Localization of Lutein in Monkey Brain and its Potential Role in Brain Health

A thesis presented to the faculty of the Gerald J. and Dorothy R. Friedman School of Nutrition Science and Policy

By

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In partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biochemical and Molecular Nutrition

Tufts University

April 2016

Acknowledgements

There are many individuals I would like to acknowledge as I would not have been able to complete my thesis without their guidance and support. First and foremost, I would like to thank my thesis committee of Dr. Elizabeth Johnson, Dr. John Erdman Jr., Dr. Oliver Chen, and Dr. Nirupa Matthan for their invaluable insight and advice. All have helped me become a better scientist and have prepared me for the next stages of my career. A special thanks to my chair, Dr. Johnson, whose enthusiasm for research has been an inspiration to me. I am so grateful for her dedication to my growth as a person, scientist, and student of nutrition. I would also like to thank current and former members of the Antioxidants Research Laboratory, particularly Dr. Jeffrey Blumberg, Dr. Diane McKay, Dr. Rohini Vishwanathan, Dr. Jason Walsh, and Jordan Haddock, for their assistance. The opportunity to work with such wonderful people on a daily basis made my pursuit of a PhD a truly positive experience. I would also like to thank Dr. Martha Neuringer for lending her time and expertise to the study design and data interpretation for this project. Last, but not least, I would like to thank my family, boyfriend, and friends for their encouragement and unwavering faith in me throughout this process. Your support has meant the world to me.

Abstract

Lutein, a carotenoid with antioxidant and anti-inflammatory properties, selectively accumulates in primate brain and may be beneficial for cognition. The combination of lutein with docosahexaenoic acid (DHA), a long-chain n-3 polyunsaturated fatty acid (PUFA), may provide additional cognitive benefits. Lutein is known to incorporate into membranes. However, its distribution in the brain, its interactions with nutrients important for brain health (such as DHA and vitamin E), and its role in brain function remain unclear. Cognitive impairment is caused by brain cell death, which can result from impaired cell signaling, oxidative stress, and gene dysregulation. Furthermore, it is known that brain cell membranes (myelin, neuronal, mitochondrial, and nuclear) are a critical determinant of cell function and viability. Thus, maintaining structurally healthy and functional membranes is essential for cognition.

The objective of this thesis was to determine the relationship between lutein and membrane composition and cell viability in regions of the brain known to control different domains of cognitive function. This objective was met through two aims using brain tissue from 11 adult rhesus monkeys (age 7-20 y) obtained from the Oregon National Primate Research Center. Nine monkeys consumed standard chow (containing 16.4 μmol/kg lutein) while two monkeys consumed the same chow plus a daily oral lutein supplement (91.16 μmol/g lutein) for 7-12 months prior to termination. This was done in order to increase the amount of lutein in the brain, thus, increasing the range of lutein concentrations across brain samples. Lutein concentrations and fatty acid profiles were determined in isolated nuclear, myelin, mitochondrial, and neuronal membranes from the striatum, cerebellum, prefrontal cortex, and hippocampus. The association of

lutein and DHA levels within membranes and the subcellular deposition of vitamin E (α-tocopherol) in relation to lutein were also evaluated. Secondly, the relationship between region and membrane-specific lutein and brain cell viability was investigated.

Membrane lutein concentrations, which did not preferentially accumulate in one membrane over another, were not driven by total membrane PUFA content as is the case with α-tocopherol, an antioxidant found in significantly higher concentrations in the brain. Membrane lutein accumulation was, however, related to DHA accumulation within membranes, lending support to the hypothesis that the two may function together. Lutein was inversely related to DHA in myelin and neuronal plasma membranes but positively related in mitochondrial membranes, indicating the nature of this relationship may be membrane-specific. Lutein concentrations were inversely associated with DHA oxidation more so than arachidonic acid oxidation. Furthermore, this relationship was observed in mitochondrial membranes only, whereas total α-tocopherol (not membrane-specific) was related to PUFA oxidation. Nuclear lutein content was not related to DNA damage, however, nuclear α-tocopherol concentrations were. Lutein in the neuronal plasma membrane was not related to ERK activation, while PUFA concentrations in this membrane were positively associated with activation of this pathway. Although no cause-and-effect conclusions can be drawn from this thesis, the collective findings suggest that lutein may act as an antioxidant in the brain but its role may be related to functions other than that of a free radical scavenger. Results from this thesis provide a critical first step toward understanding how lutein functions in the brain and have generated hypotheses for future studies investigating the mechanism underlying the beneficial effect of lutein on cognition.

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1. Introduction

1.1 General statement of problem

It has been well established that lutein is selectively deposited in the human retina where, along with its isomer zeaxanthin, it forms macular pigment (MP) (1). As MP, lutein and zeaxanthin, another carotenoid, carry out two important functions. First, they absorb high energy blue light to protect the eye from damage (2,3). Second, they act as antioxidants to directly scavenge free radicals to inhibit oxidative stress in the eye (2). In addition to these functions, *in vitro* and *in vivo* studies indicate lutein may also play a role in neural cell viability and survival at both the gene (3,4) and protein (5) expression level. More recent evidence demonstrates that lutein is also the predominant carotenoid in the primate brain, indicating preferential uptake of lutein into non-ocular neural tissue as well (6–10). Both MP density and brain concentrations of lutein are associated with better cognition (10–14), and supplementation with lutein improves cognitive performance in both younger and older adults (15,16).

The mechanisms underlying the role of lutein in cognition are unclear. Although lutein is known to incorporate into membranes, its subcellular distribution among brain membranes, its interactions with membrane-associated nutrients important for brain health (such as DHA and vitamin E), and its role in brain cell viability have not been investigated. Therefore, studies aiming to answer these questions are necessary to fill the gaps in knowledge regarding lutein and cognitive performance in humans.

1.2 Significance and impact of work

Alzheimer's Disease (AD) is the sixth leading cause of death in the United States (US), with 1 in 3 seniors (>65 years of age) dying with AD or another form of dementia (17).

The financial burden of caring for those with AD and other dementias in the US totaled approximately $226 billion in 2015 (18). AD is the most common form of dementia, which is characterized as a cognitive impairment disorder. It is the only cause of death among the top 10 in the U.S. without a known way to prevent or delay its progression (19). Mild cognitive impairment (MCI) is thought to be a transitional stage between normal aging and the earliest symptoms of AD. It has been estimated that the incidence of MCI is 14-18% in older adults (>70 years) (20). The incidence of MCI and dementia will likely continue to increase, given the increasing number of people aged 65 and older in the population, unless preventative measures can be discovered and put into action. Prevention through nutrition interventions may be the most cost-effective strategy. Therefore, *there is a strong need to fill knowledge gaps regarding the mechanisms by which nutrients, phytochemicals, and dietary patterns affect brain viability, function and, ultimately, cognition.*

Given the emerging evidence consistently demonstrating a beneficial effect of lutein on cognition, studies investigating lutein's function in the brain are warranted. To date, a significant obstacle in studying the function of lutein in the brain is that typical small animal models, such as mice and rats, are very poor models of xanthophyll carotenoid absorption, transport, and storage (21,22). However, recent studies conducted in rhesus macaque have demonstrated that these non-human primates selectively deposit lutein in the eye and brain similar to humans (7,23). Non-human primates are the closest species to humans in terms of physiological and neurological characteristics (22), making them invaluable for scientific research.

The use of these animals for studying lutein in the brain provides new opportunities for elucidating its mechanism of action to benefit cognitive function. The work described in this thesis was performed using rhesus monkey brain tissue to provide novel insight into the role of lutein in brain viability and provide a foundation for future work investigating the role of lutein in cognition.

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2. Statement of Hypothesis

The overall objective of this thesis is to determine the relationship between lutein and membrane fatty acid composition and cell viability in regions of the brain known to control different domains of cognition. The central hypothesis is that lutein content in isolated nuclear, mitochondrial, myelin, and neuronal plasma membranes from different brain regions is related to cell health and viability as measured by markers of oxidative stress, cell signaling, and gene expression.

The objective of this study was pursued using 11 rhesus monkey brains obtained from the Oregon National Primate Research Center at Oregon Health and Science University. The adult primates (7-20 y) were fed chow (n=9) containing lutein (16 μmol/kg diet) or chow and a daily lutein supplement (91.16 μmol/g lutein, gelatin beadlet, DSM Nutritional Products) (n=2) for 7-12 months prior to termination in order to increase the range of brain lutein concentrations among monkeys. The central hypothesis of this thesis was tested through two aims.

2.1 Specific aims and hypotheses

Aim 1a: Measure lutein concentrations in myelin, mitochondrial, nuclear, and neuronal plasma membranes from brain regions controlling different domains of cognition (prefrontal cortex, cerebellum, striatum, hippocampus). Lutein accumulation in a particular brain region and membrane may be indicative of its function(s).
Hypothesis: lutein accumulation is region and membrane-specific.

Aim 1b: Determine the relationship between brain region and membrane-specific lutein levels and brain cell viability.

Hypothesis: Our lab has demonstrated that brain and serum lutein concentrations are more consistently related to better cognition scores in humans compared to other antioxidants, such as vitamin E (1), despite it being found in significantly lower amounts. Therefore, lutein may function in cell processes in addition to its role as a free radical scavenger. We hypothesize that lutein concentrations are positively associated with brain cell viability as measured by markers of oxidative stress, cell survival signaling, and gene regulation.

Aim 2: Determine the association between lutein and docosahexaenoic acid (DHA) levels and DHA oxidation in the brain.

Hypothesis**:** We have observed that the association between brain lutein levels and cognition is dependent on brain DHA levels in the elderly and vice versa (2). Furthermore, supplementation with both lutein and DHA may provide additional cognitive benefits compared to supplementation with either alone (3). DHA is highly susceptible to oxidation; however, the anti-oxidant capacity of lutein may inhibit this from occurring. We hypothesize that lutein and DHA associate in membranes, and membrane lutein is inversely related to DHA oxidation.

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3. Review of the Literature

3.1 Lutein: dietary sources, absorption and storage in primates, structure, function, and health benefits

3.1.1 Carotenoids

The term *carotenoid* refers to a large family of fat-soluble compounds synthesized by plants that are responsible for their red, orange and yellow colors (1). There are over 600 of these plant pigments found in nature, many of which are consumed in fruits and vegetables (2). Research on carotenoids has shown that these compounds impart beneficial effects on human health and decrease the risk of certain age-related chronic diseases, such as cancer, cardiovascular disease, and eye disease. Of the hundreds of carotenoids found in nature, approximately 20 have been detected and identified in human blood and tissues (1). Of those detected in the body, the major carotenoids are β-carotene, α-carotene, lycopene, lutein, zeaxanthin, and cryptoxanthin. The latter three carotenoids are known as xanthophylls, or oxygenated carotenoids, because they possess hydroxyl groups in their structures. This structural characteristic makes them more polar than the former three carotenoids, which are classified as carotenes (2).

Carotenoids can be further categorized as provitamin A or non-provitamin A. β-carotene, α-carotene, and cryptoxanthin can be converted to vitamin A in the body and, therefore, are known as provitamin A carotenoids. β-carotene possesses the highest provitamin A activity among the three (12 µg β-carotene = 1 µg vitamin A compared to 24 µg for α-carotene and cryptoxanthin) and is the most widely studied (3). In addition to its ability to form vitamin A, β-carotene can also act as an antioxidant. β-carotene consumption from fruits and vegetables is associated with a decreased risk for many different cancers, with most research focused specifically on lung cancer (4,5). High dose supplementation of β-

carotene, however, may increase the incidence of lung cancer in those already at a high risk, such as smokers and asbestos workers (5,6).

Lutein, zeaxanthin, and lycopene cannot be converted to vitamin A, therefore, they are classified as non-provitamin A carotenoids (2). Research on lycopene indicates that this carotenoid may function to decrease the risk of prostate and colon cancer (7,8). Lutein and zeaxanthin are best known for protecting the retina from damage and decreasing the risk for age-related eye diseases, such as macular degeneration and cataracts (2). However, more recent evidence on the role of lutein in human health indicates that this carotenoid may impart neural health benefits beyond those in the eye.

3.1.2 Lutein absorption, transport and storage in primates

Lutein is found in a number of foods. Dark, leafy greens such as kale and spinach are particularly high sources of lutein (~50-100 µg/g fresh weight) (9), while avocados and eggs provide more bioavailable sources of lutein in our diet due to their high fat content (10,11). Given that carotenoids are fat-soluble, they are absorbed in the small intestine by the same mechanisms as dietary fat. Therefore, at least some consumption of this macronutrient must occur for lutein to be efficiently absorbed. Secretion of fat into the small intestine stimulates the release of bile, which emulsifies fat for digestion by pancreatic lipase. Lutein, along with other carotenoids, and fat-soluble vitamins are incorporated into micelles, along with the digested fat, for absorption into intestinal cells, or enterocytes (2,12). In addition to fat content in the diet, the food matrix, as well as cooking method, influence absorption of lutein (12). The carotenoid profile of a food may also influence how much lutein is taken up since *in vitro* studies have shown that carotenoids may potentially compete with one another for absorption (13).

Once in enterocytes, lutein is incorporated into chylomicrons, released into the lymphatic system, and eventually transported through the blood where chylomicron remnants are taken up by the liver (2). In the liver, carotenoids are repackaged into lipoproteins and released into the circulation. Studies have shown that lutein and zeaxanthin are primarily transported on high-density lipoproteins (HDL), while the carotenes and lycopene are mainly transported on low-density lipoproteins (LDL) (14). This difference in lipoprotein transportation, along with specific binding proteins present at the blood-retina barrier, is thought to be one of the reasons why lutein and zeaxanthin are selectively taken up into ocular neural tissue (12).

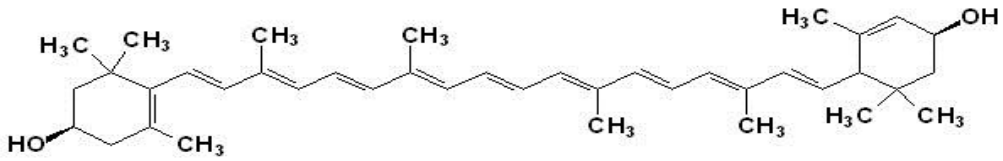
It has been well established that, once in the eye, lutein accumulates in the macula of the retina where, along with zeaxanthin, it forms macular pigment (MP) (15). However, more recent evidence demonstrates that lutein is also the predominant carotenoid in the primate brain even when it is not the major carotenoid in the diet, indicating preferential uptake of lutein into both ocular and non-ocular neural tissue (16–20).

3.1.3 Structure, function, and known health benefits of lutein

The structure of lutein consists of a long, highly unsaturated carbon chain, with one six-carbon ring attached at each end (21). Both rings possess a polar hydroxyl group (**Figure 1**). The structure of lutein causes the molecule to incorporate into cell membranes, with the lipophilic carbon chain situating into the membrane and the polar hydroxyl groups sticking out at each end (21). Investigation of the membrane distribution of lutein in retinal membranes has shown that lutein is particularly concentrated in rod outer segment membranes in both the perifoveal region of the retina, which surrounds the center of the

macula, as well as the peripheral retina (22). These membranes are rich in polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA) (23).

Figure 1. Structure of lutein (24)



In the retina, lutein (and zeaxanthin), as MP, carry out important functions to protect the eye from damage. First, these xanthophylls can absorb high energy blue light (~450 nm wavelength), which prevents the generation of reactive oxygen species (ROS) in this lipid-rich tissue (25,26). Given the high amount of light exposure in the eye, along with its rich PUFA content, this is a particularly critical function of these carotenoids. Second, lutein and zeaxanthin act as antioxidants to directly scavenge free radicals to inhibit oxidative stress in the eye (25). In addition to these established functions, evidence also exists for an anti-inflammatory effect of lutein. Cell culture studies have shown that supplementation with lutein directly inhibits damage-induced gene expression of interleukin-8 (IL-8) in photooxidative retinal pigment epithelial (RPE) cells (27), as well as nuclear levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), interleukin-1 beta (IL-1β), and cyclooxygenase-2 (COX-2) in Müller cells, which are the principal glial cells in the retina, after hypoxic injury (28). Results from these studies must be tested and confirmed *in vivo*, however, these initial findings indicate a potential role of lutein in gene regulation and protein expression to prevent chronic inflammation in the eye.

In vivo evidence for a direct effect of lutein on eye health has been observed in non-human primates. A series of studies in rhesus monkeys fed a xanthophyll-free diet from birth through their lifespan has demonstrated the importance of lutein and zeaxanthin in retinal structures (29,30). The foveal density of RPE cells was morphologically different in xanthophyll-free monkeys, with a dip at the center of the fovea compared to control monkeys, who had peak RPE cell density in this part of the retina (30). RPE cells have many important functions including maintenance of the blood-retinal barrier and recycling of the tips of photoreceptor outer segments. RPE cells are also important for the visual cycle, as the isomerization of *trans*-retinol to *cis*-retinol takes place there (31). Loss of RPE cell density compromises these functions. Current evidence indicates that dysfunctional RPE cells likely play a role in the development of age-related macular degeneration (AMD) (31). Additionally, monkeys devoid of lutein and zeaxanthin also developed drusen (deposits under RPE cells), a hallmark of AMD, at an accelerated rate compared to control monkeys (32). Collectively, these results indicate a direct role of lutein in prevention of AMD in primates.

AMD is thought to be a multi-factorial disease caused by inflammation, oxidative stress, and aberrant immune activation (33). The evidence showing a beneficial effect of lutein in the eye, and damage in its absence, has led lutein to become a focus in the field of AMD research. Additionally, supplementation with lutein has been shown to inhibit systemic activation of the complement pathway, a pro-inflammatory immune response associated with AMD (34,35). Based on this collective evidence, a number of studies investigating the effect of lutein on AMD risk have been performed. However, results from these analyses have been mixed with some, but not all, studies examining dietary

lutein/zeaxanthin or blood levels showing an association with a decreased risk of AMD (36,37). However, in a large, randomized, double-blind clinical trial, supplementation with lutein and zeaxanthin, in combination with a number of other antioxidants (vitamin C and E, zinc), showed a greater reduction in AMD progression compared to supplementation with the antioxidant formula alone (38). This result was more pronounced in individuals with lower lutein status at the start of the study. Therefore, lutein may play a role in delaying AMD progression.

Similar to the retina, lutein is the predominant carotenoid found in the primate brain (16,19). Furthermore, MP density is positively correlated to lutein in matched brain tissue (17,18). These results indicate that selective uptake of lutein into the eye and brain may occur through similar mechanisms. Analysis using rhesus monkeys indicates concentrations of lutein tend to be higher in occipital and frontal cortices compared to cerebellum and pons. Therefore, lutein may not be evenly distributed among different regions of the brain (17). However, there are an extremely limited number of studies in primates evaluating this and a lack of uniformity among them regarding brain regions analyzed (16,17,20). Therefore, it is not clear across the whole brain, which regions lutein preferentially accumulates in over others. Furthermore, *the distribution of lutein among membranes within brain regions has not been investigated.*

This accumulation of lutein into certain regions of neural tissue, namely the frontal and occipital cortices, may be indicative of an important function there. While the occipital lobe is involved in visual functioning (39), the frontal lobe is central to executive function (including language, working memory, decision-making, planning, and problem-solving) (40). Accumulation of lutein in the occipital cortex is a consistent finding with

what is known about the importance of lutein in vision; however, accumulation of lutein in the frontal cortex provides evidence for a role of lutein in cognition beyond visual processing. This result, in addition to evidence that cognitive impairment is associated with age-related eye diseases (41,42), supports the possibility that lutein plays a role in maintaining cognitive function.

3.1.4 Beneficial effects of lutein on cognitive function in humans

Several cross-sectional studies have analyzed the relationship between dietary and/or blood concentrations of lutein, as well as MP density, and cognitive function in humans. Similar to the relationship between serum lutein and AMD, serum lutein is inconsistently related to cognitive function/cognitive status in humans (43–46). Studies assessing MP and cognition, however, show a consistent, positive association between MP density and cognitive function (46–49). Furthermore, consumption of avocado (a highly bioavailable source of lutein) for 6 months increased MP density which was related to improved spatial working memory and problem approaching efficiency test scores in healthy older adults (10). Given that MP density and brain lutein are highly correlated in matched retina and brain tissue (17), these are unsurprising results. Therefore, measuring MP density as a biomarker for brain concentrations of lutein will be a vital tool moving forward with lutein and cognition research. Despite the usefulness of MP, however, it is still only a surrogate biomarker of brain lutein content. Therefore, using this marker in studies limits the conclusions that can be drawn regarding a direct effect of lutein on cognition, and the potential mechanism underlying this relationship.

