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EFFECTS OF DIET AND BEHAVIORAL ENRICHMENT ON FREE FATTY ACIDS IN THE AGED CANINE BRAIN

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Abstract—Despite several recent studies suggesting that dysregulation of brain lipid metabolism might contribute to the mechanisms of aging and Alzheimer's disease (AD), lipid metabolism has not been evaluated extensively in the aging brain. Here, we use a lipidomic approach to demonstrate that antioxidants plus mitochondrial cofactors treatment, either alone or in combination with behavioral enrichment, attenuates lipid abnormalities in the frontal cortices of aged canine in a manner correlated with cognitive scores. Our analyses revealed that the levels of free palmitoleic acid and nervonic acid were decreased in frontal cortices of aged dogs (n=5–6/group) treated with antioxidant compared with the control group. The monounsaturated/saturated fatty acid ratio, also known as "desaturation index"—an *ex-vivo* indicator of stearoyl-CoA desaturase activity, was also reduced in the frontal cortex of dogs treated with antioxidants compared with control groups. Increased palmitoleic acid levels and desaturation index were positively correlated with increased reversal learning errors and decreased cognitive performance. In conclusion, our study indicates that the addition of antioxidants and mitochondrial cofactors to the regular diet alters the composition of free fatty acids in the aged brain. Together with data showing increased palmitoleic acid levels in AD patients, our data suggest that reducing palmitoleic acid levels and desaturation index in the brain may be associated with improved cognitive performance. Published by Elsevier Ltd on behalf of IBRO.

Key words: lipids, canine, aging, Alzheimer's disease, SCD, frontal cortex.

Aging is a complex process that makes most cellular components such as proteins, DNA, and lipids susceptible to damage. Although the effect of age on protein and DNA in the brain have been extensively studied, investigations on the effects of lipids have been fewer and far in between and are largely limited to lipid peroxidation (Montine et al., 2002; Ou et al., 2002). However, evidence from recent studies strongly suggests that abnormalities in brain lipid metabolism might contribute to the mechanisms of aging and Alzheimer's disease (AD) (Cutler et al., 2004; Grimm et al., 2005; Lukiw et al., 2005; Green et al., 2007; Piomelli et al., 2007; Hooijmans and Kiliaan, 2008; Sanchez-Mejia et al., 2008). Lipids play a critical role in brain structure and function through two general mechanisms: (1) they affect the cellular membrane structures and protein-membrane interactions; (2) they serve as signaling molecules, binding to plasma membrane or nuclear receptors and mediating transmembrane signaling and cell-to-cell communication (Adibhatla and Hatcher, 2007). Furthermore, several lipid constituents of the cellular membranes have been shown to have a significant impact on receptors, glutamate transporters, and ion channels (Gegelashvili and Schousboe, 1997; Giusto et al., 2002; Meves, 2008).

Although the role of membrane bound fatty acids is indeed critical, recent evidence suggest that functional effects of lipids may not be limited to phospholipids alone. For instance alterations in levels of free fatty acids have been associated with cognitive deficits in aging and AD (McNamara et al., 2008; Astarita et al., 2010). In addition, we have very recently identified a previously unrecognized elevation in stearoyl-CoA desaturase (SCD) activity (ratio of palmitoleic to palmitic acid—16:1/16:0) and a measure of desaturation index (Flowers, 2009)—in the brains of subjects with AD compared with control subjects (Astarita et al., 2011). Although the role of SCD in the brain is still not completely understood, in peripheral tissues desaturation index is considered a potential biological marker of metabolic syndrome (Sampath and Ntambi, 2008). SCD activity is positively correlated with insulin resistance, abdominal adiposity, and hyperlipidemia (Attie et al., 2002; Warensjö et al., 2005; Corpeleijn et al., 2006; Warensjo et al., 2007; Mar-Heyming et al., 2008; Paillard et al., 2008). Recently, adipose tissue-derived palmitoleate has been proposed to act as a "lipokine," a lipid-derived circulating factor that controls energy homeostasis and insulin resistance in mice (Cao et al., 2008). As such, SCD is emerging as a promising therapeutic target for the treatment of diabetes, hyperlipidemia, and obesity (Li et al., 2002; Doherty et al., 2008; Farr et al., 2008; Guo et al., 2008; Kalra, 2008; Signore et al., 2008; Holden et al., 2009; Lieb et al., 2009; Moult et al., 2009; Solovyova et al., 2009).

