31, 32]. All experiments were repeated in triplicate using β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The forward and reverse primers **(Table 1)** were purchased from Integrated DNA technologies (San Diego, CA). The expression of HO1, FTH, IRP1, and ASN mRNAs were normalized to the geometric means of β-actin and GAPDH mRNAs, and the $2^{-\Delta Ct}$ method was used to calculate the relative mRNA expression level [31, 32].

Immunoblotting analysis. Hippocampus protein was extracted via homogenization in Pierce tissue protein extraction reagent buffer (T-PER; Thermo Fisher Scientific Inc., Rockford, IL) containing protease inhibitors (#P8340; Sigma-Aldrich Inc., St. Louis, MO) and phosphatase inhibitors (#P5726; Sigma-Aldrich Inc., St. Louis, MO) as previously detailed [5, 33]. The total protein concentration of each homogenate was determined using the Pierce 660 nm protein assay (Pierce, Rockford, IL). Samples containing equal amounts of protein (to a final concentration of 2.0 μg/μL) were boiled in SDS-loading buffer (Sigma-Aldrich Inc., St. Louis, MO), and then separated on precast 10 or 15% SDS-PAGE gels (Lonza Rockland, Rockland, ME) before being transferred to nitrocellulose membranes as described elsewhere [5, 33]. Gels were stained with RAPID protein reagent (G-Biosciences, St. Louis, MO) to confirm equal protein loading and the transfer efficiency of proteins onto the membranes.

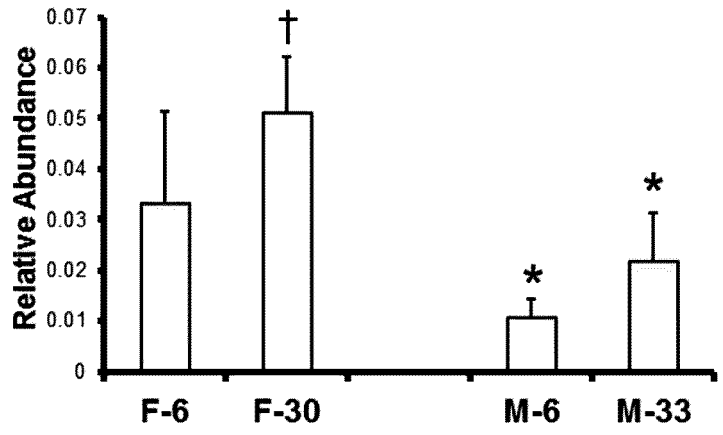

Figure 1 *Heme oxygenase 1 (HO1) mRNA expression in the hippocampus in adult and aged female and male F344BN rats (F-6 and F-30: 6- and 30-month-old female rats; M-6 and M-33: 6- and 33-month-old male rats). Data are means ± SEM (n = 6 per group). There are gender ($p \leq 0.05$) and age ($p \leq 0.05$) differences, but there is no interaction between gender and age ($p = 0.47$). †: age difference ($p \leq 0.05$) within the same gender; *: gender difference ($p \leq 0.05$) within the same age.*