Very few studies have looked directly at brain concentrations of lutein and its association with cognition in humans. However, one study has assessed the relationship between

brain carotenoid and tocopherol concentrations and cognitive test scores from decedents in the Georgia Centenarian Study (GCS). Results from this study showed that among the carotenoids, lutein was significantly related to cognitive status in centenarians with normal cognition and mild cognitive impairment (20). Furthermore, lutein was consistently related to cognitive test scores compared to the other carotenoids present in the brain, as well as vitamin E, an antioxidant found in significantly higher concentrations in this tissue (20).

Additional evidence supporting a role of lutein in cognition comes from two intervention studies, one in younger healthy adults and one in older adults. In young adults, supplementation with lutein for 4 months increased MP density, which resulted in significant improvements in neural processing speed (50). In another intervention trial, researchers aimed to determine whether supplementation with lutein (also for 4 months) could improve cognitive performance in older women (51). Results from this study found that supplementation with lutein improved verbal fluency scores in these subjects. Collectively, the evidence from these studies suggests lutein is important for cognitive function. However, *it remains unclear as to how lutein functions in the brain to influence cognition*.

Similar to AMD, the pathophysiology underlying cognitive decline is multi-factorial and complex (ex. oxidative stress, inflammation), making it challenging to elucidate how certain bioactives, such as lutein, influence cognition and its decline. However, it is known that cognitive performance is highly dependent on brain health, specifically brain cell viability. Cell death and loss of neurons are considered hallmarks of cognitive impairment and Alzheimer's disease (52). Therefore, *it is possible that lutein functions to*

maintain cell viability, thereby maintaining cognitive function. Factors that influence brain cell viability are discussed in the following section.

3.2 Relationship between membrane composition and function and brain health: Implications for cognitive function

3.2.1 Membrane composition varies among subcellular compartments, cell types, and brain regions

It has been well established through research on brain lipids that membranes vary in their composition among subcellular compartments, cell types, and brain regions (53–56). Much of what is known about differences in lipid composition among organelles in brain cells comes from studies analyzing rats and mice. To date, there have been extremely limited data comparing lipid profiles of organelles in human brain. **Table 1** illustrates the differences in phospholipid, sphingolipid, and cholesterol content (expressed as weight percentages) in plasma membrane, nuclear membrane, and mitochondrial membranes of rat brain (57).

Table 1. Organelle membrane lipid distribution in rat brain*

	Chol	**PC**	**PE**	**PS**	**PI**	**PG**	**CL**	**SM**
Plasma membrane	30	18	11	9	4	0	0	14
Nuclear membrane	10	55	20	3	7	0	0	3
Mitochondrial membrane								
Inner	3	42	24	1	6	2	18	3
Outer	5	45	23	2	13	3	4	5

* Values are given as weight percentages. Chol, cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG phosphatidylglycerol; CL, cardiolipin; SM, sphingomyelin (57)

Cholesterol and sphingomyelin are more concentrated in plasma membrane than nuclear or mitochondrial membranes, while this membrane tends to have less phosphatidylcholine and phosphatidylethanolamine relative to the other membranes. The nuclear membrane is richest in phosphatidylcholine, while the mitochondrial membrane, particularly the inner membrane, is rich in cardiolipin. Differences in lipid composition have important effects on membrane fluidity, symmetry, and cell signaling.

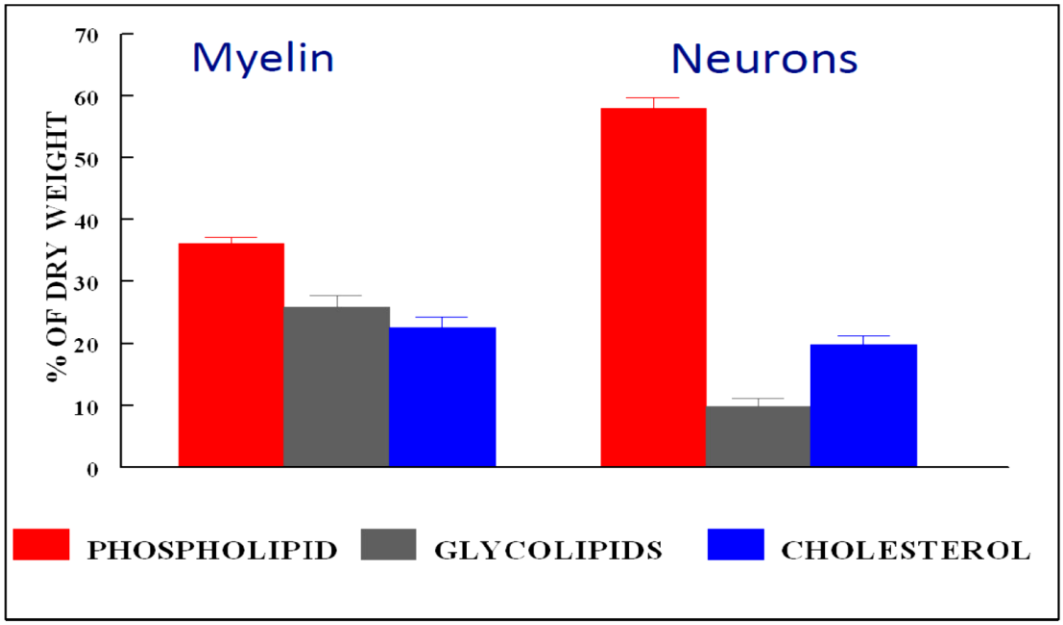
Accumulation of particular lipids in one subcellular compartment versus another provides insight into the function of those membranes in a given organelle. For example, cardiolipin is found almost exclusively in the mitochondria because it functions to help maintain membrane potential and provides structural and functional support to mitochondrial respiratory proteins (58). Sphingomyelin, on the other hand, accumulates more so in the plasma membrane because this lipid is critical for signal transduction, or the transmission of extracellular signals into a cell to influence metabolism, apoptosis, proliferation, and migration (59).

Membrane composition can also vary across cell types in the brain. Namely, it differs between myelinated and non-myelinated neurons. **Figure 2** illustrates the difference in phospholipid, glycolipid, and cholesterol content between myelin and neuronal plasma membranes in rat brain (60). While myelin contains more glycolipids, neurons have higher phospholipid content. Cholesterol content is similar between them.

Glycolipids are essential to the insulating function of the myelin sheath and impairment of glycolipid synthesis in myelin sheath formation leads to a significant decrease in nerve conduction velocity (61). Phospholipids, however, are more concentrated in the plasma

membrane of neurons as they serve as both a barrier between the extracellular and intracellular space, but also as secondary messengers in cell signaling (62).

Figure 2. Lipid distribution in myelin and non-myelinated neurons in rat brain

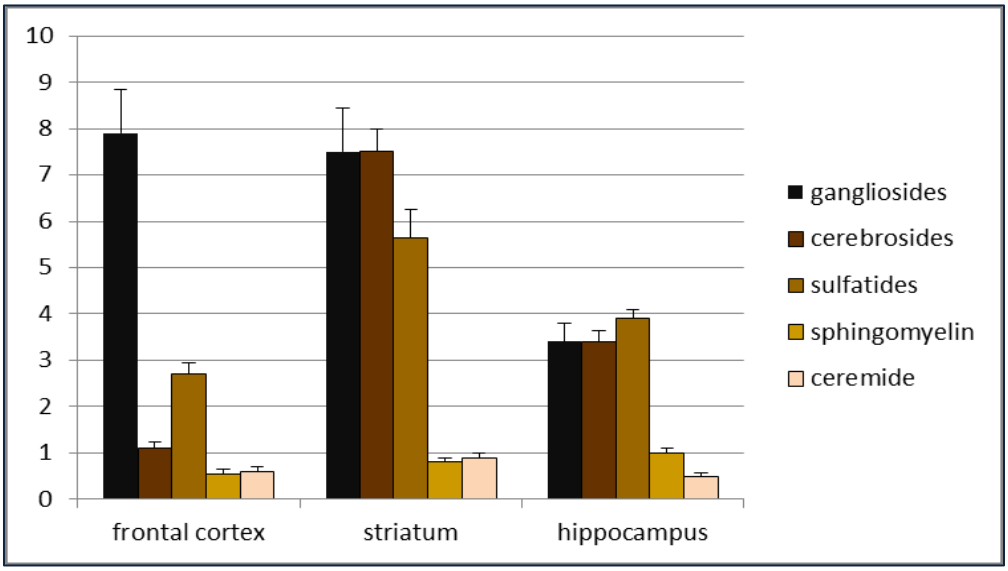


Unlike lipid composition of organelles, a few studies have been performed in primates to determine differences in lipid composition between white and grey matter. Results from analyses on the cerebral cortex of both squirrel monkey and humans are consistent with what is observed in the rat brain (54,63). Specifically, phospholipids, including phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol are significantly higher in non-myelinated neuronal membranes compared to myelin. Conversely, sphingomyelin is found in significantly greater quantities in the myelin sheath (54,63).

Membrane composition is also known to vary across different regions of the brain. **Figure 3** illustrates differences in various sphingolipids across the frontal cortex,

striatum, and hippocampus of rats (64). The frontal cortex consists primarily of gangliosides, with lower amounts of cerebrosides and sulfatides compared to the other two brain regions. Sphingomyelin content is highest in the hippocampus, while ceramide was highest in the striatum.

Figure 3. Sphingolipid distribution differs in brain regions of rats



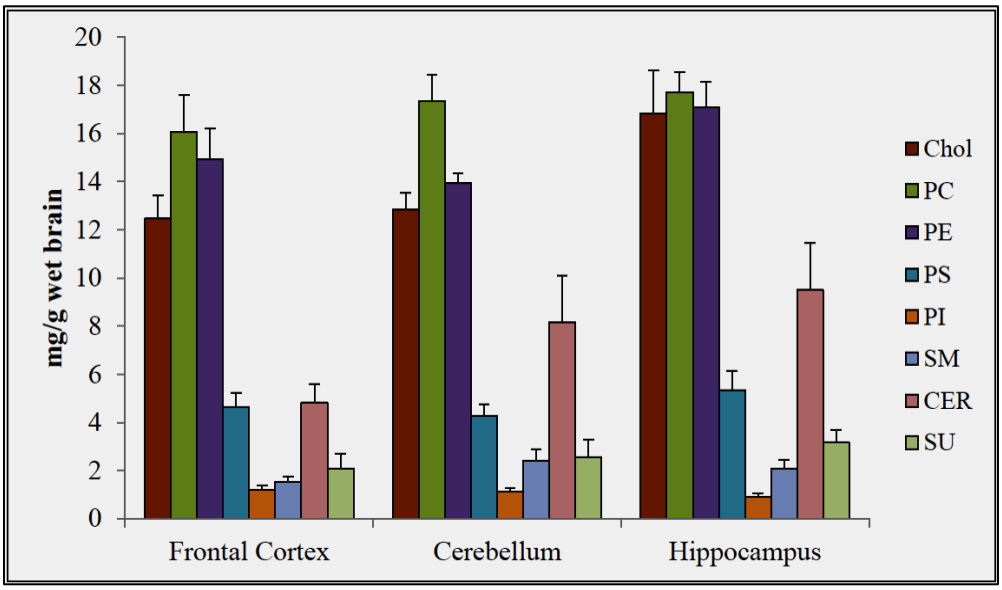
Gangliosides (µmol sialic acid/g); sulfatides (µmol cerebroside sulfate/g); cerebrosides, sphingomyelin, ceramide (µmol sphingosine/g)

Figure 4 illustrates differences in cholesterol, phospholipid, and sphingolipid composition across the frontal cortex, cerebellum, and hippocampus in a different rat study (65). Phosphatidylcholine and phosphatidylserine were similar across the regions; however, cholesterol and phosphatidylethanolamine were highest in the hippocampus. Phosphatidylinositol was lowest in the frontal cortex.

It has been hypothesized that differences in lipid composition are driven by total lipid content in each region (which also can differ significantly) as well as the proportion of white and grey matter in a region (65). Since the functional activity of neurons is highly

dependent on membrane properties, differences in function across brain regions can be attributed, in part, to differences in membrane lipid composition (65).

Figure 4. Lipid distribution differs among brain regions of rats



Chol, cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; CER, cerebroside; SU, sulfatide

Studies analyzing the human brain have determined that differences in lipid composition across brain regions are also influenced by aging (53,55,56). In one such study looking at brain tissues from individuals 33-92 years old, researchers observed that phospholipids and cholesterol were significantly different between cerebellum and hippocampus, with both lipids decreasing with age in each region (53). However the magnitude of loss over the years was dependent on the brain region. For example, phospholipid content decreases with aging much more significantly in hippocampus compared to cerebellum. However, cerebellum cholesterol decreases more significantly with age than in the hippocampus (53). Since membrane composition is important for neuronal cell function,

it is possible that age-related changes in lipid composition contribute to decline in brain cell function over time.

In summary, membrane composition exerts certain physical properties allowing it to perform unique, specialized functions for the cell and region it is located in (66,67). Therefore, membrane composition is an important determinant of brain cell and brain region function. Given the limited number of studies assessing lipid composition of membranes in primate brain across different brain regions, *there is a need to assess the composition of membranes as it relates to brain cell function and viability in order to obtain a better understanding of how these membranes influence cognition in humans.*

3.2.2 Role of subcellular membranes in brain cell viability

Each subcellular membrane plays a unique role in influencing the viability of a cell. For example, the neuronal plasma membrane plays a critical role in cell survival/death signaling pathways in the brain (68). Alterations in plasma membrane fluidity and composition are linked to changes in apoptosis (69,70). Membrane composition, fluidity, and symmetry, dictated by the packaging of membrane lipids, can modulate the accessibility of membrane receptors to other molecules, or to the membrane itself. Consequently, membrane composition and fluidity can influence aggregation of membrane receptors and transporters or regulate protein expression at the cell surface (69,71,72), thereby leading to changes in cell signaling pathways that determine cell viability. One such pathway that is modulated by the plasma membrane, and has been shown to be dysregulated in cell death, is the mitogen-activated protein (MAP) kinase pathway. In general, the MAP kinase pathway contains three sequentially activated protein kinases that regulate processes such as cell proliferation, cell differentiation, and

cell death in eukaryotes (73). One of these protein kinases is extracellular signal-regulated kinase (ERK). Transient ERK activation is necessary for cell survival, however, sustained over-expression of ERK has been observed during apoptosis in both the eye and the brain (68,74). Therefore, the neuronal plasma membrane plays a role in brain cell viability through its influence on MAP kinase signaling, and particularly ERK activation.

Mitochondria are the primary endogenous sources of reactive oxygen species (ROS). ROS are defined as chemically unstable oxygen-containing molecules that react with and damage lipids, proteins, and DNA in the cell (66,75). Examples of ROS include superoxide (O_2^-) and hydroxyl radicals (OH·). Under normal conditions, most ROS produced from energy metabolism are reduced to stable products through the antioxidant activity of endogenous enzymes (ex. superoxide dismutase) as well as small, molecular antioxidants. However, an imbalance between the two processes, due to increased ROS production or decreased capacity of the antioxidant defense network, leads to oxidative stress and cell damage (75). Dysfunctional mitochondria are thought to have elevated production of ROS as a byproduct of less efficient ATP synthesis. This decline in mitochondrial efficiency is known to occur with aging (76). ROS produced from these mitochondria, in turn, damage mitochondrial proteins in the electron transport chain as well as mitochondrial DNA, which lacks protective histones (77). Thus, a vicious cycle is created with oxidative stress being both a cause and effect of dysfunctional mitochondria. This cycle is particularly dangerous for the brain since this tissue is highly susceptible to oxidative stress due to its high energy demand, high oxygen consumption, and rich abundance of easily oxidized PUFAs (76). Damaged, dysfunctional mitochondria, as determined by changes in energy metabolism rates, expression/activity of oxidative

phosphorylation enzymes, and mitochondrial DNA damage, have been observed in the brains of those with very early-stage Alzheimer's disease and cognitive impairment (76–81). Therefore, maintaining healthy mitochondria is critical for cell viability and cognition.

Gene regulation and DNA damage dictate cell health and are linked to cognitive function (82). A study performed by Lu et al. observed that gene expression profiles involved in synaptic plasticity and mitochondrial function were altered with age. This study also determined that alterations in gene expression were followed by an increased stress response. Furthermore, genes down-regulated with aging had increased DNA damage in their promoter regions (82). Microarray studies looking at genome wide expression profiles in healthy, cognitively impaired, and demented brains show significantly different expression profiles across neurodegenerative disease progression (83). Genes related to synaptic signaling (such as vesicle trafficking and neurotransmitter receptors), energy production, and protein homeostasis are down-regulated in Alzheimer's disease brains, but are up-regulated in mild cognitive impairment, suggesting both hypo- and hyperexpression of genes involved in synaptic activity and cell metabolism lead to a decline in cognition (84,85). It also suggests that the progression from normal cognitive function to dementia may not be linear. Additionally, microarray studies have shown that dysregulation of genes involved in innate immunity occur in the brain with aging and increase the vulnerability of the brain to cognitive decline (86). Given that the nucleus is the site of transcription in the cell, the integrity and function of the nuclear compartment may play an important role in preventing aberrant gene expression, thus maintaining cell viability and normal cognition.

Myelin, which forms layers around neurons to increase nerve impulse speed, is vital for neuronal communication (87). Thicker myelin sheaths provide faster communication between neurons. Myelin abnormalities, such as changes in structural integrity and thickness, are linked with disorganized thinking and impaired memory (88). It has been well established that myelination declines as we age. Studies in monkeys have demonstrated that age-related breakdown of myelin is an underlying factor in age-related cognitive impairment in primates (89,90). Current studies using MRI techniques are being used to demonstrate a role of myelin breakdown in age-related cognitive decline and Alzheimer's disease in humans (91,92).

In summary, brain viability is affected by the structural integrity and function of brain membranes, namely nuclear, myelin, mitochondrial, and plasma membranes (93). This is especially critical in brain regions controlling cognition (52,94). Thus, maintaining the structural integrity and health of these membranes is essential for brain health and cognition.

3.2.3 Role of the striatum, cerebellum, prefrontal cortex, and hippocampus in cognitive function

In the brain, the striatum, cerebellum, prefrontal cortex, and hippocampus are known to control different domains of cognitive function. The striatum controls working memory, abstract rule learning, and attention control (95). Striatal dysfunction has been observed in numerous neurodegenerative diseases, including Alzheimer's disease, with noticeable changes in motor and non-motor cognitive function (95). The cerebellum, best known for its role in motor function, is also involved in learning, attention, executive control, and language (96,97). Evidence over the past decade has identified morphological changes,

such as neuronal loss and atrophy, in the cerebellum of individuals with Alzheimer's disease (98). The prefrontal cortex plays a key role in memory, attention, perceptual awareness, thought, language, and consciousness (99). The prefrontal cortex is known to be particularly susceptible to stress and is extremely vulnerable to degeneration in Alzheimer's disease (100). Finally, the hippocampus, which is located under the cerebral cortex, is responsible for the consolidation of information from short-term memory to long-term memory, as well as spatial memory (101). This brain region is one of the first to suffer damage in Alzheimer's disease (101).

3.3 Potential functions of lutein in the brain

3.3.1 Role of lutein in cell viability in the eye

Since brain cell viability is essential for maintaining cognitive function, and membranes play a critical role in determining cell viability, it is possible that lutein accumulates in cell membranes to influence brain cell viability and therefore cognition. However, *studies investigating this potential mechanism of action in the brain have not been performed*.

While information on the function of lutein in the brain is lacking, there has been a great deal of research focused on the molecular mechanisms underlying the effects of lutein on cell viability in ocular neural cells. In addition to the evidence from cell culture studies of the ROS quenching role and potential modulator of inflammation and immune-related genes discussed in section 3.1.3 of this review, evidence for a role of lutein in inhibiting neural cell death in the retina through the down-regulation of ERK expression in a diabetic mouse model has also been observed (102). Mice fed high doses of lutein (0.1% wt/wt) showed a reduction in aberrant phosphorylated ERK protein expression, leading to

reduced neural damage and enhanced cell survival (102). Given the importance of ROS, MAPK signaling, and gene regulation in cell viability, this evidence supports a role of lutein in neural cell health. Although mice injected with lutein were shown to have reduced ERK activation in the brain of an ischemic stroke model (103), further studies must be performed to determine if lutein consumed from the diet functions in a similar role in the primate brain. Given the importance of the mitochondria in ROS production, the nuclear membrane in gene expression, and the plasma membrane in ERK activation, *the investigation of lutein's distribution among these membranes could shed light on its potential role in these cellular processes in the brain*.

3.3.2 The potential role of brain lutein in metabolism

With studies consistently showing a clear link between lutein and cognition at different ages, but no clear mechanism identified for this beneficial effect, an exploratory metabolomic analysis was performed to elucidate potential relationships between brain lutein and metabolic pathways in regions controlling cognition in infant brain (104). Lutein concentrations correlated with lipid pathway metabolites, an expected association given that lutein accumulates in cell membranes. Most of these correlations were observed in the frontal cortex, rather than the occipital cortex and hippocampus. Lutein also correlated with energy pathway metabolites, such as NADH, brain osmolytes, amino acid neurotransmitters, namely aspartate and γ-aminobutyrate, and homocarnosine in this study. The observed relationships between lutein and metabolites occurred in a region-specific manner (104). Findings from this exploratory analysis provide a rationale for investigating the contribution of lutein to energy metabolism regulation, with a specific focus on lipid metabolism.

3.3.3 Lutein, vitamin E, and DHA in the brain

Vitamin E is another fat-soluble compound that accumulates within membranes of brain cells. Cross-sectional studies indicate that α-tocopherol, the major form of vitamin E in the body, is positively related to cognitive function, however results from intervention studies have been mixed (105–107). Although α-tocopherol is not as strongly or consistently related to cognitive function as lutein, this vitamin is thought to be beneficial due to its potent anti-oxidant capabilities (107). Given that lutein and α-tocopherol are both membrane-associated antioxidants, it is currently unclear why lutein seems to be more beneficial for cognition in humans. Therefore, this observation has generated hypotheses regarding additional functions of lutein beyond that of a free radical scavenger in the brain.

It is known that vitamin E is found in significantly higher concentrations in the brain compared to lutein (16,20), however studies comparing subcellular membrane content of these two fat-soluble compounds have not been performed. Since α-tocopherol and lutein accumulate in membranes and possess anti-oxidant functions, it is possible they are both involved in promoting cell viability. However, the relationship between lutein and vitamin E membrane distribution and its association with cell viability in the primate brain has not been characterized. *Comparing their subcellular deposition in brain membranes and their respective associations with cell viability may shed light on why their relationships with cognitive function differ in strength.*

Similar to lutein and vitamin E, docosahexaenoic acid (DHA) is known to accumulate in the brain (108). Low intake of DHA or deficiency of this nutrient has been shown to be related to cognitive decline, whereas weekly consumption of fish, a rich dietary source of

DHA, has been shown to decrease the risk of Alzheimer's disease and cognitive impairment (109–112). Furthermore, brain DHA content, determined in brain samples from subjects who participated in the Georgia Centenarian Study (GCS), was positively associated with cognitive scores in the elderly (**Table 2**).

Table 2. Cross-sectional relationship between fatty acids (mole %) in the frontal and temporal cortices of the brain and pre-mortem measures of cognition in decedents with normal cognition, mild memory loss, or MCI.

(n)	**SIB** (22)	**BDS** (22)	**Verbal fluency** (20)	**BNT** (18)	**WL MT** (15)	**CP** (19)	**WL recog** (17)	**DAFS** (22)	**CERAD total** (15)
Frontal Cortex									
SFA	-0.15	0.20	0.14	0.41	0.32	0.56**	0.34	0.18	0.38
MUFA	-0.006	-0.45**	-0.30	-0.40	-0.30	0.56**	-0.33	-0.36	-0.34
n-6 FA	-0.47**	-0.04	0.24	0.08	0.08	0.19	-0.11	-0.17	0.37
n-3 FA	0.51**	0.69***	0.42*	0.42*	0.28	0.40	0.41	0.58***	0.13
Trans	-0.41*	-0.22	-0.19	0.10	0.48*	0.02	-0.21	-0.29	0.36
DHA	**0.41***	**0.71*****	**0.47****	**0.53****	0.36	**0.50****	**0.45***	**0.57*****	0.26
Temporal Cortex									
SFA	0.45**	0.59***	0.58***	0.35	0.33	0.24	0.44*	0.34	0.37
MUFA	-0.45**	-0.61**	-0.70***	-0.36	-0.35	-0.31	-0.59**	-0.43*	-0.45
n-6 FA	0.07	0.09	0.16	-0.12	-0.26	0.02	0.02	0.19	-0.13
n-3 FA	0.38*	0.45**	0.52**	0.37	0.54*	0.18	0.55**	0.28	0.50*
Trans	-0.36	-0.24	-0.14	0.21	0.35	0.004	-0.12	-0.20	0.24
DHA	**0.47****	**0.56*****	**0.63*****	0.41	**0.58****	0.20	**0.60****	0.38	**0.58****

Values are partial correlation coefficients adjusted for sex, education, diabetes, and hypertension *P≤0.1, **P≤0.05, ***P≤0.01. SFA: saturated fatty acid, MUFA: monounsaturated fatty acids, n-6 FA: n6 polyunsaturated fatty acid, n-3 FA: n3 polyunsaturated fatty acid. DHA: docosahexaenoic acid. SIB: Severe Impairment Battery; BDS: Behavioral Dyscontrol Scale; BNT: Boston Naming test; WLMT: Word List Memory Test; CP: Constructional Praxis; WL recog: Word List Recognition Test; DAFS: Direct Assessment of Functional Status; CERAD: Consortium to Establish a Registry for Alzheimer's disease

Given what is known about DHA and cognition, the lutein intervention trials in both young adults and elderly women described in section 3.1.3 of this review (50,51) also tested whether supplementation with both DHA and lutein could improve cognitive performance in these subjects. Results from the intervention in younger adults found that supplementation with the mixed formula improved processing speed (as did lutein alone) but also visual motor reaction time (50). In older adults, supplementation with DHA alone improved verbal fluency, just as supplementation with lutein alone did. However, supplementation with both lutein and DHA improved not only verbal fluency, but also memory scores and the rate of learning (51). Collectively, results from both studies demonstrate that lutein and DHA may function together to impact cognitive performance. However, *how lutein and DHA interact in the brain remains unknown*.

Since lutein is known to accumulate in PUFA-rich domains of membranes in the retina, it is possible that lutein and DHA also co-accumulate and function together in the brain. To investigate a potential interaction between the two compounds in brain tissue, multivariate regression analysis using data from the GCS cohort was performed in order to test for an interaction between brain levels of lutein and DHA as a predictor of cognitive test score (**Table 3**).

A statistically significant interaction was observed for several cognitive tests in the Consortium to Establish a Registry for Alzheimer's Disease (CERAD), indicating that both brain lutein and DHA levels are predictors of cognitive performance and that the amount of one influences the association of the other with cognitive score.

Table 3. Significant interactions between lutein (pmol/g) and DHA (mole %) content in the frontal and temporal cortices with cognitive function. Adjusted for age, sex, education, hypertension, and diabetes

Cognitive Tests	Frontal Cortex	Temporal Cortex
Controlled word association test**	NS	NS
Verbal Fluency**	NS	**S (p<0.05)**
Boston Naming test	NS	NS
WAIS III similarities**	NS	NS
Consortium to establish registry for AD	**S (p<0.05)**	**S (p<0.05)**
Behavioral dyscontrol scale	NS	NS
Word list memory	NS	NS
Word list recognition	**S (p<0.01)**	**S (p<0.01)**
Word list recall	**S (p<0.05)**	**S (p<0.01)**

** log transformed
S: significant; NS: not significant

These results provide preliminary evidence that lutein and DHA may function together in the brain to maintain cognitive function. Given that lutein is an antioxidant, and DHA is a PUFA highly susceptible to oxidation, it is possible that lutein functions to inhibit the oxidation of DHA. This would simultaneously reduce lipid peroxidation/oxidative stress and preserve DHA for metabolism into anti-inflammatory resolvins and protectins when needed. However, *no studies, to date, have investigated the relationship between lutein and DHA oxidation in the primate brain.*

3.4 Summary: gaps in knowledge and future directions

Current evidence supporting the connection between lutein and cognition justifies the need to further evaluate the relationship between brain lutein concentrations and brain health. However, questions regarding the function of lutein in the brain continue to linger. A significant obstacle in lutein research is that typical small animal models are very poor models of xanthophyll carotenoid absorption, transport, and storage (113,114) and must be fed or injected with supraphysiological doses of lutein to mirror human physiology

(103,115). This is particularly challenging for brain research as even extreme doses of lutein lead to only small amounts of lutein in the brain, if any (113). Because these animals are not suitable models for lutein and cognition research, the ability to obtain brain tissue for experimentation is very challenging. However, recent studies conducted in rhesus macaque have demonstrated that these non-human primates respond to dietary lutein with increases in serum lutein and MP density similar to humans (17,29). Additionally, numerous studies have shown that the macaque brain is a close approximation to the human brain in terms of both basic anatomy and connectivity (116). Since these non-human primates are well-accepted models of both human carotenoid absorption/storage and brain physiology, they are excellent models for studying the role of lutein in brain function and cognition.

Given the substantial evidence that membrane composition is an important determinant of brain cell function and lifespan (66,117), and poor cell function and cell death lead to cognitive decline and dementia (117), there is a strong need to determine the distribution of lutein in brain membranes. This will provide the first steps toward understanding how lutein plays a role in cognitive function. However, to date, *studies investigating lutein and cognition have not considered the relationship between membrane type and lutein with regard to brain function.*

Another important step in elucidating lutein's mechanism of action is to determine potential interactions between this phytochemical and nutrients in the brain. Given that lutein has been shown to accumulate in PUFA-rich areas of the retina, it is possible lutein behaves similarly in the brain. Furthermore, previous findings from lutein and DHA cognition studies indicate the two compounds may work together to impart beneficial

effects. Therefore, further evaluation of the relationship between lutein and DHA content in the brain is warranted. Specifically, *there is a need to analyze the relationship between brain membrane distribution of lutein and DHA.* Similarly, given that both lutein and vitamin E are membrane-associated, lipophilic antioxidants, but their relationship to cognitive function is different with regard to strength and consistency, *studies comparing their subcellular deposition and relationship to cell viability are warranted*.

Finally, previous evidence showing a potential function of lutein in cell viability in the eye justifies the need to investigate whether lutein possesses a similar function in the brain. Assessing the relationship between membrane and brain region-specific lutein concentrations and markers for cell survival signaling, oxidative stress, and gene regulation will provide insight into the role of lutein in maintaining cell survival and function. The work described in this thesis aims to answer some of these questions in an effort to lay the groundwork for future studies investigating the role of lutein in cognition. Results from these experiments can be used to design future studies investigating the effect of lutein on cell viability and brain function, thus filling in the knowledge gaps regarding the role of lutein in cognitive function.

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4. Articles/Chapters

4.1 Subcellular Localization of Lutein in Brain Regions of Adult Rhesus Macaque and its Association with Docosahexanoic Acid in Membranes

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Funding source: This work was supported by a grant from Abbott Nutrition through the Center for Nutrition, Learning, and Memory at the University of Illinois, DSM Nutritional Products, and USDA (58-1950-0-014).

Abbreviations used: DHA, docosahexaenoic acid; PFC, prefrontal cortex; CER, cerebellum; ST, striatum; HC, hippocampus; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; *trans*, *trans* fatty acids; MP, macular pigment; CV, coefficient of variation; BCA, bicinchoninic acid; HPLC, high performance liquid chromatography; ANOVA, analysis of variance; AA, arachidonic acid; reactive oxygen species, ROS

Abstract

Lutein, a xanthophyll, preferentially accumulates in primate brain and is associated with better cognition in humans. However, the mechanism underlying the role of lutein in cognition remains unknown. Recent studies suggest that the relationship between brain lutein content and cognition may be dependent on brain concentrations of docosahexaenoic acid (DHA), an omega-3 fatty acid important for cognition. The study objectives were to determine the subcellular localization of lutein in membranes from brain regions controlling cognition and determine whether lutein and DHA co-localize in these membranes. Nuclear, myelin, mitochondrial, and neuronal plasma membranes were isolated from prefrontal cortex (PFC), cerebellum (CER), striatum (ST), and hippocampus (HC) of adult rhesus monkeys (n=11) using differential centrifugation with a Ficoll density gradient. Carotenoids and fatty acids were measured using high performance liquid chromatography and gas chromatography, respectively. Among brain regions, lutein concentrations (ng/mg protein) were highest in HC, a region rich in polyunsaturated fatty acids but low in monounsaturated fatty acids ($P<0.05$). Lutein content did not differ among membranes in any region. DHA content (mole %) was the lowest in nuclear membranes for all regions ($P<0.05$). Within membranes, lutein and DHA (ng and μg per mg protein, respectively) in PFC, CER, and ST tended to be inversely related in myelin ($P<0.1$) and neuronal membranes ($P<0.05$) but positively related in mitochondrial membranes of HC ($P<0.01$). Co-localization of lutein and DHA in some membranes and regions, but not others, may be indicative of unique and specific functions of lutein in different parts of the brain. These findings provide the first steps toward understanding the role of lutein in the brain and its association with DHA.

Introduction

Lutein, a dietary xanthophyll found in foods such as spinach, kale, eggs, avocados, and corn (1), is known to accumulate in the retina of primates where, along with zeaxanthin (another xanthophyll) it forms macular pigment (MP) (2). These xanthophylls are the only carotenoids selectively deposited in the macula of the retina. MP protects the eye from harmful blue light by acting as a filter. Lutein and zeaxanthin also act as antioxidants to protect retinal photoreceptors, which contain a high amount of polyunsaturated fatty acids (PUFAs), from peroxidation (3,4). In addition to its ability to cross the blood-retina, lutein and zeaxanthin also cross the blood-brain barrier in primates along with other carotenoids, cryptoxanthin, β-carotene, and lycopene (5–7). Studies in both infants and adults have shown that, despite not being the predominant carotenoid in the diet, lutein is found in the highest concentrations in the human brain compared to the other carotenoids (6,7). Furthermore, MP density has been shown to be positively correlated to brain lutein concentrations in matched retina and brain tissue of both humans and non-human primates (8,9). Together, these results indicate that lutein selectively accumulates in non-ocular neural tissue. This preferential accumulation of lutein into the brain during multiple life stages may be indicative of an important function in this tissue. Therefore, there is a growing interest in studying the potential role of lutein in brain health and cognition.

Over recent years, accumulating evidence demonstrates that serum and brain lutein concentrations, as well as MP density, are positively related to cognitive function in humans (7,10–12). In older adults, serum lutein was related to better verbal fluency while MP density was related to better global cognition, verbal learning and fluency, recall, and

processing and perceptual speed (11). Similarly, in a centenarian population, brain concentrations of lutein were positively related to global cognition and language, and inversely associated with depression scores (7). Several studies have also demonstrated a beneficial effect of lutein on cognitive performance in humans. Consumption of avocados, a highly bioavailable source of lutein (13), for 6 months increased MP density which was related to improved spatial working memory and problem approaching efficiency in healthy older adults (14). Additionally, supplementation with lutein has been shown to increase MP density and improve different aspects of cognitive function in both young (15) and older adults (16). In these studies, supplementation with both lutein and docosahexaenoic acid (DHA), a n-3 PUFA known to be beneficial for cognition (17–19), resulted in additional cognitive benefits beyond supplementation with either alone. Consistent with this observation, a significant interaction between brain concentrations of lutein and DHA as a predictor of cognitive scores was documented in a centenarian population (20). Together, these results indicate that lutein may function to improve cognition and that this effect may be influenced by DHA levels in the brain, but the exact mechanism of action remains unclear.

Lutein accumulates within membranes due to its amphipathic structure (21), however, its concentration among various membranes within brain regions is currently unknown. The distribution of lutein among membranes may be indicative of its function in brain cells. Furthermore, given the evidence that lutein and DHA may function together to promote brain health, determining whether the two compounds co-localize in membranes will provide valuable information towards understanding if and how they interact in the brain.

Therefore, the objective of the present study was to determine the distribution of lutein in subcellular membranes from various brain regions and characterize the fatty acid composition, specifically DHA content of membranes in which lutein accumulates. Brain regions of interest include the prefrontal cortex (PFC), cerebellum (CER), striatum (ST) and hippocampus (HC). The PFC plays a key role in memory, attention, perceptual awareness, thought, language, and consciousness (22), while the CER is involved in learning, attention, executive control, and language (23,24). The ST controls working memory, abstract rule learning, and attention control (25), and the HC consolidates information from short-term memory to long-term memory (26). This study was performed in rhesus monkeys because these non-human primates are a well-accepted model for human brain physiology and have been shown to absorb and store lutein similar to humans (27–29).

Methods

Animals and Diet

Eleven Rhesus monkeys (*Macaca mulatta*) ranging from 7-20 years of age were fed standard monkey chow (LabDiet, St. Louis, MO) containing 16.4 μmol/kg of lutein, 6.4 μmol/kg of zeaxanthin, 4.8 μmol/kg β-carotene, 1.0 μmol/kg α-carotene, and 0.1 μmol/kg cryptoxanthin throughout the lifespan along with a variety of different fruits and vegetables. The chow contained 1.54% total saturated fatty acids and 1.68% total monounsaturated fatty acids. For PUFAs, the diet contained 1.66% linoleic acid, less than 0.01% arachidonic acid (AA), and 0.10% linolenic acid. Total PUFAn-3 content was 0.13%. Eight of the 11 monkeys were female. Two of these monkeys, female, aged 9 and 20 years, were additionally supplemented daily with a gelatin beadlet containing 51.78

mg/g lutein and 3.60 mg/g zeaxanthin (supplied by DSM Nutritional Products Ltd) for 7-12 months prior to termination to increase the range of lutein content found among the brain tissues. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Oregon National Primate Research Center. Approval was also given from Tufts IACUC to receive brain tissue for analysis.

Brain Collection

Right and left hemispheres of the PFC, CER, ST, and HC were removed for analysis. The regions consisted of both gray and white matter; however, major white matter tracts were removed from cortical samples. Each brain region was immediately put on dry ice after removal and stored at -80^{0}C. Right and left hemispheres of each region were pooled and pulverized in liquid nitrogen. Pulverized samples were then aliquoted for subsequent carotenoid and fatty acid analyses and stored at -80^{0}C.

Preparation of Brain Membranes

Differential centrifugation with a Ficoll density gradient was performed to isolate and purify nuclear, myelin, mitochondrial, and neuronal plasma membranes from each brain region using an established method (30,31). Briefly, pulverized brain tissue was homogenized in aqueous buffer (10 mM HEPES, 0.25 mM EDTA, 0.32 M sucrose, pH 7.2) containing protease inhibitors (cOmplete^{TM} protease inhibitor cocktail, Roche) and subjected to low-speed centrifugation (1000 x g, 4^{0}C) to isolate the crude nuclear membrane pellet. The resulting supernatant was removed and placed in a new tube. The homogenization and centrifugation steps were repeated with the remaining pellet. Supernatant from the second centrifugation was combined with the first. The crude nuclear membrane pellet was placed on ice. The combined supernatants were then

subjected to middle-speed (17,000 x g, 4^0C) centrifugation to obtain the crude membrane pellet (containing myelin, mitochondrial, and neuronal plasma membrane). The crude membrane pellet was re-homogenized in buffer (10 mM HEPES, 0.25 mM EDTA, pH 7.2) and applied to a Ficoll density gradient (consisting of 14% and 7% Ficoll solutions) and centrifuged at high-speed (87,000 x g, 4^0C) to obtain separated myelin, mitochondrial, and neuronal plasma membranes. All three membrane fractions, along with the crude nuclear membrane, were purified via centrifugation at 17,000 x g, 4^0C. Pure membranes were aliquoted for carotenoid and fatty acid analyses, weighed, and stored at -80^0C. Membrane recovery, determined by measuring the sum of α-tocopherol levels in all membranes and supernatants and comparing to total α-tocopherol in each brain sample analyzed, was determined to be 76% ± 1%.

Carotenoid Extraction from Brain Regions and Membranes

Extraction of carotenoids from brain regions and membranes was adapted from Park et al. (32) and has been described in detail by Vishwanathan et al. (9). Briefly, reverse-phase high-performance liquid chromatography (HPLC) was used to separate and quantify carotenoids from each region/membrane extract (33), with a semi-bore C30 carotenoid column (3 μm, 150 mm × 4.6 mm; YMC, Carotenoid). The lower limit of detection is 0.2 pmol for carotenoids. Interassay coefficients of variation (CV) were 4%. Brain region and membrane data were expressed ng per mg protein.

Fatty Acid Extraction from Brain Regions and Membranes

Lipids were extracted overnight from homogenates of the various brain regions and membranes using a modified Folch method (34). Fatty acid analysis of the various brain regions and membranes was performed using an established gas chromatography method

(35). Peaks of interest were identified by comparison with authentic fatty acid standards (Nu-Chek Prep, Inc. Elysian, MN) and expressed as both molar percentage (mole %) proportions of total fatty acids and μg/mg protein. Interassay CV was <4.5% for fatty acids present at levels >1%.

Protein Determination

Delipidated brain tissue/membranes were digested in 1N sodium hydroxide for the determination of protein using the bicinchoninic acid (BCA) assay (Pierce Inc., Rockford, IL). Brain regions and membranes were digested for 8 and 5 days, respectively.

Carotenoid Extraction from Plasma

Plasma was available for 7 of the 9 exclusively chow-fed animals, and was analyzed to assess dietary intake. Plasma was also collected from the two lutein supplemented monkeys. Carotenoids were extracted from plasma and analyzed using reverse-phase HPLC as previously described by Yeum et al. (33).

Statistical Analysis

Given the novelty of this work, no sample size calculation was performed. Carotenoid data (both plasma and brain) and fatty acids are expressed as mean ± standard error of mean. For plasma, total lutein (sum of *cis* and *trans* isomers) was used for the data analysis. In the brain, only the *trans* isomer of lutein was detected. Plasma and total brain region carotenoids were normally distributed. One-way analysis of variance (ANOVA) with Tukey's HSD to adjust for multiple comparisons was performed to determine differences in concentration of carotenoids in plasma as well as across brain regions. Brain region fatty acids were not normally distributed and were ranked prior to statistical

analysis. One-way ANOVA with Tukey's HSD was performed to determine differences in fatty acid content across regions. Membrane carotenoids and fatty acids were also not normally distributed so were log-transformed and ranked, respectively, prior to statistical analysis. Two-way ANOVA with Tukey's HSD was performed to evaluate differences in concentration of membrane carotenoids and fatty acids within and across regions of the brain (for all 4 brain regions).

Pearson correlations were performed to determine whether concentrations of lutein and PUFAn-3 fatty acids were related within different membranes for each region. For correlation analysis, lutein and fatty acids were expressed ng/mg protein and µg/mg protein, respectively. The correlation between lutein and the n-6/n-3 polyunsaturated fatty acid (PUFAn-6/PUFAn-3) ratio was also assessed since this ratio has important implications regarding neuro-inflammation in the brain (36,37). PUFAn-3 and PUFAn-6 are metabolized by the same enzymes to produce anti-inflammatory and pro-inflammatory eicosanoids, respectively. Since they compete for the same enzymes, the relative amounts of each can dictate the immunomodulatory effects of PUFAs in the brain (37). All correlations were adjusted for age. For all analyses, significance was set at the 0.05 level. Statistical analysis was performed using SAS 9.3.

Results

Serum Carotenoid Profile in Adult Rhesus Macaque

Lutein was the major carotenoid detected in rhesus monkey plasma (258 ± 69 nmol/L). Other carotenoids detected include zeaxanthin (51.5 ± 13.9 nmol/L), β-carotene (35.0 ± 13.2 nmol/L), α-carotene (12.2 ± 7.3 nmol/L), and cryptoxanthin (9.93 ± 5.09 nmol/L), all of which were significantly lower than lutein ($P<0.01$, **Figure 1a**). Lutein, zeaxanthin,

and β-carotene were detected in plasma of all animals. However, α-carotene and cryptoxanthin were detected in only 3 (2 lutein suplemented and one of the chow-fed) of the 9 monkeys. **Figure 1b** shows carotenoid profiles in plasma of chow-fed versus supplemented monkeys, stratified by age to determine if there was an age-effect on carotenoid status. There was no difference in plasma lutein concentrations between the two different age groups of chow-fed monkeys. As expected, lutein concentrations were higher in the plasma of the two supplemented animals compared to chow-fed. The 20-year-old supplemented monkey also had higher levels of the other carotenoids compared to chow-fed monkeys; however, zeaxanthin, α-carotene, and β-carotene concentrations were not as high in the 9 year old compared to the 20 year old. Therefore, differences in these carotenoids between the 9-year-old supplemented monkey and the chow-fed animals were smaller.

Carotenoid Concentrations in Different Regions of the Brain

Despite having higher lutein concentrations in plasma, supplemented monkeys did not necessarily have the highest concentrations of lutein across all brain regions. One-way ANOVA was performed twice (with and without supplemented monkeys) to determine if data from the supplemented animals would skew the brain carotenoid results. Statistics for determining differences in brain region fatty acids, as well as membrane lutein and fatty acids were also performed with and without the supplemented monkeys. For all analyses, results were similar regardless of the inclusion/exclusion of supplemented animals. Therefore, they were combined with the chow-fed monkeys for all subsequent analyses described in this chapter.

Table 1 shows the carotenoid content in PFC, CER, ST, and HC of rhesus monkeys. Similar to plasma, lutein was the major carotenoid detected in the brain and was significantly greater than all other carotenoids within each region ($P<0.05$). Matched plasma and brain lutein concentrations were positively correlated for all regions (n=9). Correlations were strongest in the PFC ($r = 0.88$, $P<0.01$) and ST ($r=0.92$, $P<0.01$), but were also significant in HC and CER ($r = 0.81$, $P<0.05$, for both). Comparing carotenoids across brain regions, lutein concentrations were significantly higher in the HC compared to the PFC and CER ($P<0.05$) and tended to be higher than levels in the ST ($P<0.1$). CER had the lowest levels of lutein compared to all regions ($P<0.05$). ST and PFC had similar concentrations of lutein. Zeaxanthin followed a similar distribution pattern across brain regions. Cryptoxanthin was not detected in the ST and β-carotene was not detected in the HC of any monkey. Cryptoxanthin concentrations were similar across PFC, CER, and HC. β-carotene concentrations were highest in the PFC and ST ($P<0.05$).

Fatty Acid Profiles in Different Regions of the Brain

As is characteristic with primates, within all brain regions, saturated fatty acids (SFA) were predominant (38), followed by monounsaturated fatty acids (MUFA), n-6 polyunsaturated fatty acids (PUFAn-6), n-3 polyunsaturated fatty acids (PUFAn-3), and *trans* fatty acids (**Table 2**).

SFA content was significantly higher in PFC and HC compared to CER and ST ($P<0.05$). Palmitic acid (16:0) and stearic acid (18:0) made up the majority of SFA in the brain, while SFAs varying in chain length from 10-15 carbons accounted for very little of the total (reported as a combined total in table 2). MUFA content was highest in CER, where lutein concentrations were lowest, followed by ST, PFC, and HC ($P<0.05$). Total MUFA

brain distribution was driven primarily by oleic acid (18:1n-9) which, along with vaccenic acid (18:1n-7), were the major MUFAs detected in the brain. Total PUFA content was highest in the HC (27.20 ± 0.81 mole %), followed by ST (26.28 ± 0.81 mole %), PFC (24.95 ± 0.82 mole %), and CER (23.87 ± 0.81 mole %). Total PUFAn-6 content was highest in HC, followed by ST, PFC, and CER ($P<0.05$). AA (20:4n-6) and adrenic acid (22:4n-6) made up the majority of PUFAn-6, with AA contributing primarily to the brain distribution pattern of total PUFAn-6. Total PUFAn-3 content was higher in the PFC compared to HC ($P<0.05$), however, neither differed in PFC and CER. Within each brain region, DHA (22:6n3) accounted for 92-93% of total PUFAn-3 content and was not significantly different across brain regions. Total *trans* fatty acid content was lowest in HC ($P<0.05$) and similar across the other three regions. Similar results were observed regarding fatty acid content differences across regions when data were expressed µg/mg protein (data not shown).

Distribution of Lutein in Subcellular Membranes of Brain Regions

Lutein was the predominant carotenoid detected in subcellular membranes within each brain region. It was the only carotenoid detected in all membranes analyzed (160 total). Lutein concentrations were highly variable among monkeys for each membrane.

Figure 2 shows the membrane distribution of lutein for each brain region (geometric mean, 95% confidence interval) expressed as ng/mg protein. There were no statistically significant differences in lutein concentration across membranes within each region. Evaluation of membrane-specific concentrations across brain regions revealed nuclear and myelin lutein concentrations in ST were significantly greater than nuclear and myelin lutein concentrations, respectively, in CER and HC ($P<0.05$). Furthermore, mitochondrial

and neuronal plasma membrane lutein concentrations were greater in ST than CER ($P<0.05$).

Distribution of Total PUFAn-3 and DHA in Subcellular Membranes of Brain Regions

Table 3 shows membrane PUFAn-3 expressed as mole percent for each brain region. Concentrations of PUFA n-3s (μg/mg protein) are presented in the next chapter (*4.2 Membrane-Specific Concentrations of Lutein, Vitamin E, and Polyunsaturated Fatty Acids Are Related to Markers of Cell Viability in Rhesus Monkey Brain*).

For all brain regions, total PUFAn-3 and DHA levels were significantly lower in nuclear membranes compared to the other membranes ($P<0.05$). In PFC, total PUFAn-3 and DHA content in myelin, mitochondrial, and neuronal plasma membranes were similar. In CER, total PUFAn-3 and DHA in mitochondrial and neuronal plasma membrane were also significantly higher than myelin PUFAn-3 and DHA ($P<0.05$). In ST, total PUFAn-3 and DHA content was highest in the mitochondrial membrane, followed by myelin, then neuronal plasma membrane ($P<0.05$). In the HC, total PUFAn-3 and DHA content was highest in myelin, followed by neuronal plasma membrane and mitochondrial membrane ($P<0.05$). For all membranes, ST and HC generally had lower membrane PUFAn-3 and DHA content than in CER and PFC.

SFA, MUFA, PUFAn-6, and *Trans* Fatty Acid Profiles in Subcellular Membranes of Different Brain Regions

Tables S1-4 show the fatty acid profiles in nuclear, myelin, neuronal, and mitochondrial membranes in PFC, CER, ST, and HC, respectively. For all regions, the nuclear membrane was highest in MUFA content ($P<0.05$) and lower in SFA and PUFA content

compared to other membranes. Conversely, mitochondrial membranes were PUFA-rich, primarily due to the PUFAn-6 fatty acids, ($P<0.05$) as well as lowest in SFA. Myelin and neuronal membranes were highest in SFA ($P<0.05$) and tended to be lowest with regard to MUFA content compared to the other membranes.

Cross-sectional Relationship between Lutein and DHA in Subcellular Membranes Across Different Brain Regions

Because of our interest in studying a possible interaction between lutein and DHA, their correlation in membranes and brain regions was evaluated (**Table 4**) along with lutein's correlation with total PUFAn-3, and the PUFAn-6/n-3 ratio within membranes for each brain region. Since PUFAn-3 content is almost entirely DHA, significant correlations with lutein were the same for both. Membrane lutein and DHA concentrations were related in a region-specific manner. In the PFC, a trend for a significant inverse correlation was observed in myelin membrane only ($r = -0.55$, $P<0.09$). In CER, an inverse association was observed in neuronal plasma membrane ($r = -0.56$, $P<0.09$). In the ST, this inverse association was significant in the neuronal plasma membrane ($r = -0.63$, $P<0.05$). Conversely, a strong positive association was observed in the mitochondrial membrane for the HC region ($r = 0.79$, $P<0.01$). Total and nuclear membrane lutein and DHA were not significantly related for any brain region.

In the PFC, lutein was significantly related to the PUFAn-6/n-3 ratio in myelin and mitochondrial membranes only. The association was positive in myelin ($r = 0.63$, $P<0.05$) but inverse in the mitochondrial membrane ($r = -0.69$, $P<0.05$). In ST, lutein was positively related to the PUFAn-6/PUFAn-3 ratio in myelin ($r = 0.68$, $P<0.05$) and there was a trend for an association in the neuronal plasma membrane ($r = 0.58$, $P = 0.08$).

Discussion

Lutein concentrations in serum, brain, as well as MP density are associated with better cognitive function in humans (7,9–11). Furthermore, our group has documented a significant interaction between brain concentrations of lutein and DHA as a predictor of cognitive performance in the elderly (20). However, lutein's mechanism of action and its relationship with DHA in the brain remain unknown.

Regional Distribution of Lutein and Fatty Acids in Rhesus Monkey Brain

Consistent with previous studies in human and non-human primates (6,7,9), lutein was the predominant carotenoid found in the brain. In our monkeys, this observation is partially due to diet since the chow contained higher amounts of lutein than other carotenoids and plasma concentrations of lutein were significantly higher than the other carotenoids. However, human studies in both adults (5,7) and infants (6) have demonstrated preferential uptake of lutein into the brain compared to the other carotenoids.

Currently, none of the published studies investigating the distribution of carotenoids in human and non-human primates have looked at the exact same brain regions, therefore it is difficult to compare our results to these studies. However, concentrations of lutein described in this study are similar to those measured in rhesus monkey brain by Vishwanathan et al., who also observed significant differences in lutein concentration across brain regions, and specifically that lutein levels in CER are lower compared to other regions, including the frontal cortex (9).

Results indicate that lutein tends to have higher accumulation in PUFA-rich brain regions (HC and ST) over those that are not (CER). This is consistent with what is known about lutein accumulation in the eye, where lutein concentrations are greatest in PUFA-rich domains of the retina (4). This result is expected based on what is known about the role of lutein as an antioxidant and lends support to the hypothesis that lutein functions to prevent lipid peroxidation in the brain. However, unlike the retina, the similarity between lutein and PUFA distribution across regions in this study was due primarily to PUFAn-6 content, not PUFAn-3. This may be due to PUFAn-6 content being greater relative to PUFAn-3 in rhesus macaque brain. Another possible explanation for this is described later in this discussion.

Membrane Distribution of Lutein and PUFAn-3 in Rhesus Monkey Brain

This study is the first to report on the membrane distribution of lutein within different regions of the primate brain. Preferential accumulation of lutein in one subcellular membrane versus another may be indicative of the primary function of lutein at the cellular level. However, lutein concentrations did not significantly differ across membranes in any region, despite differences in fatty acid profiles, specifically PUFAn-6 and PUFAn-3, among membranes. These results indicate that unlike regional accumulation of lutein, membrane distribution may not be driven by total PUFA content. Furthermore, the significant amount of variability in lutein concentrations within each membrane indicates that there are likely an extensive number of factors dictating the deposition of lutein into membranes. These factors may include, but are not limited to, polarity of membranes, expression of binding proteins, sex, and individual genetics.

Although results from this cross-sectional analysis do not answer questions regarding the function of lutein in the brain, it is possible to speculate on its mechanism(s) of action based on its subcellular distribution. Similar accumulation of lutein among different membranes may indicate that lutein possesses multiple functions in the cell. For example, mitochondria are the primary source of endogenous reactive oxygen species (ROS) in the cell. Damaged mitochondria, which have increased ROS production, and therefore, increase cellular oxidative stress, are a hallmark of cell death and cognitive impairment (39–41). Although lutein was detected in this membrane, it did not selectively accumulate there. This may suggest that while lutein may function as an antioxidant to inhibit lipid peroxidation in the brain, it may not be the only function it is related to regarding its beneficial effect on cognition. Lutein also accumulated in the nuclear membrane, therefore it may influence gene expression, which is dysregulated in cognitive decline (42,43). It was also present in neuronal plasma membranes, which influence signal transduction pathways dictating cell survival (44). Therefore, lutein may be related to cell survival regulation by influencing plasma membrane composition, fluidity, or by another mechanism (45,46). This finding that lutein may be related to functions in addition to that of a free radical scavenger supports previous observations found in cross-sectional human studies showing that lutein concentrations in the brain are more strongly associated with better cognition than antioxidants like vitamin E, which are found in much higher concentrations in the brain (7). This is also consistent with studies investigating the functions of lutein in the eye that have demonstrated that lutein modulates gene expression of pro-inflammatory genes and influence cell survival *in vitro* and *in vivo* (3,47–49).

However, it is also possible that lutein does accumulate in mitochondrial membranes more significantly that the others, but we observe similar concentrations across membranes because ROS production in this organelle causes a reduction in lutein content. Therefore, future studies looking at how membrane-specific lutein concentrations relate to markers of these different cellular processes in the brain are needed as they will provide further information regarding its function and lay a foundation for understanding the role of lutein in cognition.

Membrane Association Between Lutein and DHA

Despite co-localization of lutein and DHA in subcellular membranes in a region-specific manner, total lutein and DHA concentrations were not related in any brain region analyzed. This finding indicates that the nature of its relationship to DHA may be different among membranes. Therefore, looking at the association between their total amounts in a brain region may not accurately reflect their relationship. The positive association between lutein and DHA in mitochondrial membranes suggests that lutein may co-accumulate with DHA in this membrane. A plausible explanation for this co-accumulation is that lutein may function to protect DHA from oxidation since it is susceptible to damage from ROS produced in this organelle. Interestingly, this positive association was only observed in HC mitochondrial membranes. What makes the HC different from the other three regions is not clear. However, the HC is thought to have a particularly slow turnover of mitochondria and mitochondrial components, which makes this region more susceptible to the accumulation of dysfunctional mitochondria with aging (50,51). Therefore, keeping mitochondria healthy in this region may be particularly important for maintaining normal and healthy hippocampal function.

Conversely, lutein may interact with DHA in a different capacity in myelin and neuronal plasma membranes from the other regions since the two were inversely related in these membranes. This finding is consistent with previous results from our group where the observed interaction term for lutein and DHA as a predictor of cognitive function was negative (20). This negative association may not be indicative of an adverse relationship, but rather, may indicate that lutein is related to the release of DHA from membranes, perhaps by changing the physical properties of the membrane or through signaling and/or gene expression mechanisms, for its metabolism into its anti-inflammatory eicosanoids, protectin and resolvin (37). Therefore, higher lutein concentrations in those membranes may lead to less DHA present in the membrane. Furthermore, if more DHA is being released from membranes, it follows that less AA would be released (given that they compete for the same enzymes), thereby preventing its metabolism to pro-inflammatory prostaglandins. Therefore, the observation that lutein is also positively related to the PUFAn-6/n-3 ratio in the same membranes where lutein and DHA are inversely associated provides more evidence for this potential relationship between myelin and neuronal lutein and DHA. This may also explain why AA content (and total PUFAn-6 content) was higher in parts of the brain where lutein concentrations were highest. However, other possibilities exist for why these two compounds are inversely related in membranes. Results from this study do not exclude the possibility that lutein may be used up to protect DHA from oxidation. Therefore, more work must be done to test these hypotheses and elucidate if and how lutein and DHA interact in each membrane.

A limitation of this cross-sectional analysis is that the results do not provide information on the causative effect of lutein on membrane fatty acid composition or brain function.

Therefore, hypotheses regarding the function of lutein in the brain based on our findings must be tested. Another limitation is that protein significantly varied among different membranes. For example, protein tended to be higher in mitochondrial membranes as opposed to myelin. Therefore, this denominator may have slightly skewed the data when comparing concentrations across membranes. However, it is currently the most feasible option with regard to accuracy of measurement and consistency with the literature for this type of descriptive study. Another limitation is the small sample size, which prevented us from performing multivariate regression analysis that would have provided additional information on the independent contributions of individual fatty acids to lutein concentrations in membranes. However, the primary goal of this novel descriptive work is to provide insight for the design of future studies investigating the effect of lutein, and its possible interaction with DHA, on brain function.

In conclusion, this study is the first to determine the subcellular localization of lutein in primate brain and characterize the fatty acid profiles of each membrane. While this study does not provide causative evidence on the function of lutein in the brain, these results can be used to generate hypotheses about its mechanism of action in brain cells. Future studies assessing the relationship between membrane lutein and DHA oxidation and metabolism are warranted in order to elucidate the nature of their interaction in the brain.

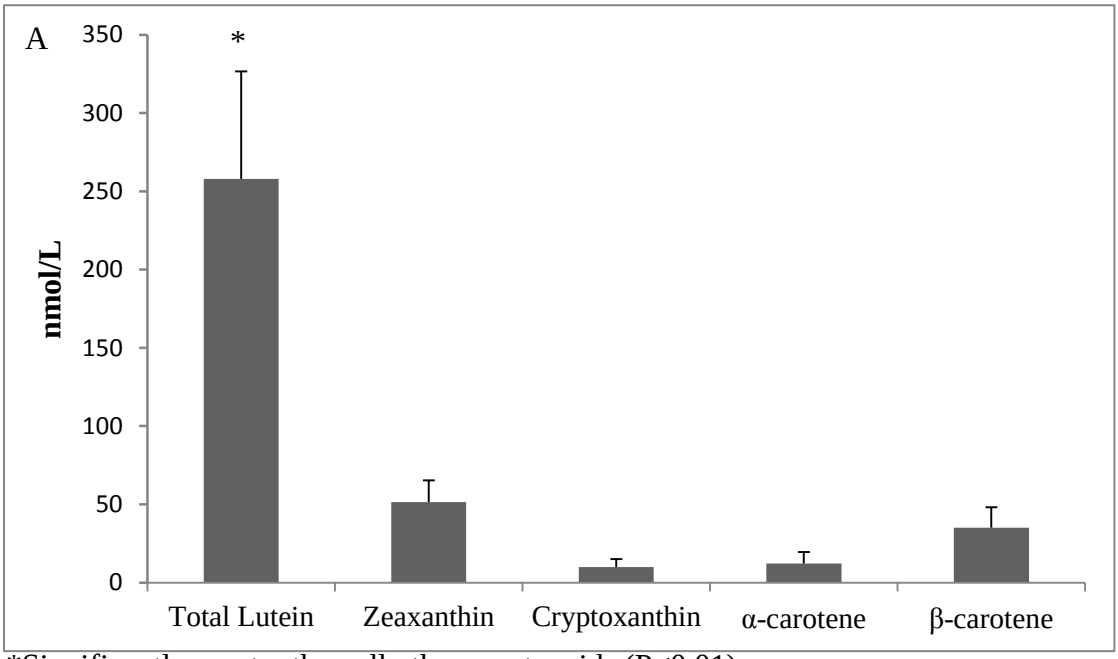
Acknowledgement

Emily E. Johnson and the ONPRC Veterinary Pathology Service assisted with brain tissue collection. Jean Galluccio, Audrey Goldbaum, and Kathryn Baldyga, from the Cardiovascular Nutrition Laboratory at Tufts University, performed brain fatty acid, and protein analysis.

Author Contributions

Conceived and designed experiments: ESM, EJJ, JWE Jr, MN, MK. Performed the experiments and analyzed data: ESM. Contributed materials/tools: EJJ, MN, NM. Intellectual contribution to manuscript: ESM, EJJ, JWE Jr. MN MK NM.

Figure 1. Serum carotenoid profile (nmol/L) in adult rhesus macaque. (A) Average carotenoid concentrations among all monkeys for which serum was available (n=9). (B) Comparison of carotenoid concentrations between two lutein/zeaxanthin (L/Z) supplemented monkeys and age-matched controls (n=7). Mean ± SEM.



*Significantly greater than all other carotenoids (P<0.01)

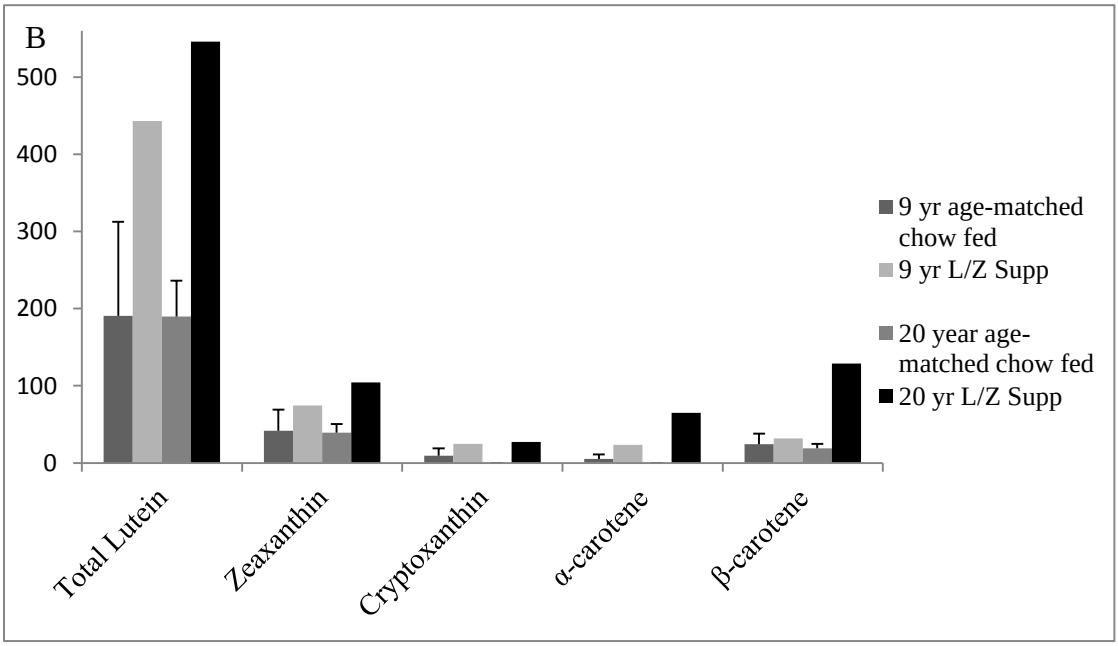


Table 1. Mean (±SEM) carotenoid concentrations (ng/mg protein) in different regions of rhesus macaque brain (n=11)

	Prefrontal Cortex	Cerebellum	Striatum	Hippocampus
Lutein	2.86 ± 0.90^{a}	1.07 ± 0.34^{b}	$3.66 \pm 0.99^{a,c}$	5.00 ± 1.08^{c}
Zeaxanthin	$0.88 \pm 0.27^{a,b}$	0.46 ± 0.12^{b}	$1.34 \pm 0.29^{a,c}$	2.34 ± 0.63^{c}
Cryptoxanthin	0.48 ± 0.30^{a}	0.23 ± 0.13^{a}	ND	0.09 ± 0.06^{a}
β-carotene	0.25 ± 0.06^{a}	0.05 ± 0.02^{b}	0.23 ± 0.06^{a}	ND

Means with different superscripts across brain regions are significantly different according to Tukey's HSD test ($P<0.05$).

Table 2. Mean (±SEM) mole percent fatty acids in different regions of rhesus macaque brain (n=11)

	Prefrontal Cortex	Cerebellum	Striatum	Hippocampus
Total SFA	**49.31 ± 0.71^{a}**	**45.75 ± 0.85^{b}**	**46.38 ± 0.58^{b}**	**48.91 ± 0.35^{a}**
10:0-15:0	0.78 ± 0.11^{a}	0.52 ± 0.11^{b}	0.54 ± 0.02^{b,c}	0.60 ± 0.09^{c}
16:0	25.12 ± 0.33^{a}	24.44 ± 0.41^{a,b}	24.12 ± 0.35^{a,b}	24.13 ± 0.24^{b}
18:0	22.78 ± 0.36^{a}	20.16 ± 0.58^{b}	21.27 ± 0.31^{b}	22.92 ± 0.17^{a}
20:0	0.14 ± 0.01^{a}	0.24 ± 0.02^{b}	0.14 ± 0.01^{a}	0.15 ± 0.01^{a}
22:0	0.09 ± 0.01^{a}	0.10 ± 0.01^{a,b}	0.07 ± 0.01^{c}	0.13 ± 0.01^{b}
24:0	0.40 ± 0.05^{a}	0.29 ± 0.04^{a,b}	0.24 ± 0.03^{b}	0.37 ± 0.08^{a,b}
Total MUFA	**25.04 ± 0.77^{a,c}**	**29.70 ± 0.84^{b}**	**26.90 ± 1.03^{a}**	**24.19 ± 0.35^{c}**
14:1n-5	0.51 ± 0.09^{a}	0.40 ± 0.10^{a}	0.09 ± 0.06^{b}	0.02 ± 0.00^{c}
16:1n-9	0.46 ± 0.03^{a}	0.29 ± 0.01^{b}	0.29 ± 0.01^{b}	0.53 ± 0.03^{a}
16:1n-7	0.47 ± 0.02^{a,b}	0.48 ± 0.02^{a}	0.42 ± 0.01^{b}	0.54 ± 0.01^{c}
18:1n-9	16.98 ± 0.55^{a,b}	20.12 ± 0.66^{c}	18.25 ± 0.69^{a}	16.40 ± 0.30^{b}
18:1n-7	4.85 ± 0.14^{a}	5.66 ± 0.0.21^{b}	6.27 ± 0.34^{c}	5.41 ± 0.07^{b}
20:1n-9	0.83 ± 0.11^{a,c}	1.56 ± 0.22^{b}	0.95 ± 0.10^{a,b}	0.64 ± 0.04^{c}
22:1n-9	0.12 ± 0.04^{a}	0.20 ± 0.02^{b}	0.10 ± 0.02^{a}	0.09 ± 0.01^{a}
24:1n-9	0.84 ± 0.11^{a}	0.98 ± 0.11^{a}	0.53 ± 0.06^{b}	0.56 ± 0.06^{b}
Total PUFAn6	**14.61 ± 0.38^{a}**	**13.78 ± 0.32^{b}**	**16.33 ± 0.20^{c}**	**17.74 ± 0.17^{d}**
18:2n-6	0.88 ± 0.04^{a,b}	1.02 ± 0.04^{a}	0.82 ± 0.04^{b}	0.81 ± 0.02^{b}
18:3n-6	0.03 ± 0.01^{a}	0.02 ± 0.01^{a}	0.03 ± 0.01^{a}	0.05 ± 0.01^{b}
20:2n-6	0.27 ± 0.13^{a}	0.37 ± 0.09^{b}	0.12 ± 0.01^{a,b}	0.10 ± 0.02^{a}
20:3n-6	1.22 ± 0.06^{a}	2.36 ± 0.10^{b}	1.69 ± 0.17^{b}	1.23 ± 0.05^{a}
20:4n-6	6.86 ± 0.27^{a}	6.37 ± 0.18^{b}	8.09 ± 0.23^{c}	9.07 ± 0.11^{d}
22:2n-6	0.07 ± 0.01^{a}	0.10 ± 0.01^{b}	0.06 ± 0.01^{a}	0.04 ± 0.01^{c}
22:4n-6	4.28 ± 0.18^{a}	2.86 ± 0.12^{b}	4.73 ± 0.11^{c}	4.97 ± 0.09^{c}
22:5n-6	1.00 ± 0.07^{a}	0.67 ± 0.08^{b}	0.79 ± 0.05^{b}	1.48 ± 0.11^{c}
Total PUFAn3	**10.34 ± 0.50^{a}**	**10.09 ± 0.41^{a,b}**	**9.95 ± 0.41^{a,b}**	**9.46 ± 0.14^{b}**
18:3n-3	0.03 ± 0.01^{a,b}	0.02 ± 0.01^{a}	0.01 ± 0.00^{b,c}	0.01 ± 0.00^{c}
18:4n-3	0.43 ± 0.05^{a}	0.31 ± 0.04^{a,b}	0.27 ± 0.03^{b}	0.36 ± 0.02^{a}
20:5n-3	0.02 ± 0.00^{a}	0.04 ± 0.00^{b,c}	0.05 ± 0.01^{b}	0.03 ± 0.00^{a,c}
22:5n-3	0.31 ± 0.02^{a,c}	0.47 ± 0.03^{b}	0.38 ± 0.03^{a,b}	0.24 ± 0.07^{c}
22:6n-3	9.55 ± 0.54^{a}	9.24 ± 0.42^{a}	9.24 ± 0.42^{a}	8.82 ± 0.15^{a}
Total *Trans*	**0.70 ± 0.36^{a,b}**	**0.69 ± 0.27^{a}**	**0.44 ± 0.08^{a}**	**0.31 ± 0.01^{b}**

Means with different superscripts across brain regions are significantly different according to Tukey's HSD test ($P<0.05$). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFAn6: n-6 polyunsaturated fatty acids, PUFAn3: n-3 polyunsaturated fatty acids, *trans*: *trans* fatty acids

Figure 2. Lutein content (ng/mg protein) of membranes for four brain regions of rhesus macaque (n=11). Geometric Mean (95% Confidence Interval).

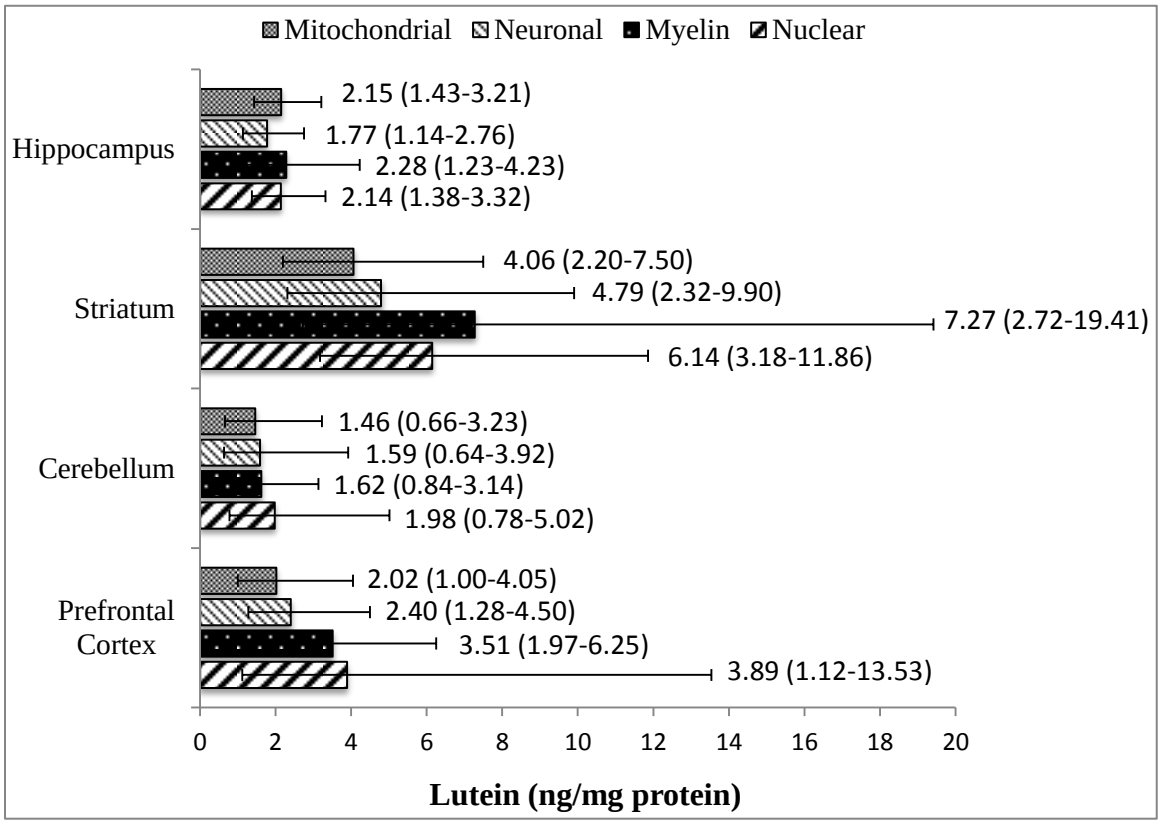


Table 3. Mean (±SEM) mole percent PUFAn-3 in different membranes within each region of rhesus macaque brain (n=11)

		Nuclear	Myelin	Neuronal	Mitochondrial
Prefrontal Cortex	**Total PUFAn3**	**8.37 ± 0.22^{a}**	**13.08 ± 0.16^{b}**	**13.12 ± 0.14^{b}**	**13.50 ± 0.31^{b}**
	18:3n-3	0.05 ± 0.03^{a}	0.05 ± 0.03^{a}	0.07 ± 0.03^{a}	0.03 ± 0.01^{a}
	18:4n-3	0.21 ± 0.07^{a}	0.10 ± 0.02^{a}	0.13 ± 0.03^{a}	0.07 ± 0.02^{a}
	20:5n-3	0.09 ± 0.07^{a}	0.05 ± 0.03^{a}	0.05 ± 0.02^{a}	0.05 ± 0.01^{a}
	22:5n-3	0.27 ± 0.05^{a,b}	0.27 ± 0.02^{a}	0.29 ± 0.03^{a}	0.20 ± 0.01^{b}
	22:6n-3	7.75 ± 0.17^{a}	12.61 ± 0.16^{b}	12.58 ± 0.17^{b}	13.16 ± 0.31^{b}
Cerebellum	**Total PUFAn3**	**8.52 ± 0.20^{a}**	**12.96 ± 0.12^{b}**	**13.75 ± 0.27^{c}**	**14.93 ± 0.39^{c}**
	18:3n-3	0.02 ± 0.00^{a}	0.07 ± 0.04^{b}	0.01 ± 0.00^{a}	0.09 ± 0.03^{b}
	18:4n-3	0.27 ± 0.04^{a}	0.09 ± 0.02^{b}	0.11 ± 0.02^{b}	0.05 ± 0.01^{b}
	20:5n-3	0.09 ± 0.04^{a}	0.06 ± 0.01^{a}	0.09 ± 0.05^{a}	0.12 ± 0.05^{b}
	22:5n-3	0.40 ± 0.02^{a}	0.32 ± 0.01^{a,b}	0.36 ± 0.03^{a}	0.30 ± 0.02^{b}
	22:6n-3	7.74 ± 0.20^{a}	12.43 ± 0.12^{b}	13.17 ± 0.30^{c}	14.38 ± 0.44^{d}
Striatum	**Total PUFAn3**	**6.87 ± 0.39^{a}**	**12.33 ± 0.18^{b}**	**11.34 ± 0.20^{c}**	**12.99 ± 0.74^{d}**
	18:3n-3	0.02 ± 0.01^{a,c}	0.01 ± 0.00^{b}	0.01 ± 0.00^{a,b}	0.04 ± 0.02^{c}
	18:4n-3	0.20 ± 0.06^{a}	0.08 ± 0.02^{a}	0.11 ± 0.03^{a}	0.07 ± 0.01^{a}
	20:5n-3	0.14 ± 0.10^{a}	0.06 ± 0.01^{a,b}	0.07 ± 0.02^{a,b}	0.07 ± 0.01^{b}
	22:5n-3	0.44 ± 0.04^{a}	0.33 ± 0.04^{b}	0.39 ± 0.03^{a,b}	0.20 ± 0.02^{c}
	22:6n-3	6.06 ± 0.41^{a}	11.84 ± 0.17^{b}	10.75 ± 0.21^{c}	12.62 ± 0.75^{d}
Hippocampus	**Total PUFAn3**	**6.80 ± 0.31^{a}**	**11.05 ± 0.49^{b}**	**9.90 ± 0.51^{c}**	**9.51 ± 0.71^{c}**
	18:3n-3	0.01 ± 0.00^{a}	0.01 ± 0.01^{a,b}	0.01 ± 0.01^{a,b}	0.03 ± 0.02^{b}
	18:4n-3	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.02 ± 0.01^{a}	0.03 ± 0.01^{a}
	20:5n-3	1.45 ± 0.21^{a}	0.60 ± 0.21^{a}	0.65 ± 0.12^{a}	0.78 ± 0.24^{a}
	22:5n-3	0.28 ± 0.07^{a}	0.23 ± 0.06^{a}	0.16 ± 0.02^{a}	0.17 ± 0.02^{a}
	22:6n-3	5.05 ± 0.25^{a}	10.19 ± 0.37^{b}	9.06 ± 0.48^{b,c}	8.50 ± 0.68^{c}

Means with different superscripts across membranes are significantly different according to Tukey's HSD test ($P<0.05$). PUFAn3: n-3 polyunsaturated fatty acids

Table 4. Partial correlations between lutein and DHA (22:6n-3) across membranes in different regions of rhesus macaque brain (n=11)

	Total	Nuclear	Myelin	Neuronal	Mitochondrial
	PUFAn3				
Prefrontal Cortex	0.35	-0.30	**-0.56***	-0.13	0.15
Cerebellum	0.37	-0.34	0.02	**-0.59***	-0.19
Striatum	-0.46	0.47	-0.01	**-0.60***	0.26
Hippocampus	0.43	-0.03	0.46	-0.01	**0.72****
	DHA (22:6n-3)				
Prefrontal Cortex	0.41	-0.28	**-0.55***	-0.11	0.15
Cerebellum	0.38	-0.35	0.03	**-0.56***	-0.19
Striatum	-0.41	0.39	0.03	**-0.63****	0.28
Hippocampus	0.44	0.02	0.44	0.003	**0.79*****
	PUFAn-6/n-3				
Prefrontal Cortex	-0.50	-0.26	**0.63****	-0.46	**-0.69****
Cerebellum	0.03	-0.11	0.36	-0.27	0.41
Striatum	0.37	0.32	**0.68****	**0.58***	-0.37
Hippocampus	0.32	0.51	0.38	0.05	0.23

Values are partial correlation coefficients adjusted for age. PUFAn3: n-3 polyunsaturated fatty acids, DHA: docosahexanoic acid.
*P≤0.1, **P≤0.05, ***P<0.01

Supplementary Data

Table S1. Mean (±SEM) mole percent membrane fatty acids in prefrontal cortex of rhesus macaque brain (n=11)

	Nuclear	Myelin	Neuronal	Mitochondrial
Total SFA	**48.38 ± 0.50^{a}**	**51.17 ± 0.88^{b}**	**50.90 ± 0.44^{b}**	**44.24 ± 0.95^{c}**
10:0-15:0	0.84 ± 0.08^{a}	1.02 ± 0.09^{b}	1.05 ± 0.09^{a,b}	1.30 ± 0.14^{b}
16:0	23.22 ± 0.29^{a}	25.60 ± 0.77^{b}	25.47 ± 0.27^{b}	19.01 ± 0.77^{c}
18:0	21.66 ± 0.22^{a}	22.84 ± 0.21^{b}	22.42 ± 0.18^{b}	22.45 ± 0.34^{b}
20:0	2.25 ± 0.49^{a}	1.59 ± 0.21^{a,b}	1.83 ± 0.41^{a,b}	1.31 ± 0.15^{b}
22:0	0.09 ± 0.01^{a}	0.03 ± 0.00^{b}	0.06 ± 0.03^{b}	0.04 ± 0.02^{b}
24:0	0.31 ± 0.04^{a}	0.08 ± 0.01^{b}	0.08 ± 0.01^{b}	0.12 ± 0.05^{b}
Total MUFA	**27.72 ± 0.50^{a}**	**20.15 ± 0.38^{b}**	**20.30 ± 0.18^{b}**	**21.81 ± 0.27^{c}**
14:1n-5	0.12 ± 0.05^{a}	0.14 ± 0.06^{a}	0.22 ± 0.07^{a}	0.22 ± 0.09^{a}
16:1n-9	0.42 ± 0.02^{a}	0.51 ± 0.02^{b,c}	0.48 ± 0.02^{a,b}	0.57 ± 0.02^{c}
16:1n-7	0.43 ± 0.02^{a}	0.52 ± 0.07^{a,b}	0.51 ± 0.03^{b}	1.24 ± 0.09^{c}
18:1n-9	20.00 ± 0.40^{a}	13.26 ± 0.20^{b}	13.45 ± 0.14^{b}	13.47 ± 0.15^{b}
18:1n-7	4.95 ± 0.08^{a}	5.12 ± 0.16^{a}	5.00 ± 0.07^{a}	5.93 ± 0.11^{b}
20:1n-9	0.89 ± 0.15^{a}	0.29 ± 0.04^{b}	0.35 ± 0.04^{b}	0.18 ± 0.03^{c}
22:1n-9	0.10 ± 0.02^{a}	0.05 ± 0.01^{b}	0.08 ± 0.02^{b}	0.12 ± 0.01^{a}
24:1n-9	0.80 ± 0.07^{a}	0.27 ± 0.08^{b}	0.21 ± 0.04^{b}	0.07 ± 0.03^{c}
Total PUFAn6	**15.19 ± 0.10^{a}**	**15.21 ± 0.50^{a}**	**15.27 ± 0.26^{a}**	**20.02 ± 0.63^{b}**
18:2n-6	0.80 ± 0.03^{a}	1.01 ± 0.19^{a,b}	1.23 ± 0.27^{b}	2.84 ± 0.23^{c}
18:3n-6	0.23 ± 0.09^{a}	0.37 ± 0.10^{a}	0.28 ± 0.12^{a}	0.39 ± 0.14^{a}
20:2n-6	0.16 ± 0.01^{a}	0.09 ± 0.01^{b}	0.11 ± 0.02^{b,c}	0.13 ± 0.01^{c}
20:3n-6	1.47 ± 0.07^{a}	1.05 ± 0.09^{b}	1.02 ± 0.04^{b}	1.32 ± 0.15^{a}
20:4n-6	6.65 ± 0.10^{a}	7.63 ± 0.41^{b}	7.35 ± 0.08^{b}	11.42 ± 0.52^{c}
22:2n-6	0.08 ± 0.02^{a}	0.03 ± 0.00^{b}	0.05 ± 0.02^{b}	0.04 ± 0.02^{b}
22:4n-6	5.11 ± 0.14^{a}	3.88 ± 0.13^{b}	4.07 ± 0.09^{b}	2.80 ± 0.14^{a}
22:5n-6	0.69 ± 0.10^{a}	1.14 ± 0.06^{b}	1.16 ± 0.06^{b}	1.08 ± 0.05^{b}
Total *Trans*	**0.35 ± 0.04^{a}**	**0.39 ± 0.04^{a}**	**0.40 ± 0.09^{a}**	**0.43 ± 0.08^{a}**

Means with different superscripts across membranes are significantly different according to Tukey's HSD test ($P<0.05$). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFAn6: n-6 polyunsaturated fatty acids, *trans*: *trans* fatty acids

Table S2. Mean (±SEM) mole percent membrane fatty acids in cerebellum of rhesus macaque brain (n=11)

	Nuclear	Myelin	Neuronal	Mitochondrial
Total SFA	**48.56 ± 0.70^{a}**	**51.28 ± 0.38^{b}**	**50.34 ± 0.66^{b}**	**43.96 ± 0.50^{c}**
10:0-15:0	1.35 ± 0.19^{a}	1.01 ± 0.11^{a,b}	0.87 ± 0.08^{b}	1.28 ± 0.17^{a,b}
16:0	23.01 ± 0.37^{a}	26.89 ± 0.20^{b}	25.92 ± 0.38^{b}	18.92 ± 0.48^{c}
18:0	19.93 ± 0.28^{a}	19.94 ± 0.15^{a}	20.13 ± 0.32^{a,b}	21.15 ± 0.21^{b}
20:0	3.89 ± 0.42^{a}	3.30 ± 0.36^{a}	3.26 ± 0.37^{a}	2.43 ± 0.28^{b}
22:0	0.09 ± 0.01^{a}	0.04 ± 0.00^{b}	0.06 ± 0.03^{b}	0.05 ± 0.02^{b}
24:0	0.30 ± 0.06^{a}	0.10 ± 0.02^{b}	0.11 ± 0.04^{b}	0.12 ± 0.04^{b}
Total MUFA	**29.41 ± 0.55^{a}**	**23.03 ± 0.41^{b}**	**21.92 ± 0.38^{c}**	**23.27 ± 0.24^{b}**
14:1n-5	0.13 ± 0.03^{a}	0.09 ± 0.03^{a}	0.18 ± 0.05^{a}	0.17 ± 0.05^{a}
16:1n-9	0.30 ± 0.02^{a}	0.33 ± 0.01^{a,b}	0.33 ± 0.02^{a,b}	0.35 ± 0.02^{b}
16:1n-7	0.46 ± 0.03^{a,b}	0.44 ± 0.02^{a}	0.50 ± 0.03^{b}	0.88 ± 0.03^{c}
18:1n-9	20.98 ± 0.57^{a}	15.75 ± 0.35^{b}	14.74 ± 0.24^{c}	14.29 ± 0.24^{c}
18:1n-7	5.38 ± 0.14^{a}	5.30 ± 0.10^{a,b}	5.11 ± 0.08^{b}	6.87 ± 0.11^{c}
20:1n-9	1.28 ± 0.23^{a}	0.83 ± 0.12^{a,b}	0.73 ± 0.07^{a,b}	0.49 ± 0.03^{b}
22:1n-9	0.22 ± 0.03^{a}	0.08 ± 0.01^{b}	0.14 ± 0.02^{c}	0.15 ± 0.02^{a,c}
24:1n-9	0.66 ± 0.11^{a}	0.22 ± 0.04^{b}	0.20 ± 0.05^{b}	0.06 ± 0.02^{c}
Total PUFAn-6	**13.02 ± 0.28^{a}**	**12.45 ± 0.27^{a}**	**13.65 ± 0.47^{a}**	**17.38 ± 0.25^{b}**
18:2n-6	0.98 ± 0.04^{a}	1.06 ± 0.04^{a,b}	1.67 ± 0.42^{b}	3.32 ± 0.20^{c}
18:3n-6	0.31 ± 0.09^{a}	0.46 ± 0.09^{a}	0.44 ± 0.09^{a}	0.54 ± 0.14^{a}
20:2n-6	0.30 ± 0.06^{a}	0.18 ± 0.01^{a}	0.17 ± 0.01^{a}	0.22 ± 0.01^{a}
20:3n-6	2.11 ± 0.08^{a}	1.53 ± 0.06^{b}	1.57 ± 0.06^{b}	2.36 ± 0.12^{a}
20:4n-6	5.69 ± 0.16^{a}	6.45 ± 0.27^{a,b}	6.83 ± 0.14^{b}	8.51 ± 0.23^{c}
22:2n-6	0.14 ± 0.01^{a}	0.04 ± 0.01^{b}	0.06 ± 0.02^{b}	0.07 ± 0.02^{b}
22:4n-6	3.06 ± 0.12^{a}	2.22 ± 0.05^{b,c}	2.33 ± 0.06^{b}	1.79 ± 0.04^{c}
22:5n-6	0.43 ± 0.06^{a}	0.52 ± 0.04^{a}	0.58 ± 0.05^{a}	0.57 ± 0.03^{a}
Total *Trans*	**0.48 ± 0.19^{a,b}**	**0.29 ± 0.02^{a}**	**0.34 ± 0.09^{a}**	**0.44 ± 0.05^{b}**

Means with different superscripts across membranes are significantly different according to Tukey's HSD test (P<0.05). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFAn6: n-6 polyunsaturated fatty acids, *trans*: *trans* fatty acids

Table S3. Mean (±SEM) mole percent membrane fatty acids in striatum of rhesus macaque brain (n=11)

	Nuclear	Myelin	Neuronal	Mitochondrial
Total SFA	**47.57 ± 0.76^{a}**	**52.11 ± 0.27^{b}**	**49.50 ± 0.30^{a}**	**45.31 ± 1.96^{c}**
10:0-15:0	0.92 ± 0.16^{a}	0.74 ± 0.07^{a}	0.70 ± 0.07^{a}	1.04 ± 0.19^{a}
16:0	22.25 ± 0.32^{a}	26.07 ± 0.12^{b}	24.04 ± 0.18^{c}	18.74 ± 0.89^{d}
18:0	20.95 ± 0.40^{a}	22.77 ± 0.21^{b,c}	22.34 ± 0.22^{b}	23.69 ± 0.73^{c}
20:0	2.91 ± 0.28^{a}	2.37 ± 0.22^{a,b}	2.17 ± 0.22^{a,b}	1.69 ± 0.28^{b}
22:0	0.12 ±0.01^{a}	0.05 ± 0.00^{b,c}	0.06 ± 0.01^{b}	0.05 ± 0.01^{c}
24:0	0.43 ± 0.05^{a}	0.13 ± 0.01^{b,c}	0.20 ± 0.02^{b}	0.10 ± 0.01^{c}
Total MUFA	**29.68 ± 0.76^{a}**	**19.99 ± 0.31^{b}**	**22.44 ± 0.43^{c}**	**21.72 ± 0.68^{c}**
14:1n-5	0.23 ± 0.06^{a}	0.06 ± 0.02^{b}	0.07 ± 0.02^{b}	0.44 ± 0.06^{a}
16:1n-9	0.26 ± 0.01^{a}	0.31 ± 0.02^{b}	0.30 ± 0.02^{b}	0.35 ± 0.02^{b}
16:1n-7	0.41 ± 0.02^{a}	0.33 ± 0.01^{b}	0.42 ± 0.01^{a}	1.01 ± 0.03^{c}
18:1n-9	20.74 ± 0.57^{a}	12.77 ± 0.25^{b}	14.72 ± 0.36^{c}	12.52 ± 0.41^{b}
18:1n-7	5.44 ± 0.14^{a}	5.86 ± 0.08^{b}	5.89 ± 0.08^{b}	6.95 ± 0.33^{c}
20:1n-9	1.30 ± 0.11^{a}	0.38 ± 0.03^{b}	0.49 ± 0.04^{b}	0.20 ± 0.02^{c}
22:1n-9	0.16 ± 0.04^{a}	0.05 ± 0.00^{b}	0.09 ± 0.01^{c}	0.18 ± 0.04^{a}
24:1n-9	1.14 ± 0.14^{a}	0.25 ± 0.02^{b}	0.46 ± 0.03^{a}	0.07 ± 0.01^{c}
Total PUFAn-6	**15.41 ± 0.28^{a}**	**15.31 ± 0.16^{a}**	**16.44 ± 0.13^{b}**	**19.38 ± 0.90^{c}**
18:2n-6	0.65 ± 0.05^{a}	0.56 ± 0.02^{a}	0.77 ± 0.03^{b}	2.08 ± 0.11^{c}
18:3n-6	0.23 ± 0.09^{a}	0.24 ± 0.10^{a}	0.13 ± 0.08^{a}	0.14 ± 0.11^{a}
20:2n-6	0.28 ± 0.12^{a}	0.10 ± 0.01^{b}	0.11 ± 0.01^{b,c}	0.26 ± 0.15^{c}
20:3n-6	1.69 ± 0.09^{a}	1.11 ± 0.06^{b}	1.41 ± 0.06^{c}	1.96 ± 0.19^{d}
20:4n-6	6.86 ± 0.25^{a}	7.82 ± 0.14^{b}	8.39 ± 0.13^{b}	11.44 ± 0.65^{a}
22:2n-6	0.09 ± 0.01^{a}	0.03 ± 0.00^{b}	0.04 ± 0.01^{b}	0.04 ± 0.01^{b}
22:4n-6	5.05 ± 0.15^{a}	4.47 ± 0.07^{b}	4.70 ± 0.06^{a,b}	2.69 ± 0.15^{c}
22:5n-6	0.56 ± 0.05^{a}	0.97 ± 0.05^{b}	0.88 ± 0.06^{b,c}	0.77 ± 0.06^{c}
Total *Trans*	**0.47 ± 0.14^{a,c}**	**0.26 ± 0.02^{b}**	**0.29 ± 0.01^{a,b}**	**0.59 ± 0.22^{c}**

Means with different superscripts across membranes are significantly different according to Tukey's HSD test (P<0.05). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFAn6: n-6 polyunsaturated fatty acids, *trans*: *trans* fatty acids

Table S4. Mean (±SEM) mole percent membrane fatty acids in hippocampus of rhesus macaque brain (n=11)

	Nuclear	Myelin	Neuronal	Mitochondrial
Total SFA	**44.26 ± 0.29^{a}**	**51.48 ± 0.53^{b}**	**50.98 ± 1.43^{c}**	**45.20 ± 1.51^{a}**
10:0-15:0	0.74 ± 0.24^{a}	0.82 ± 0.22^{a}	0.82 ± 0.13^{a}	0.89 ± 0.13^{a}
16:0	20.09 ± 0.20^{a}	26.86 ± 0.42^{b}	25.72 ± 0.86^{c}	20.33 ± 1.01^{a}
18:0	22.59 ± 0.41^{a}	23.31 ± 0.33^{a,b}	24.00 ± 0.58^{b}	23.47 ± 0.47^{a,b}
20:0	0.20 ± 0.02^{a}	0.16 ± 0.03^{a}	0.18 ± 0.03^{a}	0.15 ± 0.04^{a}
22:0	0.15 ± 0.02^{a}	0.11 ± 0.03^{a,b}	0.06 ± 0.01^{b}	0.10 ± 0.03^{b}
24:0	0.50 ± 0.08^{a}	0.23 ± 0.07^{b}	0.19 ± 0.03^{b}	0.26 ± 0.06^{b}
Total MUFA	**31.41 ± 0.45^{a}**	**20.20 ± 0.24^{b}**	**22.53 ± 0.36^{c}**	**23.97 ± 0.38^{d}**
14:1n-5	0.12 ± 0.06^{a}	0.18 ± 0.07^{a}	0.21 ± 0.07^{a}	0.14 ± 0.04^{a}
16:1n-9	0.49 ± 0.03^{a}	0.55 ± 0.03^{a,b}	0.58 ± 0.03^{b}	0.60 ± 0.03^{b}
16:1n-7	0.55 ± 0.05^{a}	0.46 ± 0.03^{b}	0.61 ± 0.04^{a}	1.62 ± 0.09^{c}
18:1n-9	22.59 ± 0.55^{a}	12.94 ± 0.17^{b}	14.86 ± 0.26^{c}	14.93 ± 0.35^{c}
18:1n-7	5.29 ± 0.08^{a}	5.39 ± 0.11^{a}	5.35 ± 0.11^{a}	6.15 ± 0.08^{b}
20:1n-9	1.09 ± 0.12^{a}	0.33 ± 0.03^{b}	0.38 ± 0.04^{b}	0.20 ± 0.01^{c}
22:1n-9	0.13 ± 0.03^{a}	0.08 ± 0.04^{b}	0.14 ± 0.03^{a}	0.14 ± 0.02^{a}
24:1n-9	1.14 ± 0.18^{a}	0.27 ± 0.04^{b,c}	0.42 ± 0.14^{b}	0.19 ± 0.05^{c}
Total PUFAn-6	**16.98 ± 0.34^{a}**	**16.66 ± 0.44^{a}**	**15.95 ± 1.20^{a}**	**20.56 ± 1.25^{b}**
18:2n-6	0.73 ± 0.03^{a}	1.09 ± 0.48^{a}	0.79 ± 0.09^{a}	2.09 ± 0.17^{b}
18:3n-6	0.10 ± 0.06^{a}	0.08 ± 0.02^{a}	0.08 ± 0.03^{a}	0.19 ± 0.09^{a}
20:2n-6	0.12 ± 0.01^{a}	0.11 ± 0.03^{a}	0.11 ± 0.04^{a}	0.10 ± 0.02^{a}
20:3n-6	1.59 ± 0.06^{a}	0.78 ± 0.09^{b}	0.96 ± 0.08^{b}	1.56 ± 0.06^{a}
20:4n-6	8.02 ± 0.22^{a}	8.09 ± 0.12^{a}	8.16 ± 0.67^{a}	11.96 ± 0.87^{b}
22:2n-6	0.06 ± 0.01^{a}	0.04 ± 0.02^{b}	0.02 ± 0.00^{b}	0.06 ± 0.03^{b}
22:4n-6	4.99 ± 0.12^{a}	4.28 ± 0.12^{b}	4.10 ± 0.29^{b}	2.80 ± 0.19^{c}
22:5n-6	1.37 ± 0.10^{a}	2.19 ± 0.19^{b}	1.72 ± 0.17^{a,b}	1.80 ± 0.20^{a,b}
Total *Trans*	**0.51 ± 0.13^{a}**	**0.52 ± 0.07^{a}**	**0.59 ± 0.08^{a}**	**0.65 ± 0.09^{a}**

Means with different superscripts across membranes are significantly different according to Tukey's HSD test (P<0.05). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFAn6: n-6 polyunsaturated fatty acids, *trans*: *trans* fatty acids

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4.2 Membrane-Specific Concentrations of Lutein, Vitamin E, and Polyunsaturated Fatty Acids Are Related to Markers of Cell Viability in Rhesus Monkey Brain

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Funding source: This work was supported by a grant from Abbott Nutrition through the Center for Nutrition, Learning, and Memory at the University of Illinois, DSM Nutritional Products, and USDA (58-1950-0-014).

Abbreviations used: DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids; PFC, prefrontal cortex; CER, cerebellum; ST, striatum; AA, arachidonic acid; ER, extracellular signal-regulated kinase; MP, macular pigment; MAP, mitogen-activated protein; reactive oxygen species, ROS; CV, coefficient of variation; BCA, bicinchoninic acid; HPLC, high performance liquid chromatography; GC, gas chromatography; ANOVA, analysis of variance; NP, neuroprostanes; IsoP, isoprostanes; PFB, pentafluorobenzyl; 8-hydroxy-2-deoxyguanosine, 8-OHdG

Abstract

Brain cell viability is critical for maintaining cognitive function. Lutein, a carotenoid with anti-oxidant properties, is beneficial for cognition. The relationship between lutein and cognition may be influenced by docosahexaenoic acid (DHA), a polyunsaturated omega-3 fatty acid (PUFAn-3) which is also related to improved cognitive function. However, the role of lutein in the brain remains unknown. Furthermore, little is known regarding how brain membrane lutein content compares to that of vitamin E (α-tocopherol), another membrane-associated antioxidant. The objective of this study was to assess the relationship of the subcellular distribution of lutein, vitamin E, and PUFAs in the brain to markers of cell viability. Nuclear, myelin, mitochondrial, and neuronal plasma membranes were isolated from prefrontal cortex (PFC), cerebellum (CER), and striatum (ST) of adult rhesus monkeys (n=11) using differential centrifugation with a Ficoll density gradient. Carotenoids/vitamin E and fatty acids were measured using HPLC and GC, respectively. DHA and arachidonic acid (AA) oxidation were measured using LC-GC/MS. Western blots were used to measure extracellular signal regulated kinase (ERK) activation. DNA damage (8-hydroxy-2-deoxyguanosine) was measured using UPLC-MS/MS. Lutein was detected in all membranes, with concentrations being ~1000 times lower than that of α-tocopherol. Mitochondrial lutein was inversely associated with DHA oxidation more so than AA oxidation, while total α-tocopherol was inversely related to PUFA oxidation in PFC and ST ($P<0.05$). Nuclear α-tocopherol was also inversely related to DNA damage in CER ($P<0.05$). Neuronal membrane AA, was related to ERK activation in ST ($P<0.05$). These findings provide the first steps toward understanding the role of lutein, and its relationship with vitamin E and PUFAs, in the brain.

Introduction

Lutein, a dietary xanthophyll found in dark leafy greens, eggs, avacados and corn (1), selectively accumulates in the primate retina where, along with its isomer, zeaxanthin, it forms macular pigment (MP) (2). As a component of MP, lutein functions to protect the eye from oxidative damage by filtering harmful blue light (2,3). Lutein also acts directly as an antioxidant to scavenge free radicals in polyunsaturated fatty acids (PUFA)-rich retinal photoreceptors (4,5). In addition to its anti-oxidant capabilities, evidence for a role of lutein in inhibiting neural cell death through modulation of extracellular signal regulated kinase (ERK) expression in mouse models have been observed (6–8). As a result of these functions, lutein has been investigated for its potential role in reducing the risk of age-related macular degeneration (3). More recently, it has been discovered that lutein also preferentially accumulates in the primate brain (9,10).

Studies have shown that lutein is found in the highest concentrations in the human brain at various life stages compared to the other carotenoids even when it is not the primary carotenoid in the diet (10,11). This suggests that lutein may have an important function in this tissue. Emerging evidence indicates that MP density, serum, and brain lutein concentrations are associated with better cognitive function in humans (11–14). Furthermore, supplementation with lutein improved memory and rate of learning in older women and neural processing speed in young healthy adults (15,16). In these studies, cognitive benefits were even greater when subjects were supplemented with both lutein and docosahexaenoic acid (DHA) (17). Consistent with this result, a significant interaction between brain concentrations of lutein and DHA as a predictor of cognitive test scores was observed in a centenarian population (18). Together, these results indicate

that lutein may function to improve cognition and that this effect may be influenced by DHA levels in the brain, or vice versa. However, the mechanism underlying the beneficial effect of lutein on cognition remains unknown. Additionally, lutein is known to accumulate within membranes due to its amphipathic structure (19), and has been shown to accumulate in polyunsaturated fatty acid (PUFA)-rich domains in the retina (5). However, membrane-specific functions of lutein within brain cells are unknown.

Each subcellular membrane uniquely influences the viability of a cell. The composition of the neuronal plasma membrane plays a role in modulating cell survival signaling in the brain (20) by influencing the aggregation of membrane receptors and transporters or regulating protein expression at the cell surface (21–23). One such cell survival pathway that is modulated by the plasma membrane is the mitogen-activated protein (MAP) kinase/ ERK pathway. Therefore, lutein in this membrane may influence cell viability through ERK modulation. Dysfunctional mitochondria have elevated production of reactive oxygen species (ROS) as a byproduct of less efficient ATP synthesis (24). ROS produced from these mitochondria, in turn, damage mitochondrial proteins, lipids, and DNA (25). This cycle is particularly dangerous for the brain due to its high energy demand, high oxygen consumption, and rich abundance of easily oxidized PUFAs (24). Damaged mitochondria have been observed in early cognitive impairment (24–29). Therefore, mitochondrial lutein may function to inhibit lipid peroxidation, thus maintaining the integrity of mitochondrial membranes and preserving normal function. Gene regulation and DNA damage dictate cell health and are linked to cognitive function (30). Genes down-regulated with aging have increased DNA damage in their promoter regions (30). Lutein concentrated in the nuclear membrane may prevent DNA damage.

Vitamin E is another fat-soluble compound that accumulates within membranes of brain cells. Cross-sectional studies indicate that α-tocopherol, the major form of vitamin E in the body, is positively related to cognitive function, however intervention studies have been mixed (31–33). Although α-tocopherol is not as strongly related to cognitive function as lutein, this vitamin is thought to be beneficial due to its potent anti-oxidant capabilities (33). Given that both α-tocopherol and lutein accumulate in membranes and possess anti-oxidant functions, it is possible they function together. However, little is known about their relationship to one another with regard to their subcellular accumulation in the brain. The study objective was to assess the relationships between subcellular deposition of lutein, α-tocopherol, and PUFAs with markers of oxidative stress and cell survival signaling in rhesus macaque brain. Brain regions analyzed include the prefrontal cortex (PFC), cerebellum (CER), and striatum (ST). PFC plays a key role in memory, attention, perceptual awareness, thought, language, and consciousness (34), while CER is involved in learning, executive control, and language (35,36). ST controls working memory, abstract rule learning, and attention control (37). Rhesus monkeys were used because they are excellent models for human brain physiology research and they selectively accumulate lutein in the retina and brain, similar to humans (38–40).

Methods

Animals and Diet

Eleven Rhesus monkeys (*Macaca mulatta*) ranging from 7-20 years of age were fed standard monkey chow (LabDiet, St. Louis, MO) containing 16.4 μmol/kg of lutein, 6.4 μmol/kg of zeaxanthin, 4.8 μmol/kg β-carotene, 1.0 μmol/kg α-carotene, 0.1 μmol/kg cryptoxanthin, 171.07 μmol/kg γ-tocopherol, and 23.55 μmol/kg α-tocopherol throughout

the lifespan along with a variety of different fruits and vegetables. The chow contained 1.54% total saturated fatty acids and 1.68% total monounsaturated fatty acids. For PUFAs, the diet contained 1.66% linoleic acid, less than 0.01% arachidonic acid (AA), and 0.10% linolenic acid (with total PUFAn-3 content being 0.13%).

Eight of the 11 monkeys were female. Two of the female monkeys, aged 9 and 20 years, were additionally supplemented daily with a gelatin beadlet containing 51.78 mg/g lutein and 3.60 mg/g zeaxanthin (supplied by DSM Nutritional Products Ltd) for 7- 12 months prior to termination in order to increase the range of lutein concentrations found in the brain tissue. All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center. Approval was also given from Tufts IACUC to receive brain tissue for analysis.

Brain Collection

Right and left hemispheres of PFC, CER, and ST were collected for analysis. The regions consisted of both gray and white matter; however, major white matter tracts were removed from cortical samples. Each brain region was immediately put on dry ice after removal and stored at -80^{0}C. Right and left hemispheres of each region were pooled and pulverized in liquid nitrogen. Pulverized samples were then aliquoted for subsequent carotenoid, tocopherol, and fatty acid analyses and stored at -80^{0}C.

Preparation of Brain Membranes

Differential centrifugation with a Ficoll density gradient was performed to isolate and purify nuclear, myelin, mitochondrial, and neuronal plasma membranes from each brain region using an established method (41,42). Briefly, pulverized brain tissue was

homogenized in aqueous buffer (10 mM HEPES, 0.25 mM EDTA, 0.32 M sucrose, pH 7.2) containing protease inhibitors (cOmplete^{TM} protease inhibitor cocktail, Roche) and subjected to low-speed centrifugation (1000 x g, 4^0C) to isolate the crude nuclear membrane pellet. The resulting supernatant was removed and placed in a new tube. Homogenization and centrifugations steps were repeated with the remaining pellet. Supernatant from the second centrifugation was combined with the first. The crude nuclear membrane pellet was placed on ice. The combined supernatants were then subjected to middle-speed (17,000 x g, 4^0C) centrifugation to obtain the crude membrane pellet (containing myelin, mitochondrial, and neuronal plasma membrane). The crude membrane pellet was re-homogenized in buffer (10 mM HEPES, 0.25 mM EDTA, pH 7.2) and applied to a Ficoll density gradient (consisting of 14% and 7% Ficoll solutions) and centrifuged at high-speed (87,000 x g, 4^0C) to obtain separated myelin, mitochondrial, and neuronal plasma membranes. All three membrane fractions, along with the crude nuclear membrane, were purified via centrifugation at 17,000 x g, 4^0C. Pure membranes were aliquoted for carotenoid and fatty acid analyses, weighed, and stored at -80^0C. Membrane recovery, determined by measuring the sum of α-tocopherol levels in all membranes and supernatants and comparing to total α-tocopherol in each brain sample analyzed, was determined to be 76% ± 1%.

Carotenoid and Tocopherol Extraction from Brain Regions and Membranes

Extraction of carotenoids and tocopherols from brain regions and membranes was adapted from Park et al. (43) and has been described in detail by Vishwanathan et al (44). Reverse-phase high-performance liquid chromatography, HPLC, (Alliance 2695 Waters, Milford, MA, USA) was used to separate and quantify carotenoids and tocopherols from

each region/membrane extract (45), with a semi-bore C30 carotenoid column (3 µm, 150 mm × 4.6 mm; YMC, Carotenoid). Lutein was detected at 445 nm and α-tocopherol was detected at 292 nm. The lower limit of detection is 0.2 pmol for carotenoids and 2.7 pmol for tocopherols. Interassay coefficients of variation (CV) were 4%. Brain membrane and brain region data were expressed ng per mg protein for lutein and µg per mg protein for α-tocopherol.

Fatty Acid Extraction from Brain Regions and Membranes

Lipids were extracted overnight from homogenates of the various brain regions and membranes using a modified Folch method (46). Fatty acid analysis of the various brain regions and membranes was performed using an established gas chromatography (GC) method (47). Peaks of interest were identified by comparison with authentic fatty acid standards (Nu-Chek Prep, Inc. Elysian, MN) and expressed as µg/mg protein. Interassay CV was <4.5% for fatty acids present at levels >1%.

Protein Determination

Delipidated brain tissue/membranes were digested in 1N sodium hydroxide for the determination of protein using the bicinchoninic acid (BCA) assay (Pierce Inc., Rockford, IL). Brain regions and membranes were digested for 8 and 5 days, respectively.

Neuroprostanes and Isoprostanes Determination

Neuroprostanes is the collective name for a group of compounds formed from the oxidation of DHA. Isoprostanes are formed from the oxidation of AA. Total neuroprostanes (NP) and isoprostanes (IsoP) were extracted and quantified using published methods (48,49) with modifications. Briefly, lipids were extracted from

homogenized brain samples using the Folch method. The lipid extract was then saponified to release esterified NP and IsoP. Neutral lipids were removed from the resulting mixture using hexane. The samples were acidified to pH 3 using HCl to protonate NP and IsoP carboxylic acid groups to allow for extraction into ethyl acetate. Addition of 1000 pg of an internal standard, [$^2H_4$] 15-F_{2t}-IsoP (Cayman Chemicals, Ann Arbor MI), was added prior to the ethyl acetate extraction. After extraction, NP and IsoP were derivitized to form pentafluorobenzyl (PFB) esters, and subjected to HPLC (Agilent 1050) to isolate NP and IsoP as PFB esters using the method described by Walter et al. (48). NP and IsoP fractions were collected, derivitized to form trimethylsilyl ether derivatives, and quantified using GC/MS (48). Selective ion monitoring was used for analysis at *m/z* 593 for NP, *m/z* 569 for IsoP and *m/z* 573 for the internal standard, [$^2H_4$] 15-F_{2t}-IsoP. Inter-assay CV was 10%.

8-hydroxy-2-deoxyguanosine (8-OHdG) Determination

Total DNA was isolated from PFC, CER, and ST using the DNA Extractor TIS kit (Wako Chemicals, USA) per manufacturer's instructions. DNA purity and concentrations were determined using a NanoDrop ND-1000. DNA purity and concentrations from ST samples were unusually low and were not adequate for subsequent analysis. Therefore, 8-OHdG was determined for PFC and CER samples only. Approximately 20 µg DNA was aliquoted for DNA hydrolysis using the 8-OHdG Assay Preparation Reagent Kit (Wako Chemicals, USA). The protocol was performed per manufacturer's instructions. Samples were filtered through 0.22 µm pores prior to injection into the UPLC-MS/MS system. Reverse-phase ultra performance liquid chromatography-tandem mass spectrometry was used to separate and quantify 8-OHdG and 2-deoxyguanosine (2-dG) with a Zorbax

Eclipse Plus C18 column (1.8 µm, 50 mm x 2.1 mm, Agilent). An isocratic method was used with 95% water with 0.1% formic acid and 5% acetonitrile with 0.1% formic acid for 3 minutes. Selected mass filters were used for analysis of 8-OHdG (Q1 284.00 Da, Q3 168.00 Da), 2-dG (Q1 268.00 Da, Q3 152.00 Da), and their respective internal standards, $^{15}N_5$-8OHdG (Q1 289.00 Da, Q3 173.00 Da) and $^{15}N_5$-2dG (Q1 273.00 Da, Q3 157.00 Da). 8-OHdG was expressed as a ratio of 8-OHdG per 10^6 2-dG.

Western Blotting

For analysis of ERK activation by western blotting, sample preparation was performed using published methods (50,51). Briefly, brain tissues were homogenized using a handheld homogenizer in buffer containing 50 mM Tris (pH 7.4), 150 mM sodium chloride, 2 mM EDTA, 2 mM EGTA and 0.1% Triton X-100, with fresh addition of mammalian protease inhibitor cocktail (1:100 dilution) (Roche Diagnostics, Indianapolis, IN, USA), 50 mM sodium fluoride, 1 mM sodium vanadate, 1 mM sodium pyrophosphate and 10 µg/ml phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1000 × g, 5 min, at 4°C and the supernatant was transferred to a fresh tube. The protein concentration in each sample was determined spectrophotometrically on a plate reader using the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Samples were subjected to western blot analysis by running equal amounts of protein (20 µg) and a pooled protein standard on 12.5% SDS-PAGE gel and electrophoretically transferring proteins to PVDF membrane. Blots were blocked with RapidBlock (Amresco, Inc Solon, OH, USA) and incubated overnight at 4°C with primary antibody diluted in RapidBlock. Primary antibodies against phosphorylated and total ERK were obtained commercially from Cell Signaling Technology (Danvers, MA, USA). Blots were washed 3-4 times with

TBST (Tris Buffered Saline/0.5% Tween-20) for 10 minutes at room temperature and incubated with the appropriate secondary antibody. Blots were washed 4-5 times with TBST for 10 minutes at room temperature. ECL development was performed for signal detection according to manufacturer's instructions (Bio-Rad Clarity Western ECL substrate). Bands were visualized using the UVP EC3 BioImaging system and UVP VisionWorks image acquisition and analysis software, which was also used to quantitate blots. ERK activation was measured as the ratio of p-ERK to ERK.

Statistical Analysis

Lutein, α-tocopherol, and fatty acid data are expressed as mean ± standard error of mean. Previous analysis with this data determined that incorporation of the supplemented monkeys with chow-fed animals did not alter the results. Therefore, data from all monkeys were analyzed together. Brain region lutein and α-tocopherol data was normally distributed; however, PUFAs were not and were thus ranked prior to analysis. One-way analysis of variance (ANOVA) with Tukey's HSD to adjust for multiple comparisons was performed to determine differences in concentration of lutein, vitamin E, and fatty acids across brain regions. For membrane analysis, regions were evaluated separately and a one-way ANOVA was performed with Tukey's HSD to evaluate differences in concentrations of lutein, α-tocopherol, and PUFAs across membranes within each region. Membrane lutein, α-tocopherol, and PUFA data were not normally distributed, therefore prior to one-way ANOVAs, lutein and α-tocopherol data were log transformed and fatty acid data were ranked.

Pearson correlations were performed to determine whether membrane concentrations of lutein and vitamin E were associated with DHA and AA oxidation, DNA damage, and

ERK activation. Pearson correlations were also performed to determine whether PUFAs were related to ERK activation and DNA damage. All correlations were adjusted for age because it is known to affect levels of oxidative stress and cell survival signaling. There were no sex differences (observation based on mean values) for brain concentrations of lutein, α-tocopherol, or fatty acids or for markers of cell viability. Therefore, it was not adjusted for in correlation analysis. Statistical analysis was performed using SAS 9.3 and significance was set at the 0.05 level for all analyses.

Results

Concentrations Across Brain Regions and Membranes

Lutein, along with α-tocopherol, was detected in all three regions analyzed (**Table 1**). Lutein concentrations were ~1000 times less than α-tocopherol and ~20,000 times less than PUFAs. Lutein and α-tocopherol concentrations, adjusted for protein, were highest in ST and PFC, followed by CER ($P<0.05$). Similar to lutein and α-tocopherol, total PUFAn-6 and AA concentrations were highest in ST, followed by PFC and CER ($P<0.05$). However, total PUFAn-3 was significantly higher in PFC compared to ST ($P<0.05$). This difference in regional concentrations was driven primarily bu α-linolenic acid content (data not shown) since DHA concentrations did not differ across regions (Table 1).

Within brain regions, lutein concentrations did not differ among membranes for any region (**Table 2**). In all three regions, α-tocopherol concentrations were generally highest in nuclear membranes, although concentrations in this membrane did not differ between myelin and neuronal membranes isolated from the PFC. Both PUFAn-6 and PUFAn-3 concentrations were lowest in the mitochondrial membrane for all three regions ($P<0.05$).

PUFAn-6 concentrations were highest in nuclear membranes ($P<0.05$), although concentrations did not differ with myelin and neuronal membranes in CER. PUFAn-3 concentrations were highest in myelin and neuronal membranes ($P<0.05$), although concentrations in neuronal membranes did not significantly differ from nuclear membranes in PFC and ST.

To further investigate the relationship between membrane lutein and α-tocopherol concentrations and membrane PUFA content, partial correlations were performed (**Table 3**). The relationship to membrane total PUFA content was stronger and more consistent for α-tocopherol than lutein within all regions. In PFC, α-tocopherol was positively related to PUFA concentrations in nuclear ($r=0.76$, $P<0.01$) and mitochondrial membranes ($r=0.81$, $P<0.01$), while lutein showed a trend for a negative association with PUFAs in myelin ($r= -0.52$, $P = 0.1$). Nuclear ($r = 0.75$, $P<0.01$) and mitochondrial ($r= 0.86$, $P<0.01$) α-tocopherol and PUFAs were also significantly related in CER, while no significant associations and trends were observed for lutein. Both lutein and α-tocopherol were most strongly related with PUFA content in ST. Nuclear ($r= 0.85$, $P<0.01$), myelin ($r= 0.76$, $P<0.01$), neuronal ($r= 0.82$, $P<0.01$), and mitochondrial ($r = 0.67$, $P<0.05$) α-tocopherol and PUFA concentrations were significantly related in this region, while only nuclear ($r = 0.86$, $P<0.01$) and myelin ($r= 0.75$, $P<0.01$) lutein and PUFA concentrations were related.

Cross-sectional Relationship between Lutein, Vitamin E, and Neuroprostane (NP) and Isoprostane (IsoP) Concentrations Across Different Brain Regions

Table 4 shows the partial correlations between total region and membrane-specific concentrations of lutein and NP and IsoP content for all brain regions. For NP

correlations, both lutein and NP concentrations were adjusted for DHA content in the brain (both expressed pg/µg DHA). For IsoP correlations, lutein and IsoP were adjusted for AA content (both expressed pg/µg AA). In PFC and ST, lutein was significantly inversely related to NP concentrations in the mitochondrial membrane only ($r = -0.69$ and $r = -0.62$, respectively) ($P<0.05$). There was a trend for a negative association between mitochondrial lutein and NP in the cerebellum ($r = -0.53$, $P =0.09$). Total lutein in PFC also showed a trend for a negative association with NP ($r = -0.51$, $P = 0.1$). For IsoP, there were no significant correlations observed with lutein. However, there was a trend for an inverse association with total region lutein ($r = -0.54$, $P = 0.09$), as well as mitochondrial and neuronal plasma membrane lutein in PFC ($r = -0.51$, $P = 0.1$ for both).

Table 5 shows the partial correlations between total region and membrane-specific concentrations of α-tocopherol and NP and IsoP. As with lutein, α-tocopherol concentrations were adjusted for DHA and AA content when performing correlations with NP and IsoP, respectively. No significant associations were observed between α-tocopherol and NP concentrations. However, a trend for an inverse association with total region α-tocopherol in PFC ($r = -0.51$) and ST ($r = -0.52$) ($P = 0.1$ for both) was observed. Total region α-tocopherol was significantly inversely associated with IsoP in PFC ($r = -0.64$, $P<0.05$) and showed a trend for an inverse association in ST ($r = -0.51$, $P = 0.1$). No significant associations with membrane-specific concentrations of α-tocopherol and IsoP were found.

Cross-sectional Relationship Between Lutein, Vitamin E, PUFAs, and DNA Damage Across Different Brain Regions

Table 6 shows the partial correlations between lutein, α-tocopherol, PUFAs, and 8-OHdG concentrations in PFC and CER. No significant correlations were observed in PFC for any of the compounds with 8-OHdG. In CER, lutein was found to be significantly associated with 8-OHdG in myelin ($r = 0.63$, $P = 0.04$), neuronal ($r = 0.73$, $P = 0.02$), and mitochondrial membranes ($r = 0.77$, $P = 0.009$). Conversely, α-tocopherol concentrations in the nuclear membrane were significantly inversely correlated with 8-OHdG concentrations ($r = -0.66$, $P = 0.03$). Although they did not reach significance, DHA and total PUFAn-3, as well as total PUFAn-6 tended to be negatively associated with 8-OHdG in the nuclear membrane ($P<0.1$).

To further investigate the positive relationship observed between lutein concentrations and DNA damage in CER, partial correlations assessing the association between concentrations of α-tocopherol and lutein in all membranes were performed. No significant relationships were observed in PFC. However in CER, nuclear α-tocopherol concentrations were strongly negatively associated with lutein concentration in all membranes (**Table 7**). Nuclear α-tocopherol concentration was most strongly inversely associated with mitochondrial lutein ($r = -0.80$, $P = 0.006$) and the weakest correlation was in the nuclear membrane ($r = -0.64$, $P = 0.05$), which corresponds to mitochondrial lutein being most strongly related to 8-OHdG concentrations and no significant association between nuclear lutein and 8-OHdG. Concentrations of α-tocopherol in other membranes were not significantly related to membrane lutein content.

Cross-sectional Relationship Between Lutein, Vitamin E, PUFAs, and ERK Activation Across Different Brain Regions

Table 8 shows the partial correlations between total region, nuclear, and neuronal plasma membrane concentrations of lutein, α-tocopherol, PUFAs, and ERK activation in all three brain regions. Myelin and mitochondrial membrane data was excluded from the table as there were no significant correlations or trends determined for any compound in any region. No significant correlations were observed in PFC and CER for any compounds. However, there were some trends observed in CER. Specifically, α-tocopherol concentrations in the neuronal plasma membrane tended to be positively associated with ERK activation ($r = 0.60$, $P = 0.07$). Conversely, total region concentrations of PUFAn-3, DHA, and PUFAn-6 all tended to be inversely associated with ERK activation ($r = -0.55$, -0.52, -0.56, respectively) ($P \leq 0.1$). In ST, total region PUFAn-6 concentrations were significantly inversely associated with ERK activation ($r = -0.70$, $P = 0.03$). Nuclear PUFAn-6 also tended to be inversely associated with ERK activation ($r = -0.56$, $P = 0.09$). However, in the neuronal plasma membrane, a positive association was observed ($r = 0.67$, $P = 0.03$). The association between AA and ERK activation was similar in that there was a trend for a negative association in the nuclear membrane ($r = -0.52$, $P = 0.1$), but a significant positive association in the neuronal plasma membrane ($r = 0.70$, $P = 0.03$). Although not statistically significant, both PUFAn-3 and DHA tended to be positively associated with ERK activation in the neuronal plasma membrane ($r = 0.55$ for both, $P = 0.09$), while total lutein tended to be inversely associated with ERK activation ($r = -0.51$, $P = 0.1$) in this region.

Discussion

Concentrations of lutein in the brain are associated with better cognition in humans (11) and supplementation with lutein can improve cognitive performance in both old and young adults (15,16). However, its mechanism of action in the brain remains unknown. DHA, an anti-inflammatory PUFA, and α-tocopherol, a fat-soluble antioxidant, also accumulate in brain membranes and are associated with cognition (17,31,32,52). Evidence indicates that brain concentrations of DHA may influence the relationship between lutein and cognition, or vice versa (18). However, little is known regarding how the membrane concentrations of lutein compare to that of vitamin E and PUFAs in the brain and how the subcellular deposition of all three relates to cell viability.

Regional and Membrane Concentrations of Lutein, α-tocopherol, and PUFAs in Rhesus Macaque Brain

Consistent with previous studies that assessed brain concentrations of carotenoids and vitamin E, lutein was found in much smaller concentrations in rhesus monkey brain compared to α-tocopherol. However, the difference was slightly larger in this study than that reported in elderly human brains where lutein was approximately 250-800 times lower than α-tocopherol concentrations (9,11). Both lutein and α-tocopherol concentrations followed the same distribution pattern across brain regions, which is different from what was observed in the human centenarian brain (11). This most likely is attributable to the different brain regions analyzed in that study. However, lutein content in the elderly human brain was significantly greater in CER compared to the frontal cortex, while α-tocopherol concentrations were lowest in CER. Our findings that total PUFAn-6 content was distributed similarly to lutein and α-tocopherol is consistent with

what is known regarding accumulation of antioxidants in PUFA-rich domains to inhibit oxidative damage (5).

To date, this study is the first to examine concentrations of lutein, α-tocopherol, and PUFAs across subcellular membranes in the primate brain. Similar to total brain region concentrations of lutein and α-tocopherol, membrane lutein concentrations were ~700-1000 times lower than α-tocopherol concentrations. The observation that α-tocopherol and PUFA concentrations but not lutein, differ across membranes within brain regions indicates that deposition of lutein into subcellular membranes may be driven by factors other than the concentrations of these nutrients, such as membrane polarity and presence of binding proteins. Additionally, there was a more consistent and stronger association between PUFA content and α-tocopherol concentrations compared to lutein concentrations in membranes. Collectively, these results indicate that an antioxidant in relatively high concentrations, like α-tocopherol, is needed in PUFA-rich membranes, most likely to protect them from peroxidation. Our findings also indicate that DHA is the predominant PUFA in brain membranes despite PUFAn-6 concentrations being greater than that of PUFAn-3s.

Mitochondrial Lutein, α-tocopherol, and PUFA Oxidation

In order to better understand how lutein may be associated with cellular oxidative stress, the relationship between membrane-specific concentrations of lutein and PUFA oxidation was investigated. Results from this correlation analysis suggest that lutein present in the mitochondrial membrane may play a more prominent role in inhibiting fatty acid oxidation compared to lutein in the other membranes. The observation that lutein is more strongly associated with DHA oxidation as opposed to AA oxidation was not a function

of concentration since isoprostane content was greater than neuroprostane content in all brain regions. Therefore, this finding lends support to the hypothesis that there may be a specific interaction between lutein and DHA.

In order to assess how lutein's relationship to PUFA oxidation compares to an antioxidant found in much higher concentrations in the brain, the association between total region and membrane-specific concentrations of α-tocopherol and NPs and IsoPs were also analyzed. The results showing that total region α-tocopherol concentrations, not membrane-specific concentrations, were inversely related to PUFA oxidation suggests that α-tocopherol may function more globally as an antioxidant compared to lutein. This is consistent with what is known about the function of α-tocopherol in the brain (53).

The strongest correlations with NP and IsoP for both lutein and α-tocopherol were in PFC. Although NP concentrations were similar across brain regions, IsoP content was higher in prefrontal cortex compared to ST and CER, even after adjusting for AA concentrations. This observation was expected given that the PFC is particularly susceptible to stress compared to other regions, ultimately making it more vulnerable to oxidative damage (54–56). Therefore, this may explain the stronger relationship between the two antioxidants and PUFA oxidation in this region compared to the others.

Lutein, Vitamin E, and Oxidative DNA Damage

Despite oxidative DNA damage being greater in PFC than CER, significant associations between lutein and α-tocopherol and 8-OHdG were only observed in CER. In this region, nuclear membrane α-tocopherol concentrations were inversely associated with DNA damage. This result lends support to the hypothesis that antioxidants accumulating in the

nuclear membrane may have a specific role in protecting DNA from damage since this organelle is the site of transcription in the cell. The strongly positive relationship observed between membrane lutein and DNA damage in this region may be partially explained by nuclear α-tocopherol concentrations. The stronger the inverse association between nuclear α-tocopherol and membrane lutein, the stronger the membrane lutein association was with DNA damage. The reason for the relationship between nuclear α-tocopherol and membrane lutein deposition is unclear; therefore, investigations into whether α-tocopherol and lutein influence each other's subcellular deposition are warranted.

Nuclear α-tocopherol was also positively associated with DHA, total PUFAn-3 and PUFAn-6 in the nuclear membrane. Therefore, it is possible that α-tocopherol may be partially responsible for the inverse association observed between nuclear PUFAs and DNA damage in CER. It is possible that α-tocopherol in this membrane may protect both fatty acids and DNA from damage.

Modulation of ERK Activation by PUFAs

Significant correlations between membrane molecules and ERK activation were observed only in neuronal plasma membranes. This result is consistent with evidence that this membrane is most critical for signal transduction (20). Trends were also observed in the nuclear membrane, which interacts with secondary messengers that influence gene expression, thus is also important for cell signaling (57). The finding that PUFA concentrations in neuronal plasma membrane, particularly PUFAn-6 and AA, were related to phosphorylation of ERK is supported by previous *in vitro* and *in vivo* studies that have shown membrane PUFAs have neuroprotective effects in the brain by

influencing cell signaling cascades related to cell survival (58–60). ERK activation in ST is thought to be important for synaptic plasticity and excitability and is one of the mechanisms underlying instrumental learning and performance (61). Given that neuronal plasma membrane PUFAs were found in concentrations approximately 40,000 times greater than membrane lutein in this region, it follows that these fatty acids would have a more significant impact on membrane composition and fluidity, thus have a potentially stronger influence on signaling molecules and membrane receptors in the brain. However, this study only investigated ERK activation in brain tissue. This particular protein was chosen because of evidence from retina and brain studies showing the effect of lutein on its expression (6–8). However, these experiments were done in rodents, not primates. Therefore, it is possible lutein functions differently in these animals. Doses of lutein in these rodent studies were also much greater (either lutein injections or 0.1% wt/wt lutein in diet) than amounts fed to monkeys in this study. Additionally, there are numerous signaling molecules that influence neuronal cell survival. Therefore, lutein may be related to the activation/inhibition of other pathways that we did not measure. Further studies investigating the role of lutein in signal transduction are needed.

A limitation of this study is the cross-sectional design. No conclusions can be made on how lutein affects cell viability or how each compound (lutein, α-tocopherol, PUFAs) affects one another in the brain. Another limitation is the small sample size which, given the number of correlation trends that did not reach statistical significane in our results, may not have been large enough to assess associations between membrane compounds and cell viability. The small sample size also prevented us from performing multivariate regression analysis that would have provided additional information on potential

interactions between lutein, α-tocopherol, and DHA as predictors of cell viability. Replication of this study with a larger sample size is needed to confirm the results. The final limitation is that NP and IsoP concentrations were determined in aliquots of whole tissue, rather than for each membrane. Currently, it is not feasible to determine membrane-specific NP and IsoP concentrations due to limitations in methodology (sample amount and instrument sensitivity).

To our knowledge, this is the first study to describe the relationship between subcellular deposition of lutein and markers of oxidative stress and cell survival signaling in the primate brain. While this study cannot provide evidence for a causative effect of lutein on brain cell health, these results do shed light on potential membrane-associated cellular processes lutein may be related to. These findings also provide insight into its associations with both DHA and α-tocopherol with regard to maintaining cell viability. Our findings indicate that the beneficial role of lutein in the brain may be more dependent on its localization in brain cells than the total amount present in the tissue. Future work investigating the relationship between lutein and DHA in non-mitochondrial membranes to understand their relationship is warranted. Additionally, further investigation into the effect of subcellular deposition of vitamin E on the deposition of lutein or vice versa may provide information on whether the two interact with one another at the subcellular level.

Acknowledgement

Emily E. Johnson and the ONPRC Veterinary Pathology Service assisted with brain tissue collection. Jean Gallucio, Audrey Goldbaum, and Kathryn Baldyga, from the Cardiovascular Nutrition Laboratory at Tufts University, performed brain fatty acid, and protein analysis. Natalia A Crivello, PhD provided guidance on optimization of the brain membrane isolation method. Greg Dolnikowski, PhD provided instruction and assistance on use of the GC/MS and UPLC-MS/MS systems for NP/IsoP and 8-OHdG assays, respectively.

Author Contributions

Conceived and designed experiments: ESM, EJJ, JWE Jr, MN, MK, OC. Performed the experiments: ESM, DFB. Analyzed data: ESM. Contributed materials/tools: EJJ, MN, NM, DFB, OC. Intellectual contribution to manuscript: ESM, EJJ, JWE Jr., MN, MK, NM, OC.

Table 1. Mean (±SEM) lutein (ng/mg protein), α-tocopherol, and PUFA concentrations (μg/mg protein) in different regions of rhesus macaque brain (n=11)

	Prefrontal Cortex	Cerebellum	Striatum
Lutein	$2.86 \pm 0.90^{a,b}$	1.07 ± 0.34^{b}	3.66 ± 0.99^{a}
α-tocopherol	2.97 ± 0.40^{a}	1.73 ± 0.21^{b}	3.19 ± 0.27^{a}
DHA	49.3 ± 1.5^{a}	47.6 ± 5.0^{a}	45.7 ± 1.3^{a}
PUFAn3	53.2 ± 1.3^{a}	$51.7 \pm 5.4^{a,b}$	49.1 ± 1.2^{b}
AA	33.0 ± 0.5^{a}	30.4 ± 3.2^{b}	37.2 ± 0.5^{c}
PUFAn6	$72.8 \pm 1.8^{a,b}$	67.4 ± 7.2^{a}	77.7 ± 1.9^{b}

Means not sharing the same superscript are significantly different according to Tukey's HSD test ($P<0.05$). DHA: docosahexaenoic acid; PUFAn3: n-3 polyunsaturated fatty acids; AA: arachidonic acid; PUFAn6: n-6 polyunsaturated fatty acids.

Table 2. Mean ± SEM lutein (ng/mg protein), α-tocopherol, and PUFA concentrations (μg/mg protein) in membranes from different brain regions of rhesus macaque (n=11)

	Nuclear	Myelin	Neuronal	Mitochondrial
	Prefrontal Cortex			
Lutein[#]	3.89[a] (1.12-13.53)	3.51[a] (1.97-6.25)	2.40[a] (1.28-4.50)	2.02[a] (1.00-4.05)
α-tocopherol[#]	2.83[a] (2.29-3.50)	2.15[a,b] (1.77-2.61)	2.22[a,b] (1.85-2.67)	1.83[b] (1.33-2.52)
DHA	150.5 ± 10.3[a]	196.7 ± 20.1[b]	181.7 ± 15.3[a,b]	44.1 ± 11.2[c]
PUFAn3	162.2 ± 12.2[a]	203.9 ± 21.0[b]	189.1 ± 16.0[a,b]	45.3 ± 11.7[c]
AA	120.2 ± 8.5[a]	107.0 ± 9.5[a]	98.5 ± 8.4[a]	35.2 ± 9.1[b]
PUFAn6	283.8 ± 21.8[a]	221.8 ± 21.3[b]	210.6 ± 18.8[b]	64.7 ± 18.7[c]
	Cerebellum			
Lutein[#]	1.98[a] (0.78-5.02)	1.62[a] (0.84-3.14)	1.59[a] (0.64-3.92)	1.46[a] (0.66-3.23)
α-tocopherol[#]	2.85[a] (2.19-3.70)	1.96[a,b] (1.66-2.32)	1.58[b] (1.36-1.83)	1.47[b] (0.92-2.36)
DHA	130.6 ± 8.1[a]	183.2 ± 16.9[b]	200.0 ± 11.3[b]	55.1 ± 27.7[c]
PUFAn3	143.1 ± 9.1[a]	190.9 ± 17.8[b]	208.3 ± 11.5[b]	59.1 ± 30.9[c]
AA	88.7 ± 5.4[a]	88.8 ± 9.5[a]	96.4 ± 5.7[a]	31.7 ± 16.8[b]
PUFAn6	206.9 ± 11.6[a]	173.1 ± 17.0[a]	193.9 ± 11.1[a]	71.5 ± 41.5[b]
	Striatum			
Lutein[#]	6.14[a] (3.18-11.86)	7.27[a] (2.72-19.41)	4.79[a] (2.32-9.90)	4.06[a] (2.20-7.50)
α-tocopherol[#]	5.04[a] (4.09-6.23)	2.78[b] (2.34-3.32)	2.73[b] (2.21-3.37)	2.23[b] (1.92-2.60)
DHA	199.7 ± 11.0[a]	260.7 ± 10.8[b]	203.0 ± 10.2[a]	62.8 ± 24.1[c]
PUFAn3	226.1 ± 12.4[a]	270.9 ± 11.0[b]	213.6 ± 10.5[a]	64.3 ± 24.4[c]
AA	216.1 ± 18.3[a]	159.4 ± 6.2[b]	147.0 ± 7.4[b]	54.9 ± 22.9[c]
PUFAn6	507.9 ± 49.8[a]	321.5 ± 13.3[b]	295.3 ± 13.9[b]	92.4 ± 36.9[c]

[#]Geometric Mean (95% Confidence Interval)

Means not sharing the same superscript are significantly different according to Tukey's HSD test ($P<0.05$). DHA: docosahexaenoic acid; PUFAn3: n-3 polyunsaturated fatty acids; AA: arachidonic acid; PUFAn6: n-6 polyunsaturated fatty acids.

Table 3. Partial correlations between lutein, α-tocopherol and total polyunsaturated fatty acid (PUFA) content in brain regions and membranes of rhesus macaque (n=11)

	Nuclear	Myelin	Neuronal	Mitochondrial
	Prefrontal Cortex			
Lutein	-0.03	**-0.52***	-0.17	0.25
α-tocopherol	**0.76*****	0.32	**0.49***	**0.81*****
	Cerebellum			
Lutein	-0.48	0.02	0.14	-0.39
α-tocopherol	**0.75*****	**0.60***	0.40	**0.86*****
	Striatum			
Lutein	**0.86*****	**0.75*****	-0.06	0.04
α-tocopherol	**0.85*****	**0.76*****	**0.82*****	**0.67****

Partial correlations adjusted for age. Lutein, mitochondrial α-tocopherol and PUFAs were log transformed

***P ≤ 0.01 **P ≤ 0.05, *P ≤ 0.1

Table 4. Partial correlations between lutein and neuroprostanes and isoprostanes in membranes from different brain regions of rhesus macaque (n=11)

	Total	Nuclear	Myelin	Neuronal	Mitochondrial
	Neuroprostanes				
Prefrontal Cortex	**-0.51***	0.01	-0.49	-0.40	**-0.69****
Cerebellum	-0.33	-0.32	-0.26	-0.31	**-0.53***
Striatum	-0.44	-0.25	-0.40	-0.37	**-0.62****
	Isoprostanes				
Prefrontal Cortex	**-0.54***	-0.12	-0.32	**-0.51***	**-0.51***
Cerebellum	-0.20	-0.28	-0.15	-0.10	-0.47
Striatum	-0.01	0.11	-0.08	-0.03	-0.28

Partial correlations adjusted for age. Lutein was log transformed

**P ≤ 0.05, *P ≤ 0.1

Table 5. Partial correlations between α-tocopherol and neuroprostanes and isoprostanes in membranes from different brain regions of rhesus macaque (n=11)

	Total	Nuclear	Myelin	Neuronal	Mitochondrial
	Neuroprostanes				
Prefrontal Cortex	**-0.51***	-0.16	0.04	-0.17	-0.37
Cerebellum	-0.17	0.06	-0.36	-0.17	-0.23
Striatum	**-0.52***	-0.03	-0.23	0.09	-0.32
	Isoprostanes				
Prefrontal Cortex	**-0.64****	-0.49	-0.13	-0.33	0.07
Cerebellum	-0.09	0.03	-0.40	-0.14	-0.48
Striatum	**-0.50***	0.17	-0.14	0.09	-0.34

Partial correlations adjusted for age.

**P ≤ 0.05, *P ≤ 0.1

Table 6. Partial correlations between lutein, α-tocopherol, n-3 polyunsaturated fatty acids (PUFAn-3), n-6 polyunsaturated fatty acids (PUFAn-6), and 8-hydroxy-2-deoxyguanosine (8-OHdG) in subcellular membranes of prefrontal cortex and cerebellum of rhesus macaque (n=11)

	Total	Nuclear	Myelin	Neuronal	Mitochondrial
	Prefrontal Cortex				
Lutein	0.23	-0.22	-0.15	0.24	-0.06
α-tocopherol	-0.11	0.21	0.48	0.48	-0.22
DHA	0.07	0.10	-0.03	-0.01	-0.35
PUFAn3	-0.04	0.13	-0.03	-0.04	-0.35
AA	**-0.51***	-0.05	-0.16	-0.07	-0.31
PUFAn6	-0.49	-0.04	-0.14	-0.13	-0.35
	Cerebellum				
Lutein	**0.56***	0.36	**0.63****	**0.73****	**0.77*****
α-tocopherol	-0.14	**-0.66****	0.42	-0.15	-0.11
DHA	-0.36	**-0.61***	0.17	-0.12	0.26
PUFAn3	-0.39	**-0.61***	0.16	-0.12	0.27
AA	-0.26	-0.43	0.27	-0.08	0.30
PUFAn6	-0.33	**-0.56***	0.20	-0.06	0.30

Partial correlations adjusted for age. Lutein, mitochondrial α-tocopherol and fatty acids, as well as 8OHdG were log transformed

***P ≤ 0.01, **P ≤ 0.05, *P ≤ 0.1

Table 7. Partial correlations between nuclear membrane α-tocopherol and lutein in subcellular membranes of cerebellum of rhesus macaque (n=11)

	Nuclear	Myelin	Neuronal	Mitochondrial
	Prefrontal Cortex			
α-tocopherol	-0.06	-0.26	0.04	0.08
	Cerebellum			
α-tocopherol	**-0.64****	**-0.71****	**-0.74****	**-0.80*****

Partial correlations adjusted for age. Lutein was log transformed

***P ≤ 0.01, **P ≤ 0.05

Table 8. Partial correlations between lutein, α-tocopherol, