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Gene Profile Analysis Implicates Klotho as an Important Contributor to Aging Changes in Brain White Matter of the Rhesus Monkey

JAMES A. DUCE,[1] SONIA PODVIN,[2] WILLIAM HOLLANDER,[1] DAVID KIPLING,[3] DOUGLAS L. ROSENE,[4,5] AND CARMELA R. ABRAHAM[1,6]*

[1]*Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts*
[2]*Department of Pharmacology, Boston University School of Medicine, Boston, Massachusetts*
[3]*Department of Pathology, Cardiff University School of Medicine, Heath Park, Cardiff, United Kingdom*
[4]*Department of Anatomy and Neurobiology, Boston University School of Medicine, Boston, Massachusetts*
[5]*Yerkes National Primate Research Center, Emory University, Atlanta, Georgia*
[6]*Department of Medicine, Boston University School of Medicine, Boston, Massachusetts*

KEY WORDS
oxidative stress; inflammation; corpus callosum; high-density oligonucleotide microarray; quantitative real time-PCR

ABSTRACT
Conventional studies of brain changes in normal aging have concentrated on gray matter as the locus for cognitive dysfunction. However, there is accumulating evidence from studies of normal aging in the rhesus monkey that changes in white matter may be a more critical factor in cognitive decline. Such changes include ultrastructural and biochemical evidence of myelin breakdown with age, as well as more recent magnetic resonance imaging of global loss of forebrain white matter volume and magnetic resonance diffusion tension imaging evidence of increased diffusivity in white matter. Moreover, many of these white matter changes correlate with age-related cognitive dysfunction. Based on these diverse white matter findings, the present work utilized high-density oligonucleotide microarrays to assess gene profile changes associated with age in the white matter of the corpus callosum. This approach identified several classes of genes that were differentially expressed in aging. Broadly characterized, these genes were predominantly related to an increase in stress factors and a decrease in cell function. The cell function changes included increased cell cycle inhibition and proteolysis, as well as decreased mitochondrial function, signal transduction, and protein translation. While most of these categories have previously been reported in functional brain aging, this is the first time they have been associated directly with white matter. Microarray analysis has also enabled the identification of neuroprotective response pathways activated by age in white matter, as well as several genes implicated in lifespan. Of particular interest was the identification of Klotho, a multifunctional protein that regulates phosphate and calcium metabolism, as well as insulin resistance, and is known to defend against oxidative stress and apoptosis. Combining the findings from the microarray study enabled us to formulate a model of white matter aging where specific genes are suggested as primary factors in disrupting white matter function. In conclusion, the overall changes described in this study could provide an explanation for aging changes in white matter that might be initiated or enhanced by an altered expression of life span associated genes such as Klotho.

INTRODUCTION

Brain aging processes are known to be highly complex, involving multiple systems of different cell types and alternate cellular pathways. Several of the well-studied biological processes linked to normal aging include inflammatory and immune responses, oxidative stress, and altered calcium regulation (Butterfield et al., 1999; Mattson, 2007; Sastre et al., 2003; Sloane et al., 1999). While precise mechanisms of these processes in brain aging are still not well understood, most hypotheses concerning age-related gene changes have been proposed based on variations in gene expression in brain samples containing unspecified mixtures of white matter and gray matter or on specific gray matter regions such as the hypothalamus, cerebral cortex, or hippocampus (Blalock et al., 2003; Jiang et al., 2001; Lee et al., 2000). As with previous protein studies, these gray matter regions showed age-related gene expression alterations corresponding to inflammatory response and oxidative stress. Gene changes in the hypothalamus and cortex also indicated evidence of irregular metabolism and protein processing.

However, strong evidence has suggested that brain white matter may also play a crucial role in age-related changes and impairment of cognitive function (Hinman and Abraham, 2007; Peters et al., 1996). As yet, pure white matter has not been genetically studied for normal age differences in any species. Gene expression profiles in the white matter have been only determined in multiple sclerosis, murine models of demyelination, or in schizophrenia (Kubicki et al., 2005; Whitney et al., 1999, 2001). This study in the rhesus monkey is the first to

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*Correspondence to: Dr. Carmela R. Abraham, Department of Biochemistry, Boston University School of Medicine, 715 Albany Street, K620, Boston, MA 02118, USA. E-mail: cabraham@bu.edu

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investigate transcriptional alterations in white matter with age. The rhesus monkey offers a valuable model of normal human aging, as the monkey brain most closely resembles that of human. Monkeys are able to perform cognitive tasks adopted from human neuropsychological test batteries (Moss et al., 1997) and age-related changes are not confounded by the potentially undetectable presence of early stage Alzheimer's disease (Kemper, 1994). Compared with human samples, RNA degradation may also be reduced as post mortem delays can be largely eliminated, enabling a more representative data set for age.

Previous studies of brain white matter from aging rhesus monkey have indicated myelin membrane changes and loss of myelinated nerve fibers (Marner et al., 2003; Peters and Sethares, 2002; Sandell and Peters, 2003). These changes were associated with the activation of microglia and astrocytes and increased expression of stress-related mediators including inducible nitric oxide synthase, peroxynitrite, calpain-1, and activated forms of complement (Duce et al., 2006; Hinman et al., 2004; Sloane et al., 1999, 2003). More recently *in vivo* studies of the aging rhesus monkey have demonstrated both loss of myelin volume using structural MRI scans (Wisco et al., 2007) and change in white matter diffusivity characteristic of white matter pathology based on MR-DTI measurements (Makris et al., 2006).

Through determining how changes in gene expression within the white matter relate to these earlier findings, a model of age-related disruption that occurs specifically in white matter can be predicted. From this model, specific molecules that may be primary factors in disrupting white matter function with the onset of age are suggested. One such molecule is the antisenescence protein Klotho, previously reported to cause several age-related disorders when mutated in a mouse model (Kuro-o et al., 1997). Both Klotho mRNA and protein expression are downregulated in the brains of monkeys, rats, and mice as a function of age. While Klotho's function in the brain remains elusive, it is known as a single-pass transmembrane protein with multiple functions in the kidney, particularly in protecting against oxidative stress. Here we propose that Klotho is implicated in the age-related disruption of brain white matter.

MATERIALS AND METHODS

Subjects

Adult male rhesus monkeys (*Macaca mulatta*) of known birth date were obtained from the colonies of the Yerkes National Primate Center (YNPRC). Prior to selection for this study, all monkeys received full medical examinations and medical records were screened to ensure that there was no previous history of severe illness or chronic drug administration. During the course of the study, all monkeys were visually inspected on a daily basis and where given general medical examinations every 4 months to ensure their continued good health. Subjects for RNA analysis were four young adult (4–7 years old) and four aged ($\geq$20 years old) rhesus monkeys. For biochemical analysis, a total of eight subjects were studied (four at 4–12 years old and four at $\geq$20 years old). Both the YNPRC and Boston University Medical Center are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care and all procedures were approved by the Institutional Animal Care and Use Committees at both facilities.

Female B6D2F1 mice, at 4, 12, 18, and 24 months (n = 3/age), and male F33 rats at 4, 18, 24, or 28 months (n = 4/age), depending on availability, were purchased from the NIA Aged Rodent Tissue Bank for confirmatory biochemical studies on Klotho.

Perfusion and Tissue Preparation

Monkeys were deeply anaesthetized and transcardially perfused with 4L of Krebs-Heinseleit buffer (6.41 mM Na_2HPO_4, 1.67 mM NaH_2CO_3, 137 mM NaCl, 2.68 mM KCl, 5.55 mM Glucose, 0.34 mM $CaCl_2$, 2.14 mM $MgCl_2$ pH 7.4) at 4°C and prepared in RNase free conditions. Following perfusion the brain was immediately dissected into neuroanatomically relevant blocks, flash frozen on dry ice, and stored at −80°C.

RNA Isolation and Affymetrix GeneChip Processing

The splenium of the corpus callosum was used for RNA isolation. First, total RNA was prepared using TRIzol reagent and following the manufacturer's protocol (Invitrogen). Briefly, 1 mL of TRIzol solution was added to 1 g of tissue and homogenized with 10 strokes of a hand-held Teflon-coated pestle followed by 10 passages through a 20-ga syringe needle. Extracted RNA was further cleaned using a Qiagen RNeasy kit and dissolved into RNase-free water. Integrity and concentration of RNA extracted was assessed by spectrophotometry and using an Agilent RNA Nano labchip read by a 2100 bioanalyser (Agilent Technologies, CA). From 10 μg of total RNA, double stranded cDNA synthesis was performed using the Superscript Choice system (Life Technologies). Biotinylated complimentary RNA (cRNA) was synthesized from cDNA using a RNA transcript labeling kit (Enzo Biochem). cRNA was further cleaned using a Qiagen RNeasy kit and then fragmented. cRNA before and after fragmentation was again assessed for purity and quantity using spectrophotometry and Agilent labchip analysis.

For protein expression analysis of monkey brain tissue, ~100 mg of medullae (rich in white matter) and Area 9 of the prefrontal cortex (rich in gray matter) were cut from frozen tissue, and mechanically homogenized in 10× (v:w) RIPA buffer with protease inhibitors (50 mM tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 5 μg/mL aprotinin, 5 μg/mL leupeptin)

at 60 r.p.s. for 6 pulses. Homogenates were incubated on ice for 1 h, then centrifuged at 10, 000*g* for 30 min. Protein concentration was determined by bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). For the mouse and rat studies, right hemispheres containing gray and white matter from three animals per age group were weighed and homogenized as above.

Affymetrix GeneChip Analysis

Fifteen micrograms of fragmented cRNA was hybridized to each U133A chip (Affymetrix) for 16 h in a 45°C rotating oven (Affymetrix). After hybridization, chips were washed and stained with streptavidin-phycoerythrin using a fluidics station (Affymetrix) and scanned using a GeneChip® Scanner 3000.

Microarray suite software (MAS 5.1, Affymetrix) calculated intensity values for each probe, identifying and removing contributions from stray hybridization signals and making absent, present or marginal calls for each mRNA. Each call was determined using algorithms within the software averaging gene intensity for each set of probe pairs. Each intensity value and presence/absence call was then copied into an Excel 11.3 workbook (SR1; Microsoft, Redmond, WA) for data transformations, filtering, and statistical analysis to observe expression patterns for the aged compared with the young monkey group.

RNA Processing and Analysis by Quantitative Real-Time PCR (qRT-PCR)

One microgram of total RNA from the corpus callosum of the same eight animals previously used for GeneChip analysis was treated with DNase (Qiagen) during further cleaning by a Qiagen RNeasy kit. DNA free total RNA was subsequently reverse transcribed using Superscript Choice system (Life Technologies).

Primers for selected genes were designed by Primer Express (PE Biosystems) (shown in supplementary Table 3). To ensure no amplification of contaminating genomic DNA, one primer from each primer pair was designed to traverse an exon boundary. Primers were initially optimized by standard PCR using Taq DNA polymerase (Qiagen) and visualized by ethidium bromide stain on a 2% low melting agarose gel. If analysis revealed a single band of the correct size the gene was further optimized with SYBR green (Applied Biosystems) detection on an Applied Biosystems ABI Prism 7900HT sequence detection system. Further optimization with SYBR green involved determining the lowest primer concentration combination and subsequent cDNA concentrations to create a concentration standard curve to validate efficiency of the assay. Triplicates of 10-μL reactions containing optimal primer and cDNA template concentrations triplicates were used for quantitation. A two-step PCR reaction was carried out as follows: 1 cycle of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 59°C for 1 min. This was followed by a dissociation curve beginning at 55°C and increasing by 1–100°C every 10 s, with SYBR green fluorescence measured at every interval. RNase P was used as a control for each target gene.

Relative quantitation of the difference between the aged and the young samples were done using the comparative C_T method ($2^{-\Delta CT}$) as comparable standard curves between target genes and RNA polymerase were obtained. Genes from each age set were tested for statistical significance ($P \leq 0.05$), relative to the control, by student *t* test.

Western Blotting and Quantitation

Twenty micrograms of protein homogenates were run on a 4–20% tris-glycine SDS-PAGE gel and transferred to PVDF membrane (Millipore). Membranes were blocked in 5% nonfat milk and incubated overnight at 4°C with 0.05 μg/mL rat monoclonal anti-Klotho (no. KM2076) (gift from Kyowa Hakko Kogyo, Tokyo, Japan) in Tris-buffered saline containing 0.05% Tween-20 (TBST). Blots were washed in TBST and incubated with goat anti-rat horseradish peroxidase (HRP) conjugated secondary antibodies (Jackson ImmunoLabs) for 1 h. Following further washes, proteins were detected on Kodak BioMax XAR film with Millipore Imobilon Chemiluminescent HRP substrate.

Klotho protein expression was quantified densitometrically (Scion Image Beta 4.0.2 software, NIH, Bethesda, MD) by normalizing to a reference protein. Rhesus monkey protein bands were normalized either against MOSP (myelin-oligodendrocyte specific protein) (0.1 mg/mL mouse anti-MOSP, Chemicon, Temecula, CA) or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (10 ng/mL mouse anti-GAPDH, Ambion, Austin, TX) and mouse bands were normalized against GAPDH.

RESULTS

Analysis of Age-Dependent Gene Expression in the Corpus Callosum by Affymetrix Microarray U133A Chips

Previous findings have indicated that the high degree of sequence identity between human and rhesus monkey genomic DNA is sufficient to allow human oligonucleotide-based microarrays to be used effectively in analyzing gene expression on the rhesus monkey (Chismar et al., 2002; Enard et al., 2002; Kayo et al., 2001; Marvanova et al., 2003). While these reports did not utilize the Affymetrix U133A human arrays, housekeeping control genes on the U133A array were all highly expressed and showed consistent expression throughout the oligonucleotide set (data not shown). Absolute measures of gene expression ("present" calls) from the monkey samples were also observed to be ~30%, a value consistent with human brain samples run on the U133A chips and the

monkey samples previously run on other types of arrays.

The corpus callosum, an area of relatively pure corticocortical white matter that originates and terminates exclusively in the cerebral neocortex, can easily be dissected without gray matter contamination, and is known to be pathologically affected with age (Peters and Sethares, 2002). To identify gene changes related to aging in the white matter while controlling for multiple comparison error, a series of filters were added to the data prior to significance testing. The number of false positives expected from multiple-comparison error has previously been shown to be directly proportional to the total number of transcript probes tested statistically (Blalock et al., 2003; Miller et al., 2001).

From the initial 22, 284 probe sets on the U133A chip, three *a priori* data filtering steps were incorporated to refine the total genes to be tested. The first filter excluded genes rated "absent" in at least five out of the eight chips used in this study. From the original probe set for the U133A chip, 14,213 genes were excluded. The second filter eliminated all present transcript sets representing unannotated "expressed sequence tags" (ESTs) and control probe sets. As explained in previous reports, although ESTs contain true positives, they would add comparisons to be managed without providing further insight into known pathways (Blalock et al., 2003; Norris et al., 2005). From the 8,071 present genes, this second filter excluded a further 1,912. The final filter eliminated genes that were different by less than 20% of the maximal difference in mean between age groups. As the maximal difference in means between young and old was 6.125, the 80% "cutoff" was determined at 1.225. This filter therefore eliminated all genes that indicated a mean difference below 1.225. This was used to eliminate genes that would not be major biomarkers for age-advancing changes in gene expression (Blalock et al., 2003). These genes of more subtle change could however represent genes changed at middle age. But it is only with the incorporation of middle-aged animals to the present dataset that these genes can be analyzed and this will be carried out within our group in the near future. This criterion excluded a further 3,960 genes and left 2,199 genes to be tested by 2-tailed homoscedastic student *t* test.

The *t* test identified 227 genes that differed significantly ($P \leq 0.05$) between aged monkeys and young adults. Of these, 118 were upregulated with age while 109 were downregulated (Fig. 1). The expected false positives at a significance value of $P = 0.05$ were 110 ($0.05 \times 2{,}199$), thus genes significantly altered with age were above the false-discovery rate. While the high false-discovery rate indicated a high noise to signal ratio, this type of multiple testing correction is often found to be overly stringent (Liang et al., 2007). It is also important to note that the filter steps used prior to this statistical analysis eliminated the possibility of obtaining too many false positives, but may have also eliminated true positive genes (termed false negatives). This would have been particularly evident for excluded ESTs and these gene sets are currently being analyzed separately.

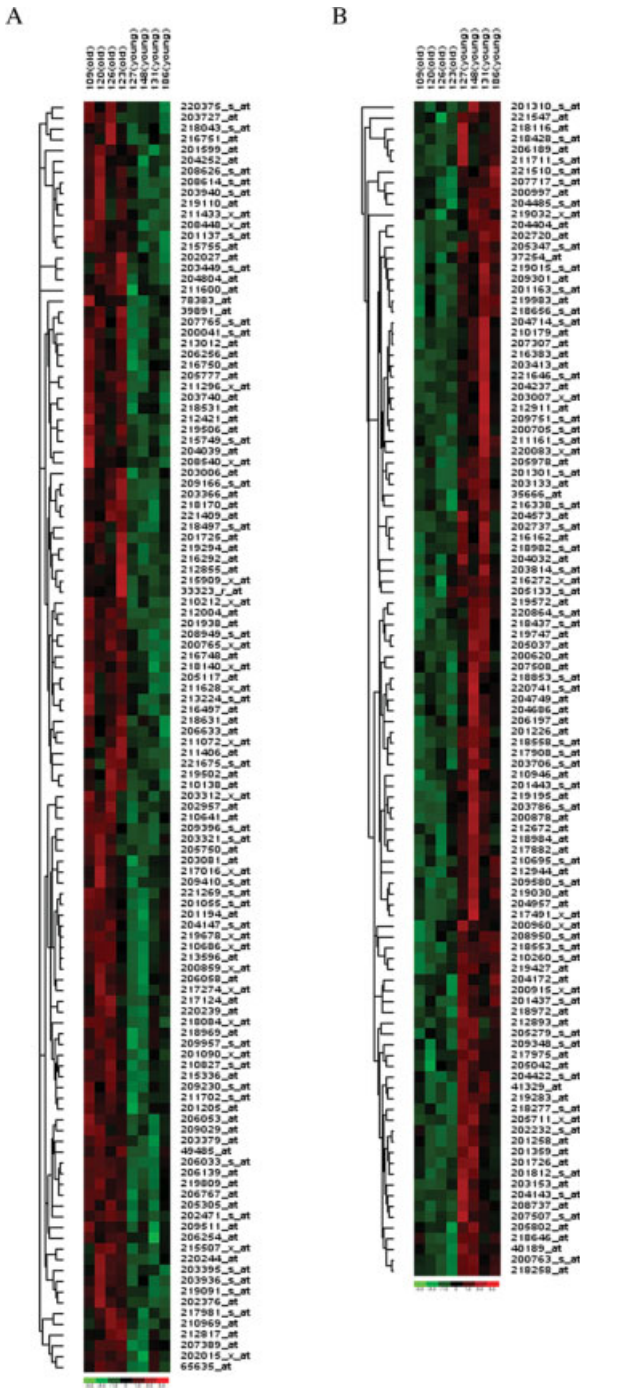

Fig. 1. Heat maps of probe sets significantly altered by age in Rhesus Monkey white matter. Significantly increased (**A**) and decreased (**B**) Affymetrix U133A probe set IDs (listed vertically) are indicated for individual tissue samples (listed across the top of each map). The color range from red (high expression) to green (low expression), as indicated by the expression color bar, represent the logged normalized expression levels used to generate each map. Fold changes for significantly altered genes and gene names are shown in supplementary Table 1.

The number of genes shown to significantly change in mRNA levels was observed to be much smaller in corpus callosum white matter than in aging prefrontal cortex of monkey (Duce and Abraham, unpublished observations)

and previously reported in humans (Lu et al., 2004). While this is an inevitable problem when using scarce biological material, which could be enhanced with the dual problems of an out-bred strain of animal and relatively small sample size, these findings may also relate to the lower numbers of cells and the relatively homogeneous composition of cell types in white compared with gray matter. It is thought, even though these results show a smaller number of significantly altered genes, they provide a more accurate representation of age changes in white matter compared with previous mixed sample reports as changes in gray matter may obscure many of the subtle changes observed in the present data. This would also explain why genes shown in the present data to have large differences in pure white matter have not been reported previously.

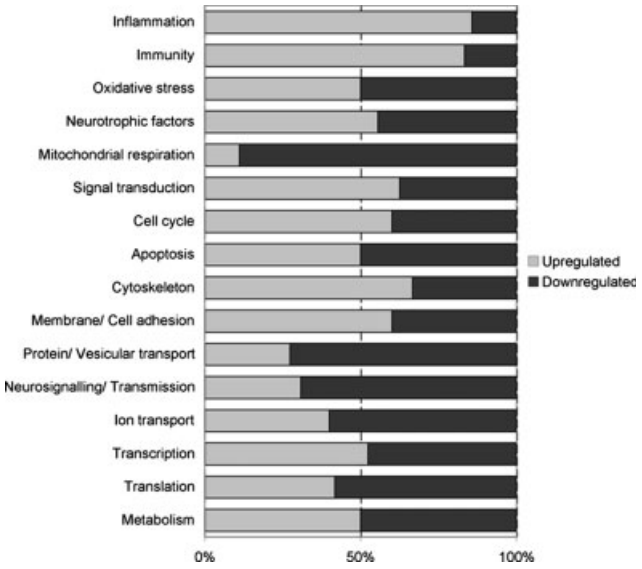

Fig. 2. Percentage of characterized genes altered with age using Type 1 analysis. Genes significantly altered with age in the corpus callosum ($P < 0.05$) were characterized and grouped into relevant categories depending on their primary function. Within each category genes were ordered dependent on regulation with age. Trends in regulation were observed within some classes shown in relation to the total genes within the group. Gene categories predominantly upregulated included inflammation and immunity, whereas downregulated groups included mitochondrial respiration, protein/vesicle transport and neurotransmission.

Functional Categorization

For functional characterization of the 227 probe sets (Fig. 1), two approaches were taken. The first approach used extensive literature searches primarily in PubMed (NCBI) but also including SwissProt (ExPASy), NetAffx (Affymetrix), InterPro (EMBL-EBI), and Ensembl (EMBI-EBI and Sanger) databases to assign genes to "functional" categories. In the second approach, genes were processed using expression analysis systematic explorer software (EASE) (http://david.niaid.nih.gov/david/ease.htm). EASE assigned genes to "biological process" and "molecular function" categories from a hierarchical database maintained by the gene ontology (GO) consortium (http://www.geneontology.org) (Ashburner et al., 2001). Functional categories of identified genes overrepresented, relative to the expected representation of identified genes for an average category, were given an EASE score by the software, based on a statistical value derived from a modified Fisher's exact test.

From the first approach, 190 genes were determined to have a relevant known function that could be involved in age-related alterations in brain function (Supplementary Table 1), while the remaining 37 genes had no known significant function within the brain (Supplementary Table 2). Based on the assignment to function we then determined the fraction of significantly altered genes either up- or downregulated within each category (Fig. 2). While a relatively large percentage of all significant age-related gene expression changes identified fell into the transcription/translation, metabolism and cell cycle categories (respectively 23.7, 13.7, and 10.5% of all significant genes), the gene categories that were predominantly upregulated included inflammation (85.7% of genes in category) and immunity (83.3% of genes in category) whereas the downregulated categories included mitochondrial respiration (88.9% of genes in category), protein/vesicular transport (72.7% of genes in category), and neurotransmission (69.2% of genes in category) (Fig. 2). In general, most upregulated genes seem likely to be related to stress responses, while the downregulated genes seem to be associated with impairment of cellular function.

EASE analysis for the second approach detected functional categories that were significantly overrepresented as age-sensitive genes (Table 1). EASE scores were observed to be lower than expected in both up and downregulated categories. This was determined to be the result of the small number of total genes obtained after filtering, from which categories overrepresented with significant genes were sought. A greater population can strengthen analyses of coregulation (Mirnics et al., 2001) and the full array set, subjected to the same analysis, yielded better significance, even if the same categories indicated a lower ratio of association in the category (data not shown). Table 1 shows that upregulated genes were predominantly involved in cell cycle and DNA replication. Closer examination of the genes within these categories found they where mostly inhibitory (e.g. CDK2 and MPP6). Interestingly, another upregulated category involved protein proteolysis. Downregulated categories were found to be similar to categories determined with the first approach. These were notably involved in mitochondrial respiration, and types of cellular transport.

Many similarities were obtained between the two approaches for assigning age-related gene changes to functional categories. Values of significance in overrepresentation were obtained by EASE analysis but it was limited by not distinguishing between inhibitor and activator genes, which only manual sorting of the genes was able to provide. Manual analysis also enabled us to

TABLE 1. Categories Overrepresented with Age in White Matter by EASE Analysis

Upregulated	(Total: 118/2,199; 5.4%)	EASE	Downregulated	(Total: 109/2,199; 5.0%)	EASE
Mitotic cell cycle	(9/58; 15.5%)	0.004	Hydrogen ion transporter activity	(6/31; 19.4%)	0.002
DNA replication	(6/29; 20.7%)	0.005	Monovalent cation transport	(7/43; 16.3%)	0.003
DNA-directed RNA polymerase II	(3/8; 37.5%)	0.027	Nucleotidyltransferase activity	(5/20; 25%)	0.008
Cell proliferation	(15/186; 8.1%)	0.030	Proton transport	(4/16; 25%)	0.013
			Cation transport	(8/59; 13.6%)	0.030
			Ion transport	(9/81; 11.1%)	0.048

Categories relating to "biological processes" and "molecular function" determined by EASE analysis to be significantly overrepresented with age in the white matter microarray analysis ($P \leq 0.05$; EASE score). After each category description is shown the number of age-changed genes compared with the total number of genes within the category, as well as the percentage represented by that ratio. The analogous ratios for the total upregulated or downregulated genes are also shown (total).

detect single genes of interest known to be associated with life span. Table 2. illustrates some of the genes of interest represented in both types of analysis and used for further discussion.

Supportive Expression Analysis for Age-Altered Genes by qRT-PCR

To confirm results from microarray analysis qRT-PCR was carried out on 12 genes. These 12 genes were chosen from the significantly altered genes of known function (Supplementary Table 1) without regard to the magnitude of age-related changes or the level of statistical significance in an attempt to validate candidate genes by alternative quantitative methods. For optimization, all 12 genes were amplified with DNA pooled from one old and one young animal, to guarantee a signal independent of expression differences with age. From the original 12 genes, 5 of the strongest amplified genes that also showed no contaminating bands at 59°C annealing temperature were continued for analysis by qRT-PCR using SYBR green. While the seven genes not quantitated by qRT-PCR may have represented false positives from microarray, further optimization by primer redesign is required to determine this and is ongoing within the study.

Triplicate PCR amplifications of each gene from DNA of each of the eight animals showed similar amplification curves in each animal and were determined to be in close enough range to RNA polymerase, the control gene, to enable the use of a comparative C_T method ($2^{-\Delta CT}$). Taking the mean from the $2^{-\Delta CT}$ calculated for each animal and then comparing old with young animals, fold differences for each gene could be determined. Variances in expression were observed between the microarray and qRT-PCR analysis (for example HLA-DPB1), but qRT-PCR significantly confirmed the microarray analysis by illustrating the same direction in up or downregulation of gene expression with age (Fig. 3).

Analysis of Age-Related Changes in Klotho

The antisenescence gene Klotho was identified in this study to be downregulated with age in the white matter ($P = 0.03$ and fold $= -1.52$). To confirm the decrease in Klotho with age at the protein level, immunoblot analysis was carried out in brain areas rich in white matter. Areas rich in gray matter were also analyzed to determine if decreased Klotho expression was an age-related phenomena in the whole brain. Previously, Klotho protein expression has been detected by immunoblot in mice as two isoforms; 130 and 65 kDa. This was confirmed in this study using mouse brain samples, however, detection in monkey brain tissue only found the 130-kDa isoform (Fig. 4).

Klotho protein expression in white matter from monkeys was significantly decreased with age by linear regression ($P = 0.007$) when compared with the white matter control protein MOSP, supporting microarray data (Fig. 4A). Moreover, comparing Klotho protein expression in gray and white matter across the age groups demonstrated that Klotho was only decreased in white matter, as prefrontal cortex showed no significant difference in expression with age (data not shown).

Expression was also determined in other species to find if this was a common occurrence in mammals. Whole hemispheres from B6D2F1 mice indicated a rise in both isoforms of Klotho from infancy until middle-age (12 months) after which Klotho was steadily reduced to below levels detected in young (Figs. 4B,C). Hence, there was a significant difference ($P < 0.05$) for the 130-kDa isoform at both the 12 months and 18 months of age compared with the oldest animals (24 months) (Fig. 4B) and the 12 months compared with 24 months for the 65-kDa isoform (Fig. 4C). A similar expression profile was also observed in rats (data not shown).

DISCUSSION

Functional Gene Groups Identified to be Involved in White Matter Aging

Expression fold change was subtle in the significant genes, but evidence of genes clustered with similarities in function has leant further support to the role of these age-related gene changes in the corpus callosum. The possible purpose of the functionally categorized genes in white matter changes with age is addressed in the following sections.

By incorporating both types of functional gene classification, the present microarray findings support and extend earlier biochemical and immunohistochemical studies of age-related changes in white matter (Butterfield et al., 1999; Hinman et al., 2004; Mattson, 2007; Sastre et al., 2003; Sloane et al., 1999). By integrating these earlier reports with the present findings a concept of white matter aging has been proposed and is illustrated in Fig. 5. Environmental and genetic factors that

TABLE 2A. Genes of Interest That May Produce Cytotoxicity in Aging White Matter

Lifespan gene	
203449_s_at	Telomeric repeat binding factor 1 (TERF1)
203366_at	Polymerase gamma (POLG)
205978_at	Klotho (KL)
Glial stress response	
208949_s_at	Lectin galactosidase-binding 3 (Galectin 3) (GALIG)
202376_at	Serine proteinase inhibitor alpha-1 antichymotrypsin (SERPINA3)
203936_s_at	Matrix metalloproteinase 9 (MMP-9)
207389_at	Glycoprotein 1B alpha (GP1BA)
219195_at	Peroxisome prolif. act. Receptor gamma coact. 1 (PPARGC1A)
201137_s_at	Major histocompatibility complex II (HLA-DPB1)
208448_x_at	Interferon, alpha 16 (INFA16)
210212_x_at	Mature T-cell proliferation 1 (MTCP1)
205133_s_at	Heat shock 10kDa protein 1 (HSPE1)
Reorganization of glial cytoskeleton	
208614_s_at	Filamin B, beta (FLNB)
200859_x_at	Filamin A, alpha (FLNA)
211072_x_at	Tubulin alpha 1 (K-alpha-1)
Oxidative and proteolytic injury	
210641_at	Calpain 9 (CAPN9)
206256_at	Carboxypeptidase N (CPN1)
213596_at	Caspase 4, apoptosis-related cysteine peptidase (CASP4)
209166_s_at	Mannosidase alpha 2B, 1 (MAN2B1)
211702_s_at	Ubiquitin specific protease 32 (USP32)
211296_x_at	Ubiquitin C (UBC)
220083_x_at	Ubiquitin carboxyl-terminal hydrolase L5 (UCHL5)
202232_s_at	Dendritic cell protein (hfl-B5)
Cell cycle inhibitor	
204039_at	CCAAT/enhancer binding protein alpha (CEBPA)
201938_at	CDK2-associated protein 1 (CDK2AP1)
203740_at	M-phase phosphoprotein 6 (MPP6)
209230_s_at	p8 protein (candidate of metastasis 1) (P8)
205777_at	Dual specificity phosphatase 9 (DUSP9)
33323_r_at	Stratifin (SFN)
204252_at	Cyclin-dependent kinase 2 (CDK2)
78383_at	Tumor protein p53-binding protein (TP53BPL)
221269_s_at	SH3 domain binding glutamic acid-rich protein 3 (SH3BGRL3)
DNA synthesis	
213012_at	Neural precursor cell expressed, develop down-reg 4 (NEDD4)
218428_s_at	REV1-like (REV1L)
40189_at	SET translocation (myeloid leukaemia-associated) (SET)
204957_at	Origin recognition complex subunit 5-like (ORC5L)
218437_s_at	Leucine zipper transcription factor-like 1 (LZTFL1)
Protein translation	
218558_s_at	Mitochondrial ribosomal protein L39 (MRPL39)
218982_s_at	Mitochondrial ribosomal protein S17 (MRPS17)
200705_s_at	Eukaryotic translation elongation factor 1 beta 2 (EEF1B2)
218258_at	RNA polymerase 1 polypeptide D (POLR1D)
212944_at	Mitochondrial ribosomal protein S6 (MRPS6)
200763_s_at	Ribosomal protein P1 (RPLP1)
201437_s_at	Eukaryotic translation initiation factor 4E (EIF4E)
201258_at	Ribosomal protein S16 (RPS16)
216383_at	Ribosomal protein L18a (RPL18A)
Signal transduction	
203081_at	Catenin beta interacting protein 1 (CTNNBIP1)
203006_at	Inositol polyphosphate-5-phosphotase (INPP5A)
203706_s_at	Frizzled homolog 7 (FZD7)
Respiratory chain	
203814_s_at	NAD(P)H dehydrogenase, quinone 2 (NQO2)
201226_at	NADH dehydrogenase 1 beta8 (NDUFB8)
220864_s_at	NADH dehydrogenase (ubiquinone) 1 alpha, sub. 13 (NDUFA13)
217491_x_at	Cytochrome c oxidase subunit VIIc (COX7C)
ATP synthesis	
207508_at	ATP synthase H+ transporting F0 complex subunit C (ATP5G3)
205711_x_at	ATP synthase H+ transporting F1 complex gamma 1 (ATP5C1)
Other ion transport	
208737_at	ATPase H+ transporting, lysosomal V1G1 (ATP6V1G1)
201443_s_at	ATPase, H+ transporting, lysosomal IP 2 (ATP6AP2)
Intracellular transport	
200960_x_at	Clathrin, Heavy polypeptide (CLTA)
219572_at	Ca2+-dependent activator protein for secretion 2 (CADPS2)
205037_at	RAB, member of RAS oncogene family-like 4 (RABL4)
Myelin and axonal injury	
209301_at	Carbonic anhydrase II (CA2)
Impairment of neurotransmission	
218553_s_at	Potassium channel tetramerization domain 15 (KCTD15)
210179_at	Potassium inwardly rectifying channel J13 (KCNJ13)

TABLE 2B. Genes of Interest That May Provide Neuroprotection in Aging White Matter

Activation of growth factors	
205117_at	Fibroblast growth factor 1 (acidic) (FGF1)
203379_at	Ribosomal protein S6 kinase, 1 (RPS6KA1)
209410_s_at	Growth factor receptor-bound protein 10 (GRB10)
206254_at	Epidermal growth factor (EGF)
211711_s_at	Phosphatase and tensin homolog (PTEN)
204422_s_at	Fibroblast growth factor 2 (basic) (FGF2)
204686_at	Insulin receptor substrate 1 (IRS1)
201163_s_at	Insulin-like growth factor binding protein 7 (IGFBP7)
Neuron axonal regeneration	
211600_at	Protein-tyrosine phosphatase receptor type O (PTPRO)
Glial proliferation; cell adhesion	
206033_s_at	Desmocollin 3 (DSC3)
211406_at	Immediate early response 3 interacting protein 1 (IER3IP1)
200775_x_at	Catenin alpha 1 (CTNNA1)
Glial proliferation; lipid turnover	
208950_s_at	Aldehyde dehydrogenase 7, A1 (ALDH7A1)
221675_s_at	Choline phosphotransferase 1 (CHPT1)
210969_at	Protein kinase N2 (PRK2)
210946_at	Phosphatidic acid phosphatase type 2A (PPAP2A)
204573_at	Carnitine O-octanoyltransferase (CROT)
203007_x_at	Lysophospholipase I (LYPLA1)
201301_s_at	Annexin A4 (ANXA4)

primarily contribute to age-related changes lead to metabolic and oxidative stress in the brain. With the common response to increased stress within the brain being activation of microglia and astrocytes, cytoskeletal reorganization is apparent from this study in the aged white matter. Glial activation leads to an increased expression of inflammatory and oxidative stress-related mediators, as confirmed in Table 2, and stress responses from microglial and astrocytic activation are postulated to cause oxidative and proteolytic-induced white matter injury through these pro-oxidant and pro-inflammatory responses (Fig. 5).

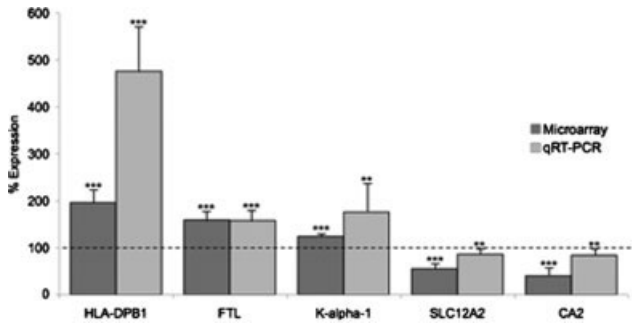

Fig. 3. Quantitative real time-PCR amplification of selected genes. Confirmation of significant difference in age of five genes indicated by microarray was carried out by qRT-PCR using SYBR green. While variances in expression values were observed between the microarray and qRT-PCR, all selected genes indicated the same highly significant difference in white matter mRNA levels due to age. Values represent the mean expression ± SD ($n = 4$) compared with young (represented at 100% expression) (**, $P \leq 0.05$; ***, $P \leq 0.01$).

White matter injury, as proposed by this model and observed postmortem, would be expected to produce a number of different effects, including depression of general cell function, and injury or loss of myelinated axons. Most of the genes altered in the aged white matter studies here represented an overall depression of cell function. The most significant decreases were associated with mitochondrial respiration and ion transport, while the most significant increases were in categories related to the inhibition of cell cycle and DNA replication (Table 1). The depressed expression of the Class 1 and 4 complexes of the electron transport respiratory chain were clearly evident as well as genes that regulate the coupling of oxidative phosphorylation to ATP synthesis. Peroxynitrite, iron as well as reactive oxygen and nitrogen species are all highly expressed in aging white matter and have been demonstrated to impair mitochondrial respiration (Lu et al., 2004; Mecocci et al., 1993; Vatassery, 2004).

While at first glance, the EASE analysis indicated an increase of cell cycle and DNA replication, further investigation showed that the increased cell cycle genes were predominantly inhibitors. Consistent with these cell cycle alterations is the suggested slowing of cell replication with genes associated with DNA synthesis. Inhibition of cell cycle and DNA replication would have also lead to the repression of genes involved in biosynthesis and protein translation. This included mRNA levels of the cytosolic ribosomal 40S and 60S subunits as well as the 28S and 39S subunits of mitochondrial ribosomal proteins. Signal transduction was also associated with depression of cell function as several G-protein coupled signaling pathways show a downregulation including the cAMP, phosphoinositide and Wnt pathways. These gene changes could operate to inhibit cell replication and differentiation. Other subtler, but still important, cellular functions were also evidently depressed, including other types of ion transport as well as endocytosis and intracellular transport (Table 2).

Myelin and axon injury may occur directly through oxidation and proteolysis from the white matter injury or via the depression of cell function (Fig. 5). An illustration of such injury is shown in the downregulation of carbonic anhydrase II (CA2), a myelin and oligodendrocyte constituent and regulator of intracellular pH and ion flux. A deficiency in CA2 has already been implicated in impairment of signaling and cognition (Sun and Alkon, 2002).

With myelin and axonal damage being present with age (Peters and Sethares, 2002; Sandell and Peters, 2003) impairment of neurotransmission will inevitably occur. While neurotransmitter-related proteins are not thought to have a major presence in white matter, there was a clear population of these genes decreased in aged white matter. While this may indicate a major transport of RNA from the neuronal cell body to the axons in a compensatory process activated with age, it is more likely to reflect glial plasticity in which these neurotransmitters play roles in intra- and intercellular signaling, cell survival, and myelin responses to injury (Charles et al. 2003; Schousboe et al., 2004; Wilke et al., 2004). Through impairment of this form of neurotransmission, cognitive impairment would be an unavoidable result. However, it was also clear from this study that neuroprotective events were occurring within the aged white matter as many genes known to be neuroprotective or associated with regeneration are significantly altered.

Neuroprotective Events Present in Aged White Matter

One of the biggest neuroprotective events shown to occur in the aging white matter was an increase in genes involved in growth factor pathways. Pathways involving insulin-like growth factor (IGF-1), fibroblast growth factor, and epidermal growth factor were all increased and have previously been reported to promote glial proliferation and formation of myelin as well as axonal regrowth (Mason et al., 2000; Xu et al., 2004). IGF-1 has an important role in nonneuronal modulation and numerous studies have confirmed that astrocytes are the main target of this growth factor by modulating their response to tissue damage (Fernandez et al., 2007). Acute stimulation of the IGF-1 receptor by IGF-1 elicits cell proliferation through activation of the PI3 kinase—Akt pathway and deactivation of PTEN (Virolle et al., 2001), the latter also shown to be decreased in white matter with age. Epidermal growth factor (EGF) and fibroblast growth factor 1 (FGF1) increases were directly shown, as was the decrease of fibroblast growth factor 2 (FGF2). Several studies suggest that the downregulation of FGF2 may enhance remyelination of white matter by FGF1 and neuroprotection by IGF-1 (Armstrong et al., 2002; Diemel et al., 2003; Russo et al., 2004). EGF promotes *in vitro* proliferation of neuroglia, astrocytes and oligodendrocytes, and is important for proliferation of progenitor cells in the aged brain (Alonso, 1999; Benoit et al., 2001). Interestingly, this does not seem to be a global phenomenon, as EGF is known to be reduced in aged forebrain (Enwere et al., 2004). Glial proliferation in aging white matter has already been suggested to reflect remyelination in the rhesus monkey (Peters and Sethares, 2004) and results have previously

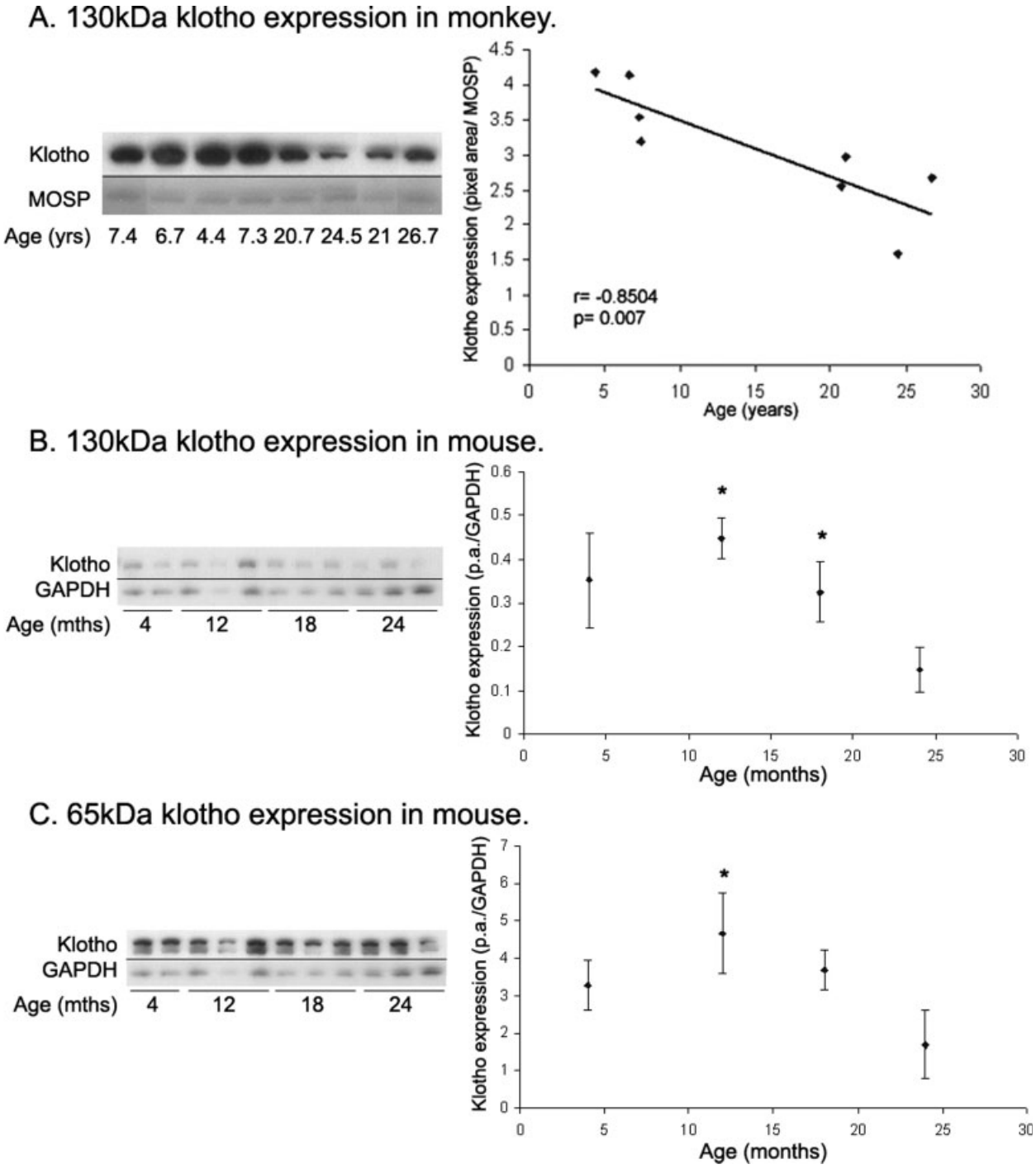

Fig. 4. Klotho protein analysis in white matter from monkey and mouse. An age related decrease of Klotho protein expression in Medullae white matter from monkey ($n = 8$) (**A**). Only a 130-kDa isoform was detected in monkey tissue, which was significantly decreased with respect to age when tested by linear regression ($P = 0.007$). An age-related decrease of Klotho protein expression was also observed in whole hemispheres of B6D2F1 mice, aged at 4, 12, 18, and 24 months (**B**,**C**). The 130-kDa isoform showed an age difference at 12 and 18 months compared with 24 months ($P \leq 0.025$) (B), while the 65-kDa isoform was only altered at 12 months compared with 24 months ($P \leq 0.05$) (C).

demonstrated that the aged brain still retains the capacity to respond to exogenous growth factors (Jin et al., 2003). Active myelin repair may also explain the upregulation of genes associated with cell adhesion as well as lipid transfer and turnover.

Role of Lifespan Genes in Aging of the White Matter

On the basis of previous literature alone, many genes have provided potential associations with white matter

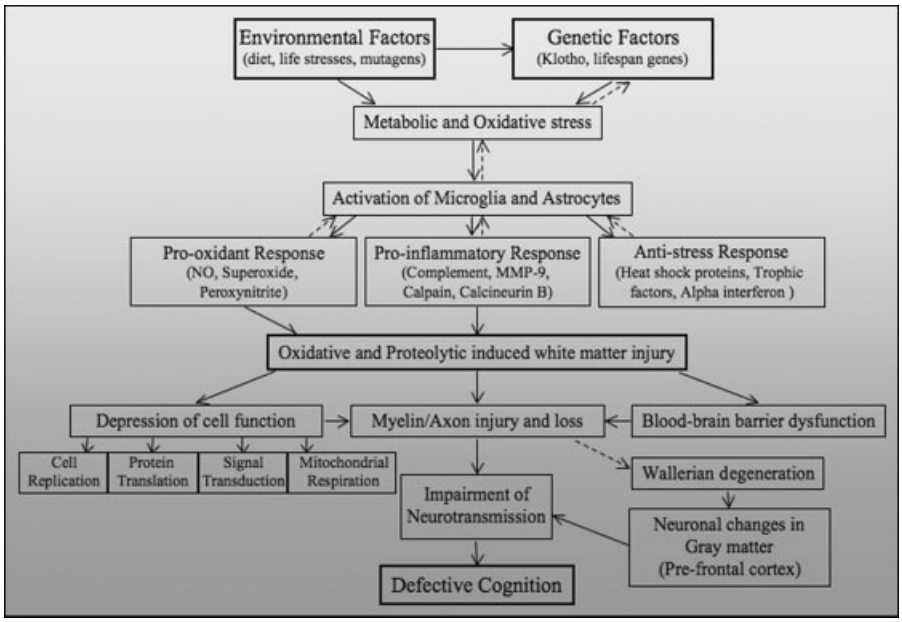

Fig. 5. Integrated concept of aging changes in brain white matter. The aging process in brain white matter is initiated by the interactions of environmental and genetic factors. As a result of oxidative and metabolic changes, as well as stress induced by these interactions, both microglia and astrocytes undergo activation thereby overexpressing and releasing reactive oxygen species, in addition to proinflammatory mediators. This stress response is followed by an anti-stress or neuroprotective counter. The inadequate protective response allows oxidative and proteolytic injury to continue in white matter leading to depression of cell function, injury of myelinated fibers and disruption of blood-barrier. Among the major cellular functions compromised are: mitochondrial respiration, cell replication, signal transduction, and protein translation. Finally, an impairment of neurotransmission and cognition occur as a consequence of these adverse changes, also compounded by neuronal changes in gray matter.

senescence. Microarray analysis enabled a method of refining many potential candidates into a few genes worthy of additional study in this model. The most striking finding to emerge from this gene array analysis was the altered expression of several genes associated with metabolic disorders causing degenerative changes in the brain, decline in cognition, and shortened life span. These genes included telomeric repeat binding factor 1 (TERF), mitochondrial polymerase gamma (POLG), and Klotho. All three have previously been investigated within the whole brain and have shown to play important causal roles in regulating the aging process, but have not been directly linked with age-related changes in white matter.

TERF is known to bind and inactivate telomerase, a reverse transcriptase that prevents telomere or chromosomal shortening and replicative senescence (Cong et al., 2002), and was found to be upregulated. Telomere shortening associated with changes in TERF1 is inducible by oxidative stress (von Zglinicki, 2002; Zhang et al. 2005), occurs with advancing age (Flanary and Streit, 2003) and shortening of telomeres may also give rise to impaired proliferation of microglia (Flanary and Streit, 2004).

POLG, a regulator of mitochondrial DNA synthesis, also displayed an increased expression. Studies in mice have demonstrated that mitochondrial DNA mutations induced by defective POLG are associated with accelerated aging and shortened lifespan (Kujoth et al. 2005; Trifunovic et al. 2004). Point mutations and deletions of mitochondrial DNA are known to accumulate in a variety of aging tissues from humans, monkeys, and rodents (Corral-Debrinski et al., 1992; Khaidakov et al., 2003). While it is yet to be found if increases in POLG mutations occur with age in white matter, loss of vital cells in which mitochondrial DNA mutations have accumulated beyond a critical threshold may be the key process that manifests as premature aging and reduced lifespan. Examples that may increase such mutations could be through apoptosis or replicative senescence associated with telomere shortening brought on by oxidative stress.

Although both TERF1 and POLG provided strong evidence for further confirmatory study, Klotho was determined to be of particular interest due to its strong differential regulation in our system as well as robust *a priori* importance with respect to the biology of white matter. The Klotho gene was originally identified in a mouse strain that resulted in several age-related disorders, including cognitive decline, leading to a shortened lifespan of only 60 days (Kuro-o et al., 1997; Nagai et al., 2000,2003). While Klotho is expressed in limited tissues, age-related defects are demonstrated in almost all organs. In the brain, Klotho has previously been shown to be expressed in the choroid plexus, from where it is secreted to the CSF (Imura et al., 2004), and in selective neuronal populations of the hippocampus, such as CA1 (Lein et al., 2004). Klotho's decreased expression at gene ($\sim$1.5 fold) and protein ($\sim$2 fold)

level in aged white matter indicates its role as a lifespan gene in an area not previously determined.

The major function of Klotho, based on the findings in the transgenic mouse model, appears to be the defense against oxidative stress, cell injury, and death with a deficiency of Klotho resulting in a loss of this protection. In Klotho mutant mice, changes indicative of oxidative stress and apoptosis have been described in the hippocampus with accompanying cognitive impairment. As the white matter of aged monkeys has also demonstrated a strong expression of oxidative stress, the present study suggests genes altered by white matter stress may be a consequence of an inadequate antioxidant expression by Klotho.

Klotho is proposed to act as a circulating hormone that binds to a cell surface receptor and repress intracellular signaling of insulin and IGF-1 (Kurosu et al., 2005). The decrease in Klotho shown here may be related to the activation of insulin/IGF-1 signaling. Accumulating genetic evidence has shown that even moderate inhibition of insulin-like signaling is a mechanism for life span extension, thus Klotho's ability to induce insulin/IGF-1 resistance may contribute to its antiaging properties (Kurosu et al., 2005). This may be of particular importance to lipid-laden cells such as oligodendrocytes, which are vulnerable to lipid-induced programmed cell death (Unger, 2006).

Further evidence has implicated a role for Klotho with another growth factor, fibroblast growth factor (FGF). Klotho functions as a cofactor in phosphate metabolism, essential for activation of FGF signaling (Kurosu et al., 2006; Razzaque et al., 2006). FGF is also known to play an important role in regulating insulin sensitivity, glucose/lipid/energy metabolism, and oxidative stress (Cassina et al., 2005), all shown to be altered with age in white matter. Therefore, Klotho may also regulate the aging process partly through controlling the FGF signaling pathway.

A recent report demonstrated that Klotho increases calcium reabsorption by activating the transient receptor ion channel, TRVP5. Abnormalities in other types of calcium metabolism are apparent in Klotho deficient mice (Tsujikawa et al., 2003) and may be the cause of some of the irregularities observed here with calcium metabolism in aged white matter. Whether TRPV5 is expressed in white matter and regulates intracellular calcium levels remains to be determined. In humans, a functional variant of Klotho has been identified as a risk factor for cardiovascular disease, stroke, and subsequent mortality (Arking et al., 2002). Determining the exact mechanism of how Klotho causes white matter injury is now of utmost importance. Designing strategies to antagonize or delay the deleterious consequences of age-dependent decrease in Klotho expression may allow us to prevent or even reverse cognitive impairment associated with aging.

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