Similarly, free fatty acids such as arachidonic acid (20:4n6), which are abundantly expressed in the brain have been shown to be implicated in AD. Imaging of radiolabeled 20:4n6 in brains of human subjects has revealed increased 20:4n6 metabolism in patients with AD (Esposito et al., 2008). Furthermore, accumulation of 20:4n6 has been shown to induce the polymerization of tau protein (Wilson and Binder, 1997) and induces apoptosis in neurons (Lipton, 1999).

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Abbreviations: AD, Alzheimer's disease; SCD, stearoyl-CoA desaturase.

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Although increase in levels of free fatty acids, specifically those implicated in the SCD pathway, and SCD activity (desaturation index) itself have previously been identified in the aging human brain (McNamara et al., 2008) and more recently in AD (Astarita et al., 2011), to our knowledge there is no information available about effective intervention strategies. Furthermore, no studies have investigated the role of free fatty acids in higher animal models of aging.

Aged dogs provide a very useful model for human aging as they naturally develop learning and memory impairments with age (Milgram et al., 1994; Snigdha et al., 2011b). Previous work in our laboratory has shown that the aging canine brain accumulates β-amyloid (Aβ) peptides (Head et al., 2000) and shows evidence for both oxidative damage (Head et al., 2002) and neuron loss in the hippocampus (Siwak-Tapp et al., 2007). We have also shown that dogs provide a unique opportunity to study the effects of potential longitudinal interventions that improve behavioral and cognitive outcome measures (Cotman and Head, 2008). Specifically, age-dependent impairment in measures of frontal lobe dependent domains such as executive function (assessed using discrimination and reversal learning tests) can be attenuated by providing an enriched environment (C/E) or an antioxidant and mitochondrial cofactor enriched (C/A) diet (Cotman et al., 2002; Milgram et al., 2002a,b, 2005). The combination of these two interventions results in a more significant improvement in cognitive test scores when compared with either intervention alone (Siwak-Tapp et al., 2007). Thus, it is possible that the two interventions have unique mechanisms that underlie their beneficial effects. Since we have previously established that levels of some free fatty acids such as 16:1 and 20:4n6 are significantly altered in AD (Astarita et al., 2011), we have focused this study on determining potential alterations in the levels of these free fatty acids following lifestyle interventions in aged dogs. We also measured levels of nervonic acid (24:1), which is produced by SCD and is markedly increased in brain tissue of AD patients. To explore the functional significance of lipid changes, we further studied the correlation between these lipid levels and cognitive markers.

EXPERIMENTAL PROCEDURES

Subjects

Twenty-four beagles ranging in age at the start of the study from 8.05 to 12.35 years (mean=10.69 years, SE=0.25, 12 males/12 females) were obtained from the colony at the Lovelace Respiratory Research Institute. They were born and maintained in the same environment and all had documented dates of birth and comprehensive medical histories. All studies were conducted in compliance with approved IACUC protocols, consistent with the National Research Council's Guide for the care and use of laboratory animals.

Group assignments and study timeline

All dogs underwent extensive baseline cognitive testing, as previously described (Milgram et al., 1994; Head, 2009). Based on cognitive test scores, animals were ranked in order of cognitive ability and placed into one of four treatment groups such that each group contained animals with equivalent ranges of cognition (e.g. poor to good): C/C—control environment/control diet, C/E—control diet/enriched environment, C/A—control environment/antioxidant diet, E/A—enriched environment/antioxidant diet. All animals in the study were evaluated annually on a battery of cognitive tests. Cognitive results for these animals have been published previously (Cotman et al., 2002; Milgram et al., 2002a,b). At the time the animals were sacrificed, 23 of the 24 animals that began the study had received the intervention(s) for >2 years (mean$\pm$SEM, 2.69$\pm$0.04 years) and ranged in age from 10.71 to 15.01 years (mean$\pm$SEM, 13.31$\pm$0.26 years). One animal did not complete the baseline phase of the study and was not evaluated further.

Environmental enrichment

The environmental enrichment protocol has been described previously (Head et al., 2009) and consisted of housing animals in pairs (social enrichment), providing 2–20 min outdoor walks per week (physical exercise), and continuous cognitive testing (cognitive enrichment). The cognitive enrichment consisted of a landmark discrimination task (Milgram et al., 2002a), an oddity discrimination task (Milgram et al., 2002b), and discrimination learning and reversal tasks (Head et al., 1998; Milgram et al., 2005). Discrimination learning and reversal was assessed after one year of treatment with a size discrimination task (Tapp et al., 2003), and again after two years with a black/white discrimination task (Milgram et al., 2005).

Diet

The two foods were formulated to meet the nutrient profile for the American Association of Feed Control Officials recommendations for adult dogs (AAFCO 1999) and have been described previously (Head et al., 2009). Control and test diets were identical in composition, other than inclusion of a broad-based antioxidant and mitochondrial cofactor supplementation to the test diet. The control and enriched foods had the following differences in formulation on an as-fed basis, respectively: DL-alpha-tocopherol acetate (120 ppm vs. 1050 ppm), L-carnitine (<20 ppm vs. 260 ppm), DL-alpha-lipoic acid (<20 ppm vs. 128 ppm), ascorbic acid as Stay-C (<30 ppm vs. 80 ppm), and 1% inclusions of each of the following (1-to-1 exchange for corn): spinach flakes, tomato pomace, grape pomace, carrot granules, and citrus pulp. The caloric content was 3750 kcal/kg$\pm$10% for both foods. Sources of fat were pork fat and soy oil. Neither food was enriched in omega-3 fatty acids.

Tissue preparation

Dogs were ex-sanguinated under anesthesia (5% isoflurane) by cardiac puncture and within 15 min the brain was removed from the skull. The brain was sectioned mid-sagitally, with the entire left hemisphere being immediately placed in 4% paraformaldehyde for 48–72 h at 4 °C then transferred to phosphate buffered saline with 0.05% sodium azide at 4 °C for long-term storage. The remaining right hemisphere was sectioned coronally (1-cm-thick sections) and flash frozen at −80 °C. Frontal cortical samples from the frozen tissue samples were used for lipid analysis as described beneath.

Lipid analyses

Fatty acids analyses were conducted as previously described (Astarita et al., 2009). Briefly, frozen tissue samples were weighed and homogenized in cold methanol containing appropriate authentic standards (listed below). Lipids were extracted by adding chloroform and water (2/1, vol/vol) and fractionated through open-bed silica gel columns by progressive elution with chloroform/methanol mixtures. Fractions eluted from the columns were dried under nitrogen, reconstituted in chloroform/methanol (1:4, vol/vol; 0.1 ml), and subjected to liquid chromatography/mass spectrometry. Fatty acids were quantified with an Agilent 1100 liquid chromatograph coupled to a 1946D mass detector equipped with an electrospray ionization interface (Agilent Technologies, Palo Alto, CA, USA). A reversed-phase XDB Eclipse C18 column (50×4.6 mm i.d., 1.8 μm, Zorbax, Agilent Tech-

nologies) was eluted with a linear gradient from 90% to 100% of A in B for 2.5 min at a flow rate of 1.5 ml/min with column temperature at 40 °C. Mobile phase A consisted of methanol containing 0.25% acetic acid and 5 mM ammonium acetate; mobile phase B consisted of water containing 0.25% acetic acid and 5 mM ammonium acetate. Column temperature was kept at 40 °C. Mass detection was in the negative ionization mode, capillary voltage was set at 4.0 kV and fragmentor voltage was 120 V. Nitrogen was used as drying gas at a flow rate of 13 L/min and a temperature of 350 °C. Nebulizer pressure was set at 60 pounds per square inch. Commercially available fatty acids (Nu-Chek Prep, Elysian, MN, USA; Cayman Chemical or Sigma-Aldrich, St Louis, MO, USA) were used as references. For quantification purposes, the deprotonated pseudo-molecular ions $[M-H]^-$ of the fatty acids were monitored in the selected ion-monitoring mode, using $[^2H_8]$-arachidonic acid (Cayman Chemical, Ann Arbor, MI, USA) as internal standard (mass-to-charge ratio, m/z=311.3).

Reversal learning/discrimination testing outcome

To evaluate if the lipid measures were related to cognitive function, outcome measures from a black and white discrimination/reversal task that engages frontal lobe function has been selected. This task was conducted at the end of the intervention period so that the molecular state of the brain would correspond closely to that present at the time of testing. Detailed testing procedure and results from this task have been described previously (Milgram et al., 2005). Briefly test sessions consisted of placing objects over the two lateral food wells; the location of the objects varied randomly, with the constraint that each object was placed on each lateral food well on exactly 50% of the trials. For the reversal learning phase, animals were given 10 trials per day, constituting one session, with an intertrial interval of 30 s. Animals received a maximum of 40 training sessions to achieve a two-stage criterion. The first stage was successfully met once the animal either averaged 80% over two sessions or at least 90% on a single session. To complete the second stage, the dog was required to respond correctly on at least 70% of the trials over three successive sessions. Following completion of the black/white discrimination learning, the animals were started on the reversal task. The testing procedures were identical, except that the previously rewarded object now became the object associated with no reward. Thus, if an animal was rewarded for approaching the white block during the initial testing, it was now rewarded for approaching the black block.

Statistical analysis

Graphpad prism 5 was used for all statistical analysis. All results are expressed as means±SEM. Prior to statistical analysis, data were checked for normality and factorial or one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons was used to identify significant differences between intervention groups. Associations between parameters were tested by correlation analysis (Pearson's). $P<0.05$ was considered significant.

RESULTS

Effects of diet and behavioral enrichment on free fatty acid levels in the brain

We have reported that long-term dietary supplementation with antioxidants and mitochondrial cofactors or behavioral enrichment with social, cognitive, and exercise components effectively improved cognitive performance and reduced brain pathology in aged dogs (Cotman and Head,

Table 1. Effects of diet and behavioral enrichment on levels of free fatty acids in the frontal cortices of aged canine

Fatty acid	C/C (nmol/g)	C/A (nmol/g)	*P*-value	C/E (nmol/g)	*P*-value	E/A (nmol/g)	*P*-value
16:0 palmitic acid	65.07±3.07	61.49±6.77	0.602	76.27±7.01	0.153	61.96±5.11	0.614
17:0 margaric acid	2.16±0.20	1.92±0.15	0.409	2.34±0.14	0.509	2.06±0.17	0.702
18:0 stearic acid	91.17±6.04	88.22±10.88	0.803	97.49±10.59	0.601	89.73±6.87	0.878
20:0 arachidic acid	2.16±0.26	1.94±0.27	0.601	1.81±0.25	0.374	4.31±2.72	0.449
22:0 behenic acid	1.14±0.09	0.95±0.02	0.134	1.13±0.15	0.929	0.98±0.06	0.157
24:0 lignoceric acid	1.27±0.07	1.18±0.08	0.451	1.53±0.33	0.407	1.05±0.03*	0.018
26:0 cerotic acid	0.58±0.06	0.56±0.08	0.875	0.67±0.12	0.500	0.47±0.02	0.097
16:1 palmitoleic acid	2.06±0.11	1.32±0.12**	0.002	2.90±0.62	0.179	1.50±0.21*	0.039
18:1 oleic acid	47.43±5.21	35.28±2.5	0.112	65.94±15.08	0.242	40.66±4.8	0.362
20:1 gadoleic acid	2.12±0.31	1.79±0.20	0.457	3.30±1.03	0.261	1.95±0.28	0.697
22:1 erucic acid	2.18±0.21	1.58±0.16	0.077	2.35±0.55	0.768	2.02±0.20	0.596
24:1 nervonic acid	1.09±0.15	0.69±0.10	0.080	1.22±0.37	0.742	0.72±0.06*	0.038
26:1 ximenic acid	0.84±0.12	0.77±0.06	0.647	1.32±0.46	0.297	0.68±0.09	0.311
20:3 n9 mead acid	0.37±0.05	0.34±0.09	0.784	0.77±0.22	0.081	0.41±0.09	0.662
18:2 n6 linoleic acid	3.90±0.36	2.39±0.16*	0.013	10.57±4.72	0.153	3.02±0.38	0.125
20:3 n6 dihomo-gamma-linolenic acid	3.56±0.45	2.50±0.30	0.116	5.02±0.97	0.180	2.86±0.51	0.329
20:4 n6 arachidonic acid	48.87±3.86	31.74±1.21***	0.008	55.3±6.85	0.414	44.68±5.89	0.565
22:4 n6 docosadienoic acid	8.04±0.72	6.02±0.89	0.114	10.83±2.22	0.230	6.62±0.75	0.200
22:5 n6 cocosapentaenoic acid	2.61±0.32	2.32±0.27	0.542	3.22±0.37	0.247	3.00±0.42	0.479
18:3 n3 alpha-linolenic acid	0.21±0.02	0.12±0.02*	0.010	0.53±0.30	0.267	0.17±0.01	0.112
20:5 n3 eicosapentaenoic acid	0.16±0.02	0.11±0.02	0.091	0.20±0.04	0.276	0.12±0.02	0.151
22:5 n3 docosapentaenoic acid	0.76±0.12	0.45±0.09	0.102	0.82±0.10	0.715	0.52±0.05	0.096
22:6 n3 docosahexaenoic acid	14.7±1.60	10.53±1.86	0.130	16.06±1.38	0.546	12.24±1.42	0.276
SFA	163.54±9.05	156.27±17.96	0.699	181.24±16.55	0.351	160.56±11.86	0.846
MUFA	55.72±5.85	41.43±2.72	0.098	77.02±17.95	0.253	47.52±5.45	0.329
PUFA	83.19±6.78	56.51±4.32*	0.019	103.32±15.96	0.246	73.65±9.00	0.417

C/C, aged controls; C/A, antioxidant and mitochondrial cofactor; C/E, behavioral enrichment; E/A, combined treatment with antioxidants and behavioral enrichment.

Data are expressed as ±SEM.

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with C/C; n=5–6/group.

2008). To determine the effects of C/A and C/E alone or in combination (A/E) on the lipid metabolism of aged-dogs brains, we measured the levels of non-esterified ("free") fatty acids by LC/MS. In Table 1 we report the levels of fatty acids in the frontal cortex of the dogs treated with an antioxidant diet, behavioral enrichment, or the combined treatment compared with control dogs.

Levels of palmitoleic acid and ratio of palmitoleic/palmitic acid in frontal cortex of aged canine. Palmitoleic acid (16:1) is formed by insertion of a *cis* double bond between carbons 9 and 10 in palmitic acid (16:0) (Yeom et al., 2003). This reaction is catalyzed by SCD, and the ratio of 16:1/16:0 is an indicator of desaturation index for conversion of saturated fatty acids to monounsaturated fatty acids. Changes in the desaturation index can alter cell membrane fluidity and is associated with conditions such as aging, diabetes, and AD. The expression of SCD has been known to be regulated by factors such as age (Martin et al., 1999) and diet (Daniel et al., 2004; Issandou et al., 2009). Therefore we examined if dietary or behavioral enrichment alter the levels of 16:1 or the ratio of 16:1/16:0 in the aged canine. We found significant changes in expression of 16:1 and the ratio of 16:1/16:0 in the dietary intervention groups. An overall ANOVA revealed that the interventions induced a significant change in 16:1 content ($F_{3,22}$=7.61, P<0.01, one-way ANOVA). Further post hoc testing showed that 16:1 levels were decreased in the C/A group (P<0.01) and the combined treatment group (P<0.05), compared with control animals (Fig. 1A). One-way ANOVA to examine the effect of interventions on the "desaturation index" or ratio of 16:1/16:0 showed a robust effect ($F_{3,22}$=4.37, P<0.01). Post hoc tests indicated that the ratio of 16:1/16:0 was decreased following C/A treatment alone (P<0.01) and in the combined treatment group (P<0.05) (Fig. 2A). These findings are consistent with the hypothesis that dietary intervention can alter SCD activity in cells.

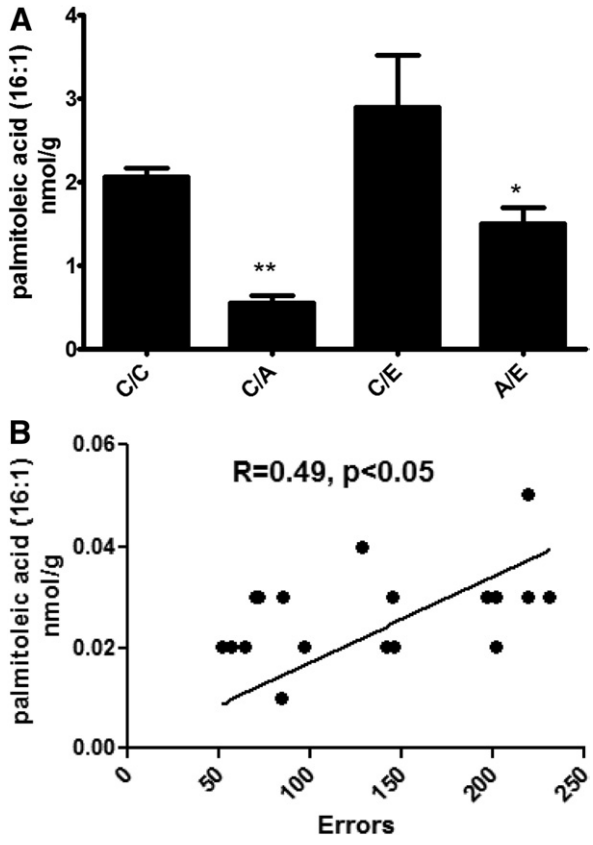

Fig. 1. (A) Effects of diet and behavioral enrichment on the levels of free palmitoleic acid (16:1) in the frontal cortices of aged canine. Average palmitoleic acid levels show significant reductions in the C/A and A/E groups receiving the antioxidant diet, but not with behavioral enrichment alone C/E when compared with aged control (C/C). Data are expressed as mean±SEM. * P<0.05, ** P<0.01, n=5–6/group. (B) Correlation between palmitoleic acid (16:1) levels and cognitive function. There was a positive correlation between cortical palmitoleic acid levels and reversal learning error scores (r=0.49, P<0.05).

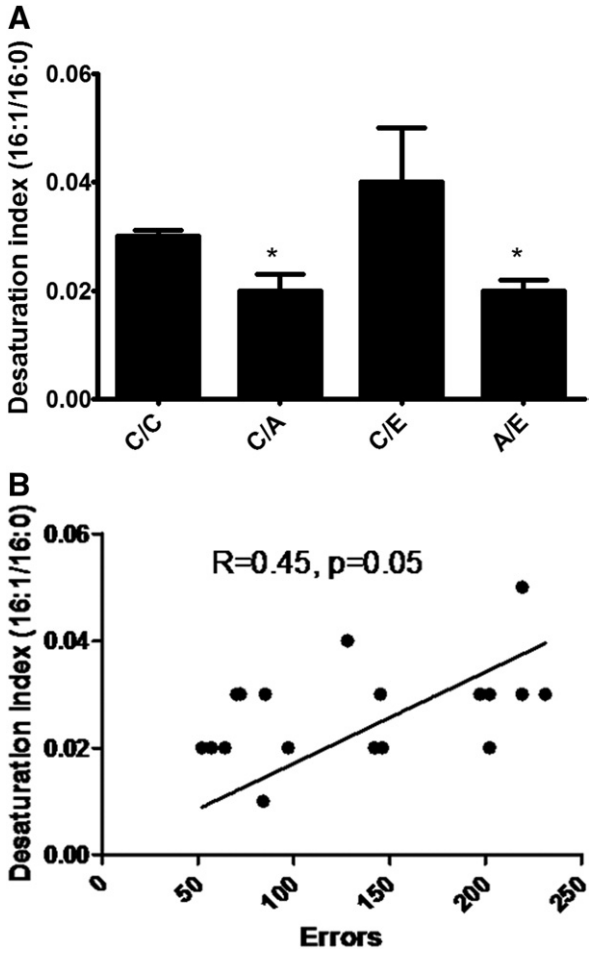

Fig. 2. (A) Effects of diet and behavioral enrichment on the desaturation index in the frontal cortices of aged canine. The desaturation index (16:1/16:0 ratio) was significantly reduced in the C/A and A/E groups receiving the antioxidant diet, but not with behavioral enrichment alone C/E when compared with aged control (C/C). Data are expressed as ±SEM. * P<0.05, n=5–6/group. (B) Desaturation index and cognitive function. There was a positive correlation between the ratio of 16:1/16:0 and reversal learning error scores (r=0.49, P=0.05).

Levels of nervonic acid in the frontal cortices of aged canine. Biosynthesis of 24:1 occurs downstream of desaturation of stearic acid (18:0) into oleic acid (18:1) and is

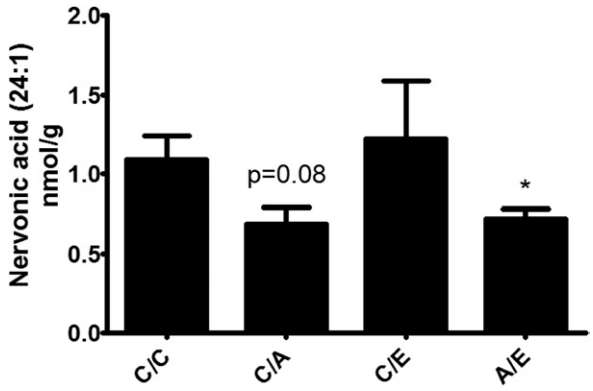

Fig. 3. Effects of diet and behavioral enrichment on the levels of nervonic acid (24:1) in the frontal cortices of aged dogs. Average nervonic acid levels show almost significant reductions in the C/A and significant reductions in the E/A groups receiving the antioxidant diet, but not with behavioral enrichment alone C/E when compared with aged control (C/C). Data are expressed as ±SEM. * $P<0.05$, $n=5$–6/group.

also catalyzed by SCD. Therefore to test the hypothesis that SCD activity is altered by the diet intervention, we assessed levels of 24:1 in the frontal cortices of the animals. There was a decrease in levels of nervonic acid (24:1) following dietary treatment. An overall ANOVA showed a significant effect of treatment on levels of 24:1 ($F_{3,22}=3.47$, $P<0.05$). Post hoc analysis showed that the combination treatment reduced the levels of 24:1compared with controls ($P<0.05$, Fig. 3).

Levels of arachidonic acid in the frontal cortices of aged canine. A factorial ANOVA showed a significant effect of intervention ($F_{3,74}=5.51$, $P<0.01$) and a significant effect of lipids measured ($F_{3,74}=315.04$, $P<0.001$). There was no effect of interaction ($F_{9,74}=1.73$). Further post hoc analysis revealed that levels of arachidonic acid (20:4n6) were significantly decreased in the frontal cortices of the C/A group compared with controls ($P<0.05$, Fig. 4). However, there were no significant changes in free fatty acid levels in the frontal cortex of C/E- or E/A-treated dogs compared with the control group.

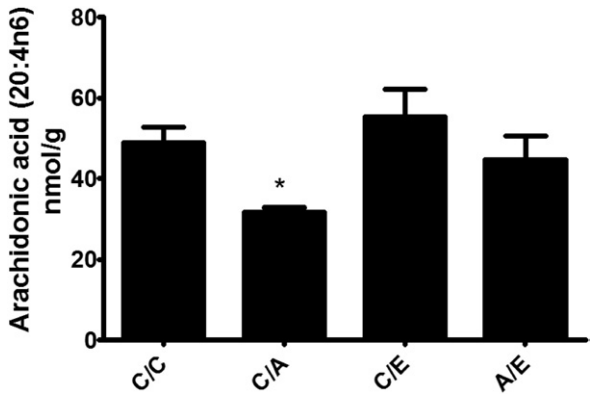

Fig. 4. Effects of diet and behavioral enrichment on the levels of arachidonic acid (20:4n6) in the frontal cortices of aged canine. Average arachidonic levels show significant reductions in the C/A group receiving the antioxidant diet but not with behavioral enrichment alone C/E or in the combination group A/E when compared with aged control (C/C). Data are expressed as ±SEM. * $P<0.05$, $n=5$–6/group.

Correlation between fatty acids and reversal learning error scores

The reversal learning task is a test of executive function, which assesses an animal's ability to distinguish two objects which differ only in a single dimension (Head et al., 1998). The reversal task used here tested the ability of the animals to switch from responding to one object that differed from the other in color. This task was selected because we have previously found it to be both age- and diet-sensitive (Milgram et al., 2004, 2005) and because it relies heavily on the frontal cortical region of the brain.

We observed a positive correlation of 16:1 ($r=0.49$, $P=0.05$, Fig. 1B) and 16:1/16:0 ($r=0.45$, $P=0.05$, Fig. 2B).

DISCUSSION

Findings from this study provide novel evidence for functional roles of free fatty acids in the frontal cortices of aged canine in a paradigm relevant to aging and age-related cognitive deficits. Results presented here show for the first time that a diet enriched in antioxidants and mitochondrial cofactors can reduce the levels of palmitoleic acid (16:1) and desaturation index (16:1/16:0 ratio)—an index for SCD activity in the canine brain. Another significant finding reported here is the reduction in arachidonic acid (20:4n6), a key marker of inflammation, following treatment with antioxidants and mitochondrial cofactors in the frontal cortices of aging canine subjects. It is noteworthy that there was no significant effect of environmental enrichment alone on any of the above-mentioned fatty acid levels. The combined treatment also did not reduce levels of 20:4n6 suggesting that the beneficial effects of the two interventions (on cognition) are mediated via distinctly different pathways.

Previous work in our laboratories has shown increased levels of 16:1 and SCD activity in the frontal cortex from subjects with AD compared with control subjects (Astarita et al., 2011). In the present study, the correlation between SCD activity and reversal learning error scores across treatment groups suggests that SCD might have a role in regulating cognitive function. Therefore, our study indicates that dietary interventions can reduce brain SCD activity in a manner correlated with behavioral scores. Furthermore, levels of another fatty acid, nervonic acid (24:1), which is formed downstream of the desaturation of stearic acid (18:0), are also shown to be reduced by the C/A and A/E treatments. This extends previous findings from our laboratory showing that levels of 24:1 is increased in AD compared with control samples (Astarita et al., 2011).

We also report reductions in 20:4n6, following treatment with antioxidants and mitochondrial cofactors in the frontal cortices of aging canine subjects. This fatty acid is a precursor for potent pro-inflammatory mediators and a key marker of inflammation. In response to inflammatory stimuli, phospholipase A_2 enzymes cleave membrane

phospholipids, releasing 20:4n6. We have previously shown that the C/A intervention described here reduces inflammatory stimuli in the canine brain (Opii et al., 2008), thus it stands to reason that effects downstream of such stimuli would be reduced. We found no significant effect of environmental enrichment alone or in combination with the diet on levels of 20:4n6 suggesting that downstream signal transduction events mobilized by antioxidants may be separable and from those engaged by environmental enrichment. Such events may include activation of protective mechanisms such neurotrophic factor accumulation or even improved synaptic connectivity. It is also important to note that the dissociation between specific pathways activated by dietary vs. enrichment interventions reported here is consistent with what we have observed and reported previously (Snigdha et al., 2011a).

20:4n6 can inhibit the activity of many K^+ channels (Honoré et al., 1994; Keros and McBain, 1997; Meves, 2008) and glutamate transporters (Gegelashvili and Schousboe, 1997) while increasing activation of NMDA receptors (Miller et al., 1992). Whereas most neurotransmitters are limited in their ability to access the core of the membrane due to their hydrophobic nature, lipids are amphipathic compounds and can function both inside and outside membrane boundaries. Thus, free 20:4n6 can also bind receptors at synapses or diffuse into the cell nucleus and interact with transcription factors to control transcription for cytokines. A decrease in expression of free 20:4n6 would hence result in decrease in pro-inflammatory signal in the brain.

Overall our results build on and extend our previous observations that lipid alterations occur in AD vs. control subjects (Astarita et al., 2011). Here we report that dietary intervention can attenuate this increase in a manner correlated with behavioral function in the aged canine. We also describe a novel finding about the expression and role of free fatty acids in the brain and underscore a functional effect of SCD activity and arachidonic acid in mediating cognitive deficits induced by aging. In view of the value of the aged canine as a higher animal model for studying human aging and cognitive impairment, these results are extremely significant. They demonstrate that levels of various free fatty acids, which are increased in the AD brain, can be reduced by lifestyle interventions such as diet.

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