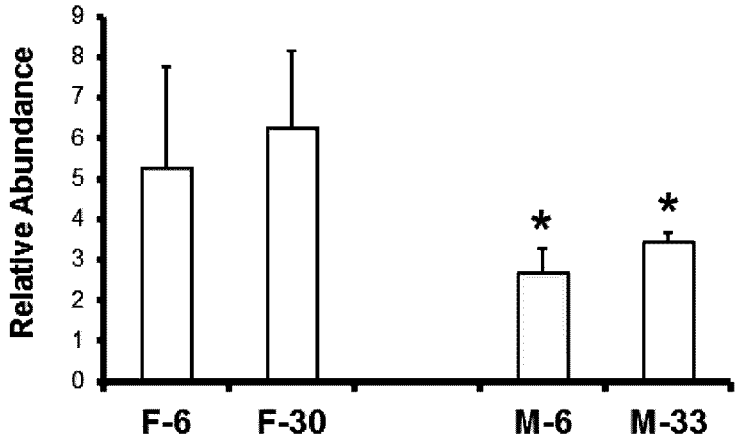

Figure 2 *Ferritin heavy chain (FTH) mRNA expression in the hippocampus in adult and aged female and male F344BN rats (F-6 and F-30: 6- and 30-month-old female rats; M-6 and M-33: 6- and 33-month-old male rats). Data are means ± SEM (n = 6 per group). There is a difference by gender ($p \leq 0.05$), but there is neither an age effect ($p = 0.20$) nor an interaction between gender and age ($p = 0.86$). *: gender difference ($p \leq 0.05$) within the same age.*

To probe for the antigen of interest, the nitrocellulose membranes were first incubated with an antigen-specific primary antibody, followed by incubation with the appropriate horseradish peroxidase (HRP)-linked secondary antibody. The primary antibody against ASN was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the GAPDH antibody and HRP-linked anti-rabbit IgG secondary antibody were obtained from Cell Signaling Technology (Danvers, MA). The expression of the target protein was visualized following incubation of the nitrocellulose membranes with ECL Western Blotting reagent (GE Healthcare Bio-Sciences, Piscataway, NJ). The amount of target protein present was quantified using AlphaEaseFC image analysis software, and was normalized to the amount of GAPDH.

Data analysis. Results are presented as the mean ± SEM. A two-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test was performed using the SigmaStat 3.5 statistical program (SPSS Science Inc., Chicago, IL) to determine the effect of sex (male versus female), age (adult versus aged), and the interaction of sex × age on both mRNA and protein expression. A value of $p \leq 0.05$ was considered to be statistically significant.

Results

The expression of HO1, FTH, and IRP1 mRNAs in rat hippocampus. The expression of HO1 mRNA was higher in the female hippocampus than that observed in the male animals ($p \leq 0.05$; **Figure 1**). The expression of HO1 mRNA in the aged female hippocampus was higher than that in the adult female rats ($p \leq 0.05$), while HO1 mRNA expression was unaltered with aging in males (**Figure 1**). The expression of both FTH and IRP1 mRNAs in the female hippocampus were higher than in male rats ($p \leq 0.05$; **Figures 2, 3**), and were unaltered with aging in rats of either sex (**Figures 2, 3**).

The expression of ASN mRNA in rat hippocampus. The expression of ASN mRNA in the female hippocampus was lower than that observed in either the adult or aged male rats ($p \leq 0.05$; **Figure 4**) and was unaltered with aging in rats of either sex (**Figure 4**).

The expression of ASN protein in rat hippocampus. The expression of ASN protein was lower in

the female hippocampus than in that of males ($p \leq 0.05$; **Figure 5**). The expression of ASN protein in the aged female hippocampus was higher than that in adult female rats ($p \leq 0.05$), and was unaltered with aging in males **(Figure 5)**.

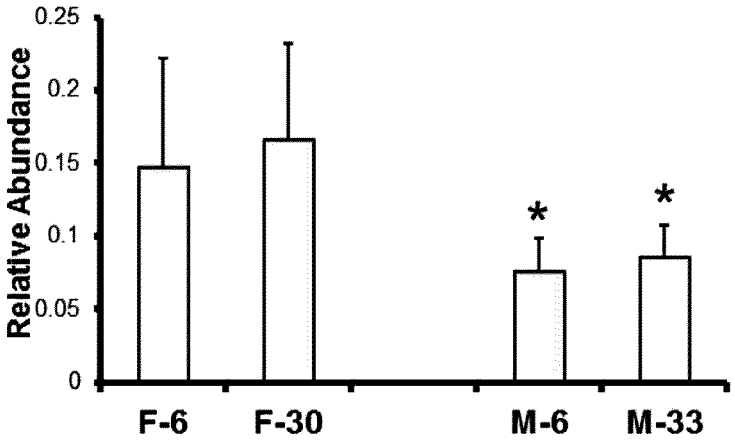

Figure 3 *Iron-regulatory protein (IRP1) mRNA expression in the hippocampus in adult and aged female and male F344BN rats (F-6 and F-30: 6- and 30-month-old female rats; M-6 and M-33: 6- and 33-month-old male rats). Data are means ± SEM (n = 6 per group). There is a gender effect (p ≤0.05), but there is neither an age (p = 0.56) nor an interaction between gender and age (p = 0.86). *: gender difference (p ≤ 0.05) within the same age.*

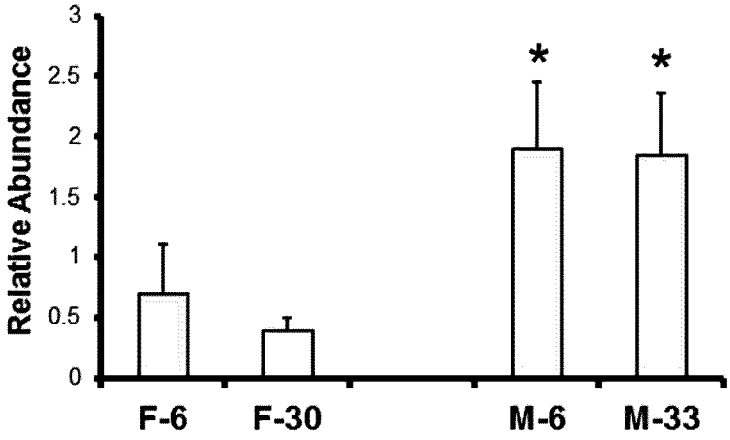

Figure 4 *Alpha-synuclein (ASN) mRNA expression in the hippocampus in adult and aged female and male F344BN rats (F-6 and F-30: 6- and 30-month-old female rats; M-6 and M-33: 6- and 33-month-old male rats). Data are means ± SEM (n = 6 per group). There is a difference by gender (p ≤ 0.05), but there is neither an age effect (p = 0.31) nor an interaction between gender and age (p = 0.49). *: gender difference (p ≤ 0.05) within the same age.*

Discussion

It is well known that females and males display different rates of aging, while others have postulated that the interaction of sex with age may play a role in the development of disease [2, 34-36]. Using the F344BN rat model [5, 10, 29], we have herein demonstrated significant sex-associated differences in the regulation of iron- and neurodegeneration-related molecules in the hippocampus. These data support the possibility that sex may play a role in the development of age- and iron-related neurodegenerative diseases.

HO1 is an inducible heme oxygenase that functions to degrade the pro-oxidant heme in response to stress. Consistent with previous work [37], we demonstrated that the expression of HO1 is increased with age **(Figure 1)**. We also found, for both adult and aged rats, that HO1 expression is higher in the female hippocampus when compared to that observed in the age-matched male hippocampus **(Figure 1)**. As an important antioxidant enzyme and a dynamic sensor of cellular oxidative stress, the increased HO1 expression in the aged female hippocampus may protect cells by degrading pro-oxidant metalloporphyrins. This finding, if present, may help to explain why some have suggested that the female rat brain exhibits better control of oxidative stress balance than does the male brain in animals of similar ages [11, 38].

Free ferrous iron generated from the HO1-induced degradation of heme is a potential stimulator of oxidative stress, as it mediates the formation of free radicals [39, 40]. Although no significant differences in total iron level between male and female brains of Sprague-Dawley rats have been reported [11], it is worthwhile to note that it is free ferrous iron that mediates the formation of free radicals [39, 40]. When bound to the iron storage protein ferritin, free ferrous iron is converted to a non-toxic form [18, 41]. Given that the expression of FTH is

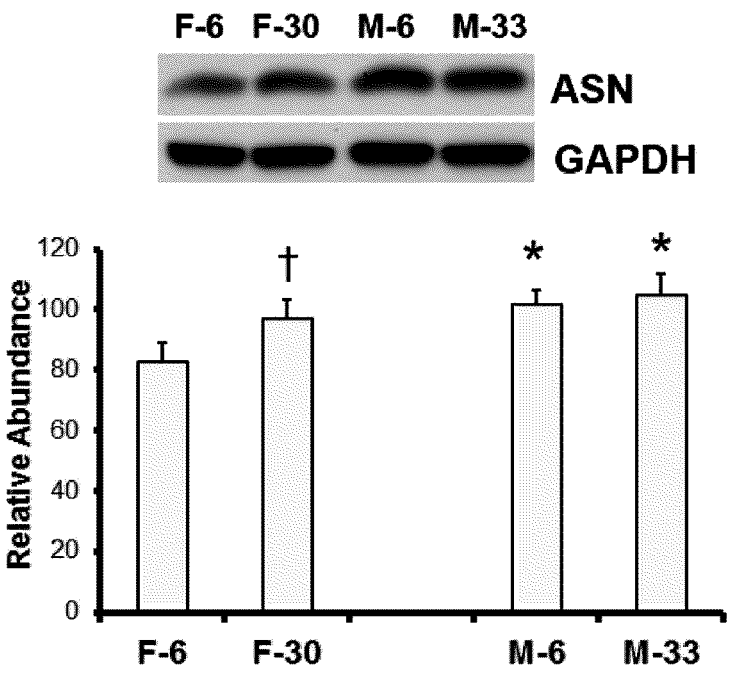

Figure 5. *Alpha-synuclein (ASN) protein expression in the hippocampus in adult and aged female and male F344BN rats (F-6 and F-30: 6- and 30-month-old female rats; M-6 and M-33: 6- and 33-month-old male rats). Data are means ± SEM (n = 6 per group). There are gender ($p \leq 0.05$) and age ($p \leq 0.05$) differences. †: age difference ($p \leq 0.05$) within the same gender; *: gender difference ($p \leq 0.05$) within the same age.*

higher in the female hippocampus than in that of age-matched males **(Figure 2)**, it is possible that the female brain is better able to "capture" free iron from tissues, thus potentially protecting against its deleterious effects and the associated oxidative damage. Future research that includes the measurement of free ferrous iron in hippocampal tissue will provide valuable information for testing this hypothesis.

It has been shown that iron can induce the misfolding and aggregation of ASN protein to form the main component of the intracytoplasmic Lewy bodies, an abnormal protein cluster found in the brains of Parkinson's patients [22-25]. Interestingly an IRE-like stem-loop structure has recently been found in the 5'UTR of ASN mRNA [26]. Studies have shown that when intracellular free iron content is low, IRP1 binds to the IRE in the 5'UTR of mRNA and inhibits its translation. Conversely, increased intracellular free iron concentration functions to inhibit the binding of IRP1 to IRE thereby allowing translation and synthesis of proteins [18, 26]. Additional data from *in vitro* experiments has suggested that knockdown of IRP1 results in increased expression of both ASN mRNA and protein in human SK-N-SH cells [42]. In the current study, we observed higher expression of HO1 **(Figure 1)** and FTH **(Figure 2)** in the female hippocampus, which is consistent with the possibility of diminished free iron content. This finding, if present, suggests a higher binding of IRP1 to the IRE in the 5'UTR of target mRNA and inhibition of target protein translation [18, 26]. This possibility is supported by our findings of decreased ASN mRNA and protein in the female hippocampus (**Figures 4 and 5**, respectively). Although the results of the present study alone are not sufficient to determine whether there is a causative link between the development of neurodegenerative diseases, ASN expression, and iron dysregulation, they may provide insight into the mechanism(s) potentially responsible for the significantly higher incidence of Parkinson's disease in men than in women [2]. Further study using pharmacological intervention and/or transgenic animals will no doubt shed light on the definitive mechanism.

In summary, this study demonstrates sex- and age-dependent differences in the expression of iron-related molecules (HO1, FTH, and IRP1) in the hippocampus of F344BN rats. These differences are linked to differences in the expression of ASN, a critical protein related to the development of neurodegenerative diseases. These data provide the molecular basis for further study to address the effects of sex, iron accumulation, and advanced age on the pathological development of neurodegenerative diseases.

Acknowledgements

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