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Implications of age and diet on canine cerebral cortex transcription

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Abstract

Mechanisms contributing to age-related cognitive decline are poorly defined. Thus, we used canine microarrays to compare gene expression profiles of brain tissue from geriatric and young adult dogs. Cerebral cortex samples were collected from six geriatric (12-year old) and six young adult (1-year old) female beagles after being fed one of two diets (animal protein-based versus plant-protein based) for 12 months. RNA samples were hybridized to Affymetrix GeneChip® Canine Genome Arrays. Statistical analyses indicated that the age had the greatest impact on gene expression, with 963 transcripts differentially expressed in geriatric dogs. Although not as robust as age, diet affected mRNA abundance of 140 transcripts. As demonstrated in aged rodents and humans, geriatric dogs had increased expression of genes associated with inflammation, stress response, and calcium homeostasis and decreased expression of genes associated with neuropeptide signaling and synaptic transmission. In addition to its existing strengths, availability of gene sequence information and commercial microarrays make the canine a powerful model for studying the effects of aging on cognitive function.

Keywords: Aging; Brain; Canine; Microarray

1. Introduction

With age, numerous anatomic and functional changes are known to occur within brain tissue and contribute to cognitive decline. Decreased brain mass, increased ventricular size, demyelination, neuroaxonal degeneration, and decreased cholinergic activity are just a few characteristics of the aging canine brain (Borras et al., 1999; Su et al., 1998). While numerous factors such as amyloid precursor protein (APP), β-amyloid (Aβ), tau protein, and lipofuscin have been linked with cognitive decline and neurodegenerative diseases, few mechanisms by which they contribute to disease have been demonstrated.

To gain a better understanding of brain aging, techniques capable of identifying specific molecular processes have been utilized recently. With the advent of microarray technology, which enables the measurement of thousands of gene transcripts simultaneously, researchers have been able to obtain a global view of brain gene expression. Thus far, microarrays have been used to study aged brain tissue of mice (Jiang et al., 2001; Lee et al., 2000; Weindruch et al., 2002) and humans (Erraji-Benchekroun et al., 2005; Lu et al., 2004). From these initial experiments, aged brain tissue has been reported to have increased expression of genes associated with inflammatory response, oxidative stress, and DNA repair. Conversely, aged brain tissue has reduced neurotrophic support, mitochondrial function, and synaptic plasticity. These initial experiments have identified several genes and biological pathways that may act as targets for dietary or drug intervention.

Dietary intervention is arguably the most important environmental factor affecting the aging process. Various changes

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to diet have been shown to impact tissue aging and life expectancy. The effects of caloric restriction, the only intervention shown to slow the intrinsic rate of aging in mammals (Weindruch and Walford, 1988), has been shown to beneficially affect age-related decline in psychomotor and spatial memory tasks (Ingram et al., 1987), reduce age-associated loss of dendritic spines (Moroi-Fetters et al., 1989), and reduce neuronal degeneration (Duan and Mattson, 1999). A major focus of the transcriptional profiling experiments reported by Lee et al. (2000) and Weindruch et al. (2002) was to evaluate the effects of caloric restriction on brain tissue gene expression profiles. In those studies, caloric restriction was reported to attenuate many of the negative changes in gene expression that occur with increased age, including inflammatory and stress response genes.

Besides caloric restriction, other dietary factors are known to affect tissue aging and may be used to maintain brain health. Dietary fat and cholesterol inclusion levels and fatty acid type may impact cognitive development and the effects of aging on brain health. It has been reported that both the generation and clearance of Aβ are regulated by cholesterol, which modulates the processing of both APP and Aβ. Elevated cholesterol level increases Aβ in cellular and most animal models and is a risk factor for Alzheimer's disease (AD) (Jarvik et al., 1995). Dietary fat intake has also been associated with psychosocial and cognitive function in young children. While total fat and saturated fat intake were unrelated to performance on achievement and intelligence tests, cholesterol and polyunsaturated fatty acid intakes were associated with decreased and increased performance, respectively (Zhang et al., 2005).

While numerous rodent models may be used to study brain aging, the utility of the aged dog model has been demonstrated by several research groups over the past decade. The dog model has several advantages, in that they: (1) share many of the same environmental conditions with humans; (2) can perform a sophisticated repertoire of complex cognitive behaviors; (3) show many of the same pathological changes as humans; and (4) develop neuropathologic changes that are significantly associated with cognitive decline (Cotman et al., 2002). Aged dogs have been shown to develop many of the same neurological features as elderly humans. Briefly, decreased frontal lobe volume (Tapp et al., 2004), ventricular enlargement (Su et al., 1998), β-amyloid accumulation (Borras et al., 1999; Tapp et al., 2004), and lipofuscin accumulation (Borras et al., 1999) have been demonstrated in aged dogs. Moreover, the neuropathologic changes that occur with age are associated with cognitive changes (Chan et al., 2002; Milgram et al., 1994; Tapp et al., 2003) and behavioral alterations (Siwak et al., 2001, 2003) in dogs as they are in elderly humans (Colle et al., 2000; Cummings et al., 1998).

To our knowledge, molecular analysis of aged versus young adult brain tissue using microarray technology has not yet been performed in the dog model. Thus, to identify potential targets for preventative or treatment therapies of cognitive decline, our primary objective of this study was to compare gene expression profiles of cerebral cortex tissue from geriatric compared with young adult canines. Previously we demonstrated that a diet based on animal protein, containing high concentrations of fat and low concentrations of fiber, altered whole body metabolism as compared with a plant protein-based diet containing moderate amounts of fat and fiber (Swanson et al., 2004). For example, blood cholesterol was affected by age and diet, with old dogs and dogs fed the high-fat diet having greater blood cholesterol concentrations. Alkaline phosphatase (ALP) and corticosteroid-induced alkaline phosphatase (CALP), an isoenzyme unique to dogs, were also influenced by age and diet. In particular, geriatric dogs fed the high-fat diet had increasing ALP concentrations over time, which was primarily due to a dramatic increase (∼5-fold) in CALP compared with geriatric dogs fed the low-fat diet. This study extends those findings to determine changes in cerebral transcriptional activity as a function of diet and age.

2. Materials and methods

2.1. Animals and diets

Six geriatric (average age = 11.1-year old at baseline; Kennelwood Inc., Champaign, IL) and six weanling (8-week old at baseline; Marshall Farms USA, Inc., North Rose, NY) female beagles were used in this experiment. Three dogs of each age were assigned to one of two dietary treatments. Diets tested in this experiment were previously shown to manipulate energy metabolism, including cholesterol metabolism, as reported elsewhere (Kuzmuk et al., 2005; Swanson et al., 2004). Dry kibble diets were manufactured by Wenger Manufacturing Company (Sabetha, KS) as described by Swanson et al. (2004) and fed for 12 months.

One diet was an animal-protein-based diet (APB) and was composed primarily of highly digestible ingredients and animal-derived protein and fat sources (brewer's rice, poultry by-product meal, poultry fat) and was formulated to contain 28% protein, 23% fat, and 5% dietary fiber. The other diet was a plant-protein-based diet (PPB) and was composed primarily of moderately digestible plant-derived ingredients (corn, soybean meal, wheat middlings, and meat and bone meal) and was formulated to contain 26% protein, 11% fat, and 15% dietary fiber. Although the two diets were very different in terms of ingredient and chemical composition, both were formulated to meet or exceed all nutrient requirements for canine growth according to the Association of American Feed Control Officials (AAFCO, 2007). Young dogs were fed ad libitum to allow for adequate growth, while geriatric dogs were fed to maintain baseline BW throughout the experiment.

To produce the desired metabolic effects, the PPB diet was formulated to contain a lower caloric density (APB = 5.38 kcal/g; PPB = 4.75 kcal/g) and have a lower nutrient digestibility than the APB diet. Thus, dogs fed the PPB diet needed to consume a greater ($P < 0.05$) quantity

of food (237 g/d; 1123 kcal/d) than dogs fed the APB diet (166 g/d; 893 kcal/d) to grow (young dogs) or maintain BW (old dogs) (Swanson et al., 2004). Even though metabolic indices were altered, mean BW among dietary treatments was not different at any time over the course of the study for young or geriatric dogs.

At the time of tissue collection, the mean age of geriatric dogs was 12-year old, which translates to a 77-year-old human according to Patronek et al. (1997). Young adult dogs were 14 months of age when tissues were collected, translating to a 20-year-old human according to Patronek et al. (1997). Dogs were housed individually in kennels (1.1 m × 0.9 m) in temperature-controlled (72 °F) rooms with a 12-h light:12-h dark cycle at the Edward R. Madigan Laboratory on the University of Illinois campus. The Institutional Animal Care and Use Committee approved all animal care procedures prior to initiation of the study.

2.2. Tissue sample collection and handling

After 12 months on experiment, animals were fasted for 12 h and then given a lethal dose (130 mg/kg BW) of sodium pentobarbital (Euthasol®, Virbac Corp., Fort Worth, TX) intravenously into the left forearm. Death was confirmed by lack of respiration and a corneal reflex, and absence of a heartbeat detected with a stethoscope placed under the left elbow. Cerebral cortex samples were collected immediately after death was confirmed, flash frozen using liquid nitrogen, and stored at −80 °C until further analysis.

2.3. RNA extraction

Total cellular RNA was isolated from cerebral cortex samples using the Trizol reagent as suggested by manufacturer (Invitrogen, Carlsbad, CA). RNA concentration was determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA integrity was confirmed using a 1.2% denaturing agarose gel. Since it was critical to measure inter-animal variation, tissue samples were not pooled in this experiment. Thus, each animal was analyzed as an individual experimental unit.

2.4. Microarray analyses

All RNA samples were prepared and hybridized to the Affymetrix GeneChip® Canine Genome Arrays (Affymetrix, Santa Clara, CA). All reactions were performed using Affymetrix GeneChip® Expression 3′-Amplification Reagents (One-Cycle Target Labeling and Control Reagents package) according to the manufacturer's instructions. Canine total RNA samples were spiked with four polyadenylated prokaryotic RNA-labeling controls and used for reverse transcription synthesis of double-stranded complementary DNA (cDNA). cDNA then was purified using the GeneChip® Sample Cleanup Module. Purified, double-stranded cDNA was used for in vitro transcription and amplification, incorporating biotin-labeled pseudouridine nucleotide analog. cDNA then was purified, fragmented, mixed with manufacturer-supplied biotin-labeled hybridization control polynucleotides (a synthetic oligonucleotide and four bacterial cDNA sequences), and hybridized to the microarray chip.

Following hybridization, chips were washed and stained with streptavidin-conjugated phycoerythrin dye (Invitrogen) enhanced with biotinylated goat anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) utilizing an Affymetrix GeneChip® Fluidics Station 450 and GeneChip® Operating Software. Finally, images were scanned using an Affymetrix GeneChip® Scanner 3000.

2.5. Microarray data analyses

Affymetrix's Canine Genome array contains 23,836 probe sets, which interrogate over ∼21,700 *C. familiaris* transcripts gleaned from GenBank® (August 2003), dbEST (October 2003), and cDNA libraries from 11 tissues, including brain, licensed from LION bioscience AG. In addition to the recommended Affymetrix quality control measures, we performed both graphical and quantitative QC assessments of the arrays using the affy (Gautier et al., 2004), made4 (Culhane et al., 2005), and affyPLM (Bolstad, 2004) packages from the Bioconductor project (Gentleman et al., 2004). All arrays passed the QC assessments.

Each probe set consists of 11 perfect match (PM) and 11 mismatch (MM) probes. The raw PM and MM probe-level data were pre-processed into one number per probe set using the GCRMA algorithm in Bioconductor's affy (Gautier et al., 2004) and gcrma (Irizarry et al., 2003) packages. GCRMA does a background correction based on GC-content, performs quantile normalization and then summarizes the PM values into one number using median polish. Because the Canine Genome array contains transcripts from other tissues besides brain, we used Affymetrix's call detection algorithm (GeneChip, 2002) to assess which probe sets were reliably detected above background on each array. A probe set was discarded from further analysis if it was not called present on at least one array or marginal on two arrays. Of the 23,836 probe sets, 14,859 passed this filter and were assessed for differential expression due to age and diet (described below). Heat maps were generated using the Heatplus (Ploner, 2006) package from Bioconductor (Gentleman et al., 2004). MetaCore (GeneGo, Inc., St. Joseph, MI) was used to build gene networks and interpret microarray data. Functional attribution was made according to the database SOURCE (http://source.stanford.edu) (Diehn et al., 2003).

2.6. qRT-PCR analyses

A subset of differentially expressed genes identified using microarrays were validated using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Primer Express 2.0 software (PerkinElmer, Boston, MA) was used to design Taqman primer-probe pairs specific for each gene

selected (Supplementary Table 1). The High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) prepared cDNA from tissue RNA samples. The cDNA samples then were evaluated using real-time two-step qRT-PCR using an Applied Biosystems Taqman Gene Expression Assay containing a FAM dye-labeled Taqman MGB probe and the Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems). Each gene was validated in triplicate using a control sample to create a standard curve. Eukaryotic 18S rRNA was amplified as a control in parallel with the gene of interest. Data were normalized to 18S rRNA and expressed as a ratio to the 18S rRNA signal.

2.7. Brain lipid analyses

Cerebral cortex samples were analyzed for long chain fatty acid (LCFA) concentrations according to Lapage and Roy (1986). Briefly, brain samples were first homogenized in water using a Fisher Powergen Model 125 tissue homogenizer (Fisher Scientific, Hampton, NH). Internal standards and 0.1 g of tissue then were put through a hexane extraction to remove the lipid portion. Individual fatty acids were determined from the extracted portion using gas chromatography (Hewlett-Packard 5890A Series II) and external standards for identification and quantification.

2.8. Statistical analyses

Differential expression of the microarray data was assessed using the limma package (Smyth, 2004). A linear model for the four age × diet groups was fit for each probe set, then differences between groups were extracted as contrasts from them model. Next, an empirical Bayes "shrinkage" method was employed on the standard errors, which improves power when sample sizes are small (Smyth, 2004). Finally, multiple test correction of P values was done using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995). qRT-PCR data were analyzed using the General Linear Models procedure of SAS (SAS Institute, Inc., Cary, NC). Brain lipid data were analyzed using the mixed models procedure of SAS. Brain lipid and qRT-PCR data were considered significant when $P < 0.05$.

3. Results

Overall, age had the strongest effect on mRNA abundance, whereas diet had a modest effect. At an FDR = 0.05, 963 transcripts were differentially expressed due to age in one or both diets, compared to only 1 gene that was differentially expressed due to diet at either age. To get a broader picture of expression patterns affected by diet, the cutoff threshold was increased to an unadjusted $P = 0.005$, which highlighted 140 differentially expressed transcripts. While these genes did not meet the more stringent FDR cutoff, they have the most evidence for differential expression due to diet. For comparison purposes, 1372 gene transcripts were identified as being different due to age using an unadjusted $P = 0.005$. The number of differentially expressed transcripts are discussed as being affected by age or diet and represent comparisons of all young ($n = 6$) and old dogs ($n = 6$) for age effects and all dogs fed APB ($n = 6$) and PPB ($n = 6$) for all diet effects. All differentially expressed genes with annotation that were analyzed using Metacore are presented in Supplementary Table 3 (age effects) and Table 4 (diet effects). All microarray data have been deposited in Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI) archives.

Heat maps representing transcript expression differences due to age and diet are presented in Figs. 1 and 2, respectively. Heat maps provide several useful functions with such vast datasets. In addition to presenting the number of up- and down-regulated transcripts in the dataset, the magnitude of change and the overall pattern of expression among groups are demonstrated. With virtually all dogs within an age group having the same expression level for any given gene transcript, Fig. 1 demonstrates the strong and consistent effect of age on gene expression regardless of diet consumed. Fig. 2, however, demonstrates the lack of consistency among dietary treatments and the diet × age interactions present in the dataset.

Selected genes having increased expression in geriatric versus young adult dogs are presented in Table 1. As noted in aged rodent and human brain samples (Lu et al., 2004; Weindruch et al., 2002), geriatric dogs had increased expression of genes associated with inflammation and protein folding. Numerous genes involved with calcium homeostasis also were greater in geriatrics versus young adults, including calponin 3, S-100A1, S-100A10, calcineurin B, calnexin, and calmodulin 3. Protein metabolism and turnover and nucleotide processing appeared to be increased in aged compared with young brain tissue, as several genes associated with translation, ribosomal proteins, and RNA processing were up-regulated in geriatric dogs. Spliceosome RNA helicase BAT1, several ATP-dependent RNA helicases (DDX17, DDX42, DDX55), eukaryotic translation initiation factors (EIF3S12, EIF3S7, EIF4A2, EIF4E), heterogeneous nuclear ribonucleoproteins (F, H, U), several ribosomal proteins of the 40S and 60S subunits, and several RNA splicing factors were up-regulated in aged dogs.

Selected genes having decreased expression in geriatric versus young adult dogs are presented in Table 2. As anticipated, the expression of genes involved in neuropeptide signaling and synaptic transmission, were decreased in geriatric dogs. Neuropeptide Y (NPY), neuromodulin, neural proliferation differentiation and control protein 1 (NPDC1), brain-derived neurotrophic factor (BDNF), BDNF/NT-3 growth factors receptor (TRKB), and others were all decreased in geriatric dogs, regardless of dietary treatment. Glutathione *S*-transferase A4 and Mu3, important responders to oxidative stress, were unexpectedly decreased in geriatric dogs.

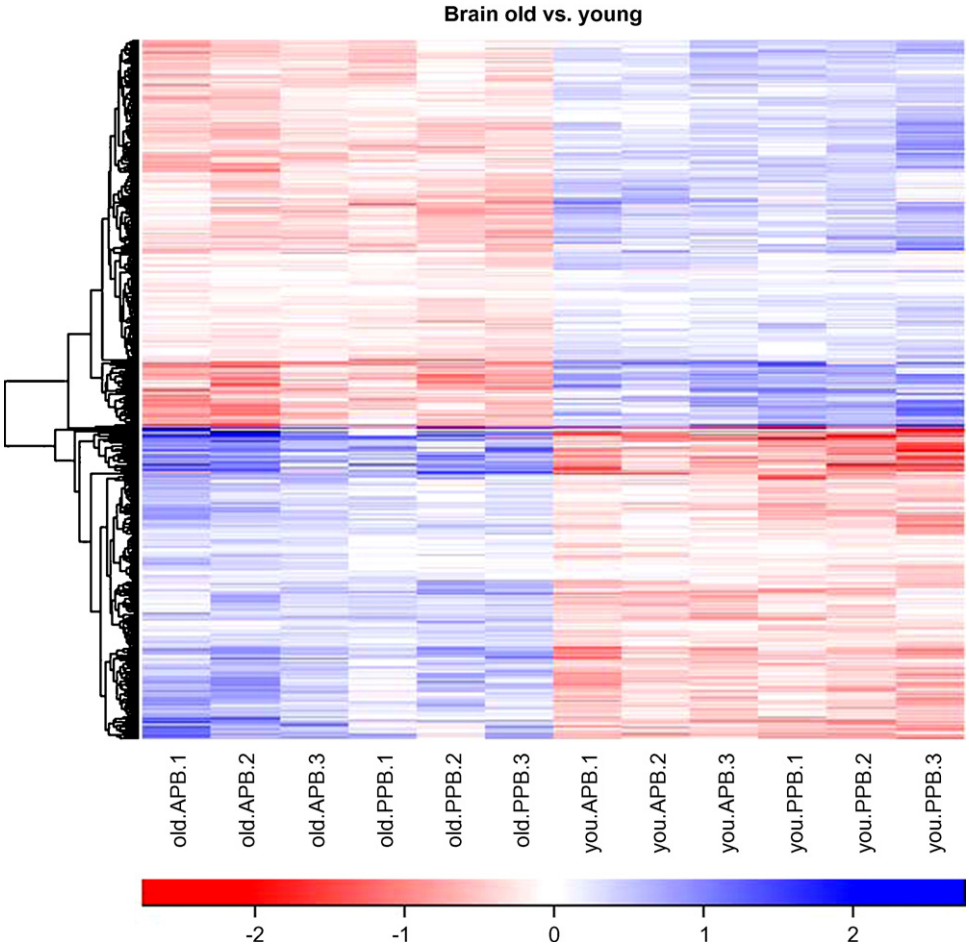

Fig. 1. Heatmap of geriatric vs. young adult pairwise comparisons. Values are the GCRMA-processed probe set value (log2 scale) minus the mean value for that probe set across all arrays. The dendrogram was created by hierarchical cluster analysis.

As reported previously (Swanson et al., 2004), dietary treatments fed in this experiment resulted in substantial differences in nutrient digestibility and metabolic status. Of interest herein were differences observed in blood cholesterol concentrations among treatment groups. Cholesterol has been shown to interfere with the processing of Aβ, is negatively associated with cognitive performance (Zhang et al., 2005), and is a known risk factor for AD (Jarvik et al., 1995). In this population of dogs, cholesterol metabolism was impacted by diet and age. Blood cholesterol concentrations were greater ($P < 0.05$) in geriatric compared with young adult dogs and greater ($P < 0.01$) in dogs fed APB versus those fed PPB (Swanson et al., 2004). Given these differences, cerebral cortex gene expression differences due to diet were of great interest.

As stated above, diet had only modest effects on mRNA abundance in the current experiment. Selected genes differentially expressed in dogs consuming APB versus those consuming PPB are presented in Table 3. Although the influence of diet was fairly minor, there were a few key differences that may require further study. For example, BDNF had decreased expression in young dogs fed APB versus PPB, but was not affected by diet in geriatrics. Toll-like receptor 4 was increased and calcineurin was decreased in geriatrics fed APB, but did not affect young adults.

Six transcripts differentially expressed according to microarray data were measured using real time qRT-PCR. After performing statistical analyses, two of the six genes were found to be different among groups and were in agreement with array data. Angiotensinogen expression was greater ($P < 0.01$) in geriatric compared with young adult dogs, but was not impacted by diet. Prosomatostatin was impacted by age and diet, having a greater expression in young compared with old dogs ($P < 0.01$) and in dogs consuming PPB ($P < 0.01$). Although the qRT-PCR and array data were in agreement, the low sample number ($n = 3$ per treatment) and variation among animals in this experiment requires a larger sampling size to achieve statistical difference with the other four genes tested using qRT-PCR. Nonetheless, the similarity between microarray and RT-PCR data provides us with confidence in our array data in this experiment (Supplementary Table 2).

Long chain fatty acid concentrations of cerebral cortex samples are reported in Table 4. Because the main difference between APB and PPB was the amount of fat, not the fatty acid source (poultry fat was the primary fat source for

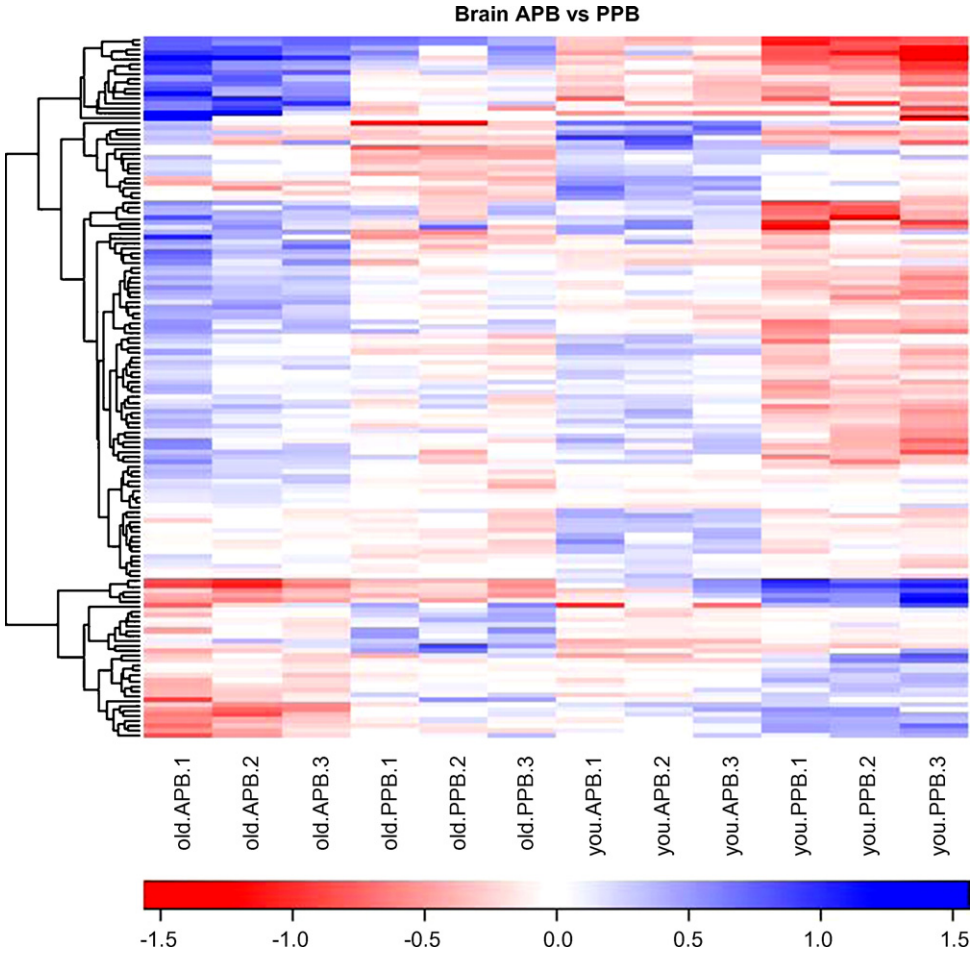

Fig. 2. Heatmap of APB vs. PPB diet pairwise comparisons. Values are the GCRMA-processed probe set value (log2 scale) minus the mean value for that probe set across all arrays. The dendrogram was created by hierarchical cluster analysis.

each diet), it was not surprising to note few differences in brain fatty acid content due to diet. The largest diet-related difference in brain fatty acid content was that of total monounsaturated fatty acids (MUFA), which tended to be greater ($P < 0.09$) in dogs fed APB versus PPB. The same numerical trend occurred with saturated fatty acid content, but was not significantly different among dietary treatments. Several age-related differences were noted in brain fatty acid concentrations. In general, old dogs tended to have greater ($P < 0.06$) brain MUFA concentrations, while young dogs tended to have greater ($P < 0.07$) saturated fatty acid concentrations. Brain polyunsaturated fatty acid (PUFA) concentrations were relatively unchanged among age groups.

4. Discussion

Over the past century, public health programs and advances in clinical medicine have dramatically increased the life span in developed countries. As average life expectancy has increased, so has the incidence of AD. In the year 2000, 7.1 million and 4.5 million cases of AD were present in Europe and the United States, respectively (Hebert et al., 2003; Wancata et al., 2003). Unless new discoveries lead to preventative strategies, the prevalence of AD is estimated to increase to 16.2 million in Europe and 13.2 million in the United States by the year 2050 (Hebert et al., 2003; Wancata et al., 2003). While aging is known to be the primary contributing factor for AD, most of the molecular events leading to disease are poorly defined. Recent advances in genomic biology, however, have provided new tools to study complex disease states. Because they provide a global view of tissue gene expression, DNA microarrays have been a popular tool for the study of aging and complex diseases. Using commercial canine microarrays, we aspired to identify genes and/or biological pathways differentially expressed in aged compared with young adult canines, many of which may play an important role in brain aging.

Over the past decade, the dog has been a useful model in the study of brain aging. Similar to humans (Colle et al., 2000; Cummings et al., 1998), aged dog brains have increased number of apoptotic cells (Kiatipattanasakul et al., 1996), plaques containing Aβ (Borras et al., 1999; Cummings et al., 1996), and lipofuscin deposits (Borras et al., 1999). Furthermore, neuropathologic changes are associated with changes in cognition and behavioral patterns (Chan et al., 2002; Siwak et

Table 1
Genes up-regulated in cerebral cortex of geriatric vs. young adult dogs

Functional classification	Gene name	Gene symbol	Fold change (APB)	Fold change (PPB)
Apoptotic pathway				
Apoptosis	B-cell lymphoma/leukemia 2	BCL2	1.8	1.8
Apoptosis	Survival of motor neuron-related splicing factor 30	SMNDC1	2.5	2.7
Cell signaling and signal transduction				
Cell signaling	S100 A1 calcium binding protein	S100A1	3.4	4.2
Cell signaling	Protein tyrosine kinase binding protein	TYROBP	2.4	2.2
Signal transduction	Annexin A1	ANXA1	1.8	1.6
Signal transduction	14-3-3 protein epsilon	YWHAE	3.4	3.0
Cell development and motility				
Cell motility	Talin-1	TLN1	2.1	2.1
Muscle contraction	Calponin 3	CNN3	3.9	3.7
Microtubule activity	Microtubule-associated protein tau	MAPT	1.7	1.5
Cellular trafficking and protein processing				
Ion transport	Chloride channel protein 3	CLCN3	3.2	3.1
Iron transport	Transferrin	TF	1.8	2.6
Phosphorylation	Dual specificity tyrosine-phosphorylation-regulated kinase 1A	DYRK1A	1.7	1.5
Phosphorylation	Serine/Threonine protein kinase	SGK	4.6	3.2
Protein folding	Calnexin	CANX	2.3	2.1
Protein folding	Alpha crystallin B chain	CRYAB	1.8	2.2
Protein folding	Heat shock 70 kDa protein 1A	HSPA1A	3.0	2.6
Proteolysis	Ubiquitin-protein ligase E3	MDM2	2.1	1.6
Transport	Lysosome-associated membrane glycoprotein 1	LAMP1	1.6	1.8
Transport	Lysosomal-associated transmembrane protein 4A	LAPTM4A	2.5	2.3
Transport	Amino acid transporter	SLC38A1	1.5	1.6
Immune function				
Immune function	Complement C1r subcomponent	C1R	2.3	1.7
Immune function	Complement C3	C3	2.3	2.7
Immune function	HLA class II histocompatibility antigen, DR alpha chain	HLA-DRA	3.5	2.7
Immune function	Toll-like receptor 4	TLR4	2.1	1.5
Metabolism				
Calcium metabolism	Calmodulin 3	CALM3	2.7	2.5
Calcium metabolism	Calcineurin B, type 1	PPP3R1	2.8	2.2
Lipid metabolism	Adiponectin receptor protein 2	ADIPOR2	1.9	1.6
Lipid metabolism	Peroxiredoxin 6	PRDX6	1.6	1.6
Retinoid metabolism	Retinoic acid receptor responder protein 2	RARRES2	3.3	2.8
Transcription–translation				
RNA processing	Spliceosome RNA helicase BAT1	BAT1	5.3	4.6
Transcription regulation	Peroxisome proliferator-activated receptor binding protein	PPARBP	2.4	1.8
Translation	Eukaryotic translation initiation factor 4E	EIF4E	7.1	4.1
Miscellaneous and unknown				
Blood pressure regulation	Angiotensinogen	AGT	2.8	4.8
Coagulation regulation	Annexin A7	ANXA7	1.7	2.2
Unknown	Glutamine-rich protein 1	QRICH1	5.6	4.6
Unknown	S100 A10 calcium binding protein	S100A10	1.4	1.6

al., 2003; Tapp et al., 2003). To our knowledge, however, the current experiment is the first to use DNA microarrays to identify gene expression differences of aged compared with young adult canine brain tissues. Although the diets fed in this experiment had different concentrations of dietary fat, which affected metabolic status in these animals (including blood cholesterol concentrations), the overall patterns of gene expression were only mildly impacted by diet. Thus, in this experiment, the majority of gene expression differences observed were due to age and are the focus of the discussion.

Many of the results from the current experiment are in good agreement with that reported in aged mice (Jiang et al., 2001; Lee et al., 2000; Weindruch et al., 2002) and humans (Erraji-Benchekroun et al., 2005; Lu et al., 2004). Oxidative stress is known to contribute to genomic instability and cellular senescence and, thus, is one of the most popular theories of aging (Finkel and Holbrook, 2000). Reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radicals are generated by metabolism and cause molecular damage to proteins, lipids, and nucleic acids. The accumu-

Table 2
Genes down-regulated in cerebral cortex of geriatric vs. young adult dogs

Functional class	Gene name	Gene symbol	Fold change (APB)	Fold change (PPB)
Apoptotic pathway				
Apoptosis	NF-kappa-B inhibitor alpha	NFKBIA	−2.3	−1.5
ATP synthesis				
ATP synthesis	ATP synthase lipid-binding protein, mitochondrial	ATP5G1	−1.5	−1.3
ATP synthesis	Vacuolar ATP synthase 16 kDa proteolipid subunit	ATP6V0C	−1.4	−1.3
Cell development and motility				
Cell adhesion	Cadherin 6 precursor	CDH6	−2.0	−3.8
Cell adhesion	Laminin subunit alpha-1	LAMA1	−1.6	−2.1
Cell adhesion	Protocadherin-8	PCDH8	−2.5	−2.4
Cell motility	Neural Wiskott-Aldrich syndrome protein	WASL	−1.8	−1.5
Cellular trafficking and protein processing				
Ion transport	Small conductance calcium-activated potassium channel protein 2	KCNN2	−2.4	−2.2
Phosphorylation	Microtubule affinity-regulating kinase 1	MARK1	−1.5	−2.0
Protein folding	Peptidyl-prolyl *cis–trans* isomerase NIMA-interacting 1	PIN1	−1.6	−1.9
Proteolysis	Neuroendocrine convertase 1	PCSK1	−1.7	−2.3
Transport	Vesicular glutamate transporter 1	SLC17A7	−1.8	−1.6
Transport	Glutamate/aspartate transporter 2	SLC1A2	−2.0	−1.8
Neurogenesis and neuropeptide signaling				
CNS development	Alpha-synuclein	SNCA	−1.7	−1.9
Neurogenesis	Brain-specific angiogenesis inhibitor 1-associated protein 2	BAIAP2	−2.6	−2.1
Neurogenesis	Brain-derived neurotrophic factor	BDNF	−1.4	−2.1
Neurogenesis	Neuromodulin	GAP43	−1.7	−2.0
Neurogenesis	Neural proliferation differentiation and control protein 1	NPDC1	−1.7	−1.5
Neurogenesis	Olfactomedin 1	OLFM1	−2.5	−2.2
Neurogenesis	Neurotrophic tyrosine kinase receptor 2	TRKB	−1.4	−1.4
Neuropeptide signaling	Neuropeptide Y	NPY	−3.0	−3.5
Neuropeptide signaling	Proenkephalin A	PENK	−2.2	−2.3
Neuropeptide signaling	Protachykinin 1	TAC1	−3.1	−3.0
Cell signaling and signal transduction				
Cell signaling	Somatostatin receptor 2	SSTR2	−1.5	−1.3
Signal transduction	Brain-specific angiogenesis inhibitor 3	BAI3	−1.8	−1.6
Signal transduction	Cholecystokinin	CCK	−1.7	−1.7
Signal transduction	Corticotropin-releasing hormone	CRH	−2.3	−3.6
Signal transduction	Insulin-like growth factor 2 receptor	IGF2R	−2.1	−1.6
Miscellaneous				
Metabolism	Glutathione *S*-transferase Mu 3	GSTM3	−1.6	−2.0
Stress response	Glutathione *S*-transferase A4	GSTA4	−1.5	−1.4
Transcription regulation	*N*-myc proto-oncogene protein	MYCN	−1.7	−2.1

lation of compounds such as lipofuscin, tau protein, and Aβ may also lead to various cellular protective responses in geriatric animals. In fact, expression of microtubule-associated protein tau (MAPT) was increased in geriatric dogs in the current study. Thus, it was not surprising that several genes that mediate stress responses and repair were up-regulated in cerebral cortex of aged dogs, regardless of dietary treatment.

Examples include genes involved with protein folding, namely alpha crystallin B and heat-shock protein 70, that have been shown to be increased in aged mouse (Lee et al., 2000) and human (Lu et al., 2004) brain tissues and were increased in geriatric dogs in the current study. Induction of alpha crystallin B is known to occur in AD patients and thought to be a direct response to cellular accumulation of Aβ and suggests that further research should be performed in this area (Link et al., 2003). In the current study, geriatric dogs had increased expression of dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A). DYRK1A has been proposed to participate in brain development, but its role in adult brain is poorly understood. Transgenic models of AD expressing hyperphosphorylated tau in cerebral cortex neurons have been shown to have increased DYRK1A expression (Ferrer et al., 2005), suggesting its role in tau turnover. Finally, serine/threonine protein kinase (SGK), a gene activated by oxidative stress and cytokines and shown to be increased in various neurodegenerative disease models (Schoenebeck et al., 2005), was increased in geriatric dogs. While the up-regulation of SGK strongly correlates with the occurrence of cell death, it is thought to play a protective role in oxidative stress situations (Schoenebeck et al., 2005).

Lysosomal-associated protein transmembrane 4A (LAPTMA4), lysosome-associated membrane glycoprotein

Table 3
Genes in cerebral cortex affected by consuming APB vs. PPB

Functional classification	Gene name	Gene symbol	Fold change (young)	Fold change (geriatric)
Neurogenesis	Brain-derived neurotrophic factor	BDNF	−1.8	–
Phosphorylation	Calcium/calmodulin-dependent serine protein kinase	CASK	−1.4	–
Amino acid metabolism	Kynurenine-oxoglutarate Transaminase 1	CCBL1	1.7	–
Unknown	Junctional adhesion molecule 3	JAM3	–	1.5
Transport	Lysosome-associated membrane glycoprotein 2	LAMP2	1.5	–
Nitric oxide synthesis	Nitric oxide synthase 3	NOS3	–	−1.7
Dephosphorylation	Calcineurin	PPP3CA	–	−1.5
Proteolysis	Proteasome subunit beta type 9	PSMB9	–	1.9
Signal transduction	Rho-related GTP-binding protein	RHOG	1.5	–
Signal transduction	Ras and Rab interactor 2	RIN2	–	2.2
Transcription regulation	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily C member 2	SMARCC2	1.5	–
Electron transport	Sulfide:quinine oxidoreductase	SQRDL	–	1.8
Iron transport	Transferrin	TF	1.9	–
Immune function	Toll-like receptor 4	TLR4	–	1.8
Muscle development	Tropomyosin 1 alpha	TPM1	–	1.7
Metabolism	Thiopurine *S*-methyltransferase	TPMT	–	−1.6

1 (LAMP1), and many other LAMP proteins and vacuolar protein sorting-associated proteins were also up-regulated transcripts in aged dog brain. In aging brain tissue, lysosomes become highly susceptible to oxidative stress, often leading to lysosomal dysfunction (Cutler et al., 2004). Elevated concentrations of cathepsin D, a lysosomal protease enzyme, have been reported in AD patients and an aged dog model (Bi et al., 2003). When functioning properly, lysosomes

Table 4
Cerebral cortex saturated (SAT), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acid concentrations in geriatric and young adult dogs[a]

Fatty acid	Geriatric		Young adults			*P* value	
	APB	PPB	APB	PPB	S.E.M.	Age	Diet
Saturated fatty acids							
6:0	1.6	1.3	0.5	1.0	0.33	0.08	NS
14:0	1.0	0.7	0.8	0.9	0.17	NS	NS
16:0	56.7	64.7	72.6	70.2	7.78	NS	NS
17:0	1.2	0.9	1.1	0.7	0.17	NS	0.05
18:0	84.2	60.7	95.0	89.8	7.91	0.04	NS
20:0	0.8	0.6	0.4	0.3	0.12	0.04	NS
22:0	0.6	0.5	0.7	0.5	0.14	NS	NS
24:0	3.8	3.3	2.5	1.7	0.23	<0.01	0.02
Total SAT	149.8	132.8	173.7	165.2	13.04	0.07	NS
Monounsaturated fatty acids							
16:1	2.2	2.2	2.5	2.4	0.27	NS	NS
18:1	100.4	93.3	92.0	64.6	9.16	0.09	NS
20:1	2.1	2.2	2.1	1.7	0.29	NS	NS
24:1	5.5	5.0	3.5	2.2	0.59	<0.01	NS
Total MUFA	110.2	102.7	100.1	71.0	9.27	0.06	0.09
Polyunsaturated fatty acids							
22:5$n-3$	0.0	0.2	0.1	0.1	0.07	NS	NS
22:6$n-3$	2.3	2.6	3.2	2.7	1.20	NS	NS
18:2$n-6$	0.8	0.6	0.7	0.4	0.12	NS	NS
20:2$n-6$	0.2	0.2	0.0	0.0	0.08	0.04	NS
20:3$n-6$	0.9	0.7	0.8	0.7	0.16	NS	NS
20:4$n-6$	3.6	2.9	4.6	4.1	0.84	NS	NS
21:2$n-6$	0.2	0.1	0.1	0.0	0.08	NS	NS
22:4$n-6$	2.3	2.4	2.7	1.9	0.40	NS	NS
22:5$n-6$	0.9	0.8	1.1	0.6	0.35	NS	NS
Total $n-3$	2.3	2.6	3.2	2.8	1.26	NS	NS
Total $n-6$	8.9	8.9	9.9	7.7	1.71	NS	NS
Total PUFA	11.3	11.5	13.1	10.5	2.88	NS	NS

[a] Values are represented as mg fatty acid/g tissue ($n=3$ per treatment).

represent a major pathway by which cells degrade and recycle cellular materials. Under conditions of oxidative stress, however, processing capacity declines. Free radical production often increases from incomplete catabolism, exacerbating the problem. Compromised or leaky lysosomes may release damaging hydrolases into the cell, leading to oxidative cell death (Thibault et al., 1998). Lysosomal enhancement has been shown to be a protective mechanism and to participate in Aβ and likely hyperphosphorylated tau turnover in AD patients (Barrachina et al., 2006). Together, these data suggest that the up-regulation of LAPTMA4 and LAMP1 may be part of a compensatory system in response to cellular accumulation of nondigested materials (Butler and Bahr, 2006).

A considerable body of evidence has demonstrated a critical role of calcium dysregulation during aging and cognitive decline. The calcium homeostasis dysregulation hypothesis of brain aging and neurodegeneration proposes that increased basal calcium levels are present in aged neurons and that restoration of calcium homeostasis is compromised (Mattson et al., 1993; Thibault et al., 1998). Calcium signaling is mediated through several calcium-binding proteins, including calmodulin, which modulates the activity of several key-signaling molecules that are crucial for synaptic plasticity (Xia and Storm, 2005). The expression of calmodulin, calbindin, calcineurin, and calmodulin kinase genes have been reported to be decreased in aged mouse (Jiang et al., 2001) and human (Lu et al., 2004) cortex tissue. Our results, however, contradict these experiments, as aged dogs had increased expression of genes associated with calcium binding and signaling (e.g., calcineurin, S-100A1, S-100A10, calmodulin 3, 14-3-3 protein). Because oxidative agents have been shown to disrupt calcium homeostasis and activate calcium-dependent proteases (Sanvicens et al., 2004), it may be that an increase in oxidative stress is, in part, responsible for the differences observed in aged dogs. B-cell lymphoma protein 2 (BCL2), an anti-apoptotic protein that improves neuronal survival following cellular insult, has been associated with mitochondrial calcium sequestration and also was increased in geriatric dogs in the current experiment. Together, these data demonstrate the need for a better understanding of calcium homeostasis and its role in cognitive health in the aged.

In the current experiment, geriatric dogs had several genes associated with neuropeptide signaling, synaptic transmission, or brain development with decreased expression. Decreased somatostatin receptor expression may be expected, as somatostatin expression has been shown to decline with age in non-human primates (Hayashi et al., 1997) and humans (Lu et al., 2004) and is decreased in AD patients (Dournaud et al., 1994). Decreased brain somatostatin has important implications on cognitive function, as it has been shown to regulate Aβ metabolism in both in vitro and in vivo systems (Saito et al., 2005). It is hypothesized that somatostatin regulates Aβ levels by modulating the activity of neprilysin, a neutral endopeptidase responsible for Aβ catabolism (Hama and Saido, 2005).

The expression of other genes involved with neuropeptide signaling, namely NPY and glutamate transporters, also were decreased in aged dogs. Abundance of NPY, which is co-localized with somatostatin in a majority of cortical neurons (Chronwall et al., 1984), has been reported to decline in rat brain tissue with age (Hattiangady et al., 2005). Decreased NPY expression may contribute to many of the behavioral changes observed in aged dogs and humans, as it has been shown to have anti-anxiety effects (Heilig et al., 1992), influence feeding behavior (Merlo Pich et al., 1992), and is thought to be involved in the pathophysiology of depression (Widdowson et al., 1992; Widerlöv et al., 1988).

In the current experiment, the vesicular glutamate transporter 1 (SLC17A7) and the glutamate/aspartate transporter 2 (SLC1A2) were decreased in the cortex of aged dogs regardless of dietary regimen. Interestingly, a transporter for glutamine (SLC38A1), an important precursor of glutamate, was increased in aged dogs. Glutamate is the principal excitatory neurotransmitter in the brain and is crucial in neuronal differentiation, migration, and survival in the developing brain. Despite its importance in neurological function, glutamate can be neurotoxic at high concentrations. Dysfunction in any of the five glutamate transporters known to exist in the mammalian central nervous system may result in neurotoxicity. Rothstein et al. (1995), for example, reported a reduction in the expression of the glial glutamate transporter in spinal cord and brain regions showing loss of motor neurons. Neurodegeneration in a variety of late onset neurological disorders (e.g., motor neuron disease, Huntington's disease, Parkinson's disease, AD) is at least partially dependent on the activation of ionotropic receptors by glutamate (Meldrum, 2000). Thus, decreased glutamate transporter expression in aged brain tissue, as reported here and in mice (Jiang et al., 2001) and humans (Lu et al., 2004), may contribute to cognitive decline.

As expected, the expression of BDNF was decreased in geriatric dogs, a response that has been observed in aged rats (Croll et al., 1998) and in AD patients (Phillips et al., 1991). BDNF plays a key role in neuronal survival and functions via the tyrosine kinase receptor TRKB, a gene also decreased in geriatric dogs of the current experiment. Numerous studies have evaluated the interaction between estrogen and BDNF signaling, demonstrating that BDNF-synthesizing neurons are co-localized with estrogen receptors (Miranda et al., 1993) and estrogen replacement increases BDNF expression in cortex tissue (Sohrabji et al., 1995). Although not measured in this experiment, decreased estrogen levels may have contributed to the decreased BDNF and TRKB expression in the geriatric dogs of the current experiment.

Expression of corticotropin releasing hormone (CRH), a neuroprotective hormone that responds to stressful stimuli and inhibits apoptosis, was decreased in aged dogs in the current experiment. Disruption of the CRH system has been shown to be associated with neurological diseases. Reduced CRH, as noted in old dogs herein, has been observed in AD patients and is considered to be a surrogate marker for

the disease (Davis et al., 1999). Expression of neuromodulin, also known as growth-associated protein (GAP43), was also decreased in geriatric dogs. Although it is known to participate in axon elongation in the developing brain, neuromodulin's role is not well understood in adult animals. Neuromodulin is thought to mediate experience-dependent plasticity and long-term potentiation in adults and may enhance the growth and retraction of presynaptic terminals in cortical brain areas (Lovinger et al., 1986).

Our analyses demonstrated that the effects of feeding a high-fat diet on cerebral cortex mRNA abundance were less consistent and prominent than those of age. However, interesting changes in mRNA abundance were noted. Expression of toll-like receptor 4 was increased in aged dogs fed APB versus PPB. Over 10 toll-like receptors exist and function by activating immune cells in response to pathogens or cell damage. Toll-like receptor 4 is thought to play a role in Aβ uptake and clearance, as mutations for the gene lead to increased Aβ deposition in rodent models (Tahara et al., 2006). Thus, increased toll-like receptor 4 expression in old dogs fed APB may be a compensatory mechanism by which to clear excess Aβ in these dogs.

Diet-induced changes in gene expression were not only present in old dogs, but young dogs as well. Similar to the effects of old age, lysosomal-associated membrane protein 2 (LAMP2) was increased in young adult dogs fed APB versus those fed PPB. Although it is only speculative at this time, increased LAMP2 expression may be an early indicator of oxidative stress or changes in Aβ metabolism and requires further study. Another gene affected by age, BDNF, was also affected by high-fat feeding in young adult dogs. Expression of BDNF was decreased in young adult dogs fed APB versus those fed PPB, a response that was similar to that reported previously in rats (Molteni et al., 2002). In that study, rats fed diets containing high saturated fat and refined sugar levels had decreased hippocampal BDNF and spatial learning performance. Because cognitive tests were not performed in the current study, the effects of high-fat feeding on learning ability in young dogs are not known. Our mRNA data suggest negative outcomes of consuming high fat and justify the use of cognitive testing in future canine studies.

Overall, the results of this experiment further justify the use of the dog as a model for age-related cognitive decline. A majority of the age-related gene expression differences observed in our geriatric dog population were in accordance with what has been reported in the human and rodent literature. In general, aged dogs had an up-regulation of genes associated with an inflammation or stress response, protein folding, calcium signaling, and down-regulation of genes associated with neuropeptide signaling and synaptic transmission. Many of the gene expression differences observed among aged and young adult dogs are not only altered with aged humans, but also in AD patients.

Using the data reported here, future experiments may be designed to pursue links between behavioral traits or cognitive test performance and molecular markers. Evaluating the effects of dietary intervention, including candidates such as antioxidants and omega-3 fatty acids, on mRNA abundance and behavioral assessment are also of interest. Identifying genotypic–phenotypic associations will promote our understanding of this complex field and may aid in the development of preventative and/or treatment therapies for age-related cognitive decline. In addition to its existing strengths, the availability of gene sequence information and commercial canine microarrays has made the canine a powerful model for studying the effects of aging on cognitive function.

Conflict of interest

All authors have no conflicts of interest pertaining to the data presented and the publication of this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2007.10.017.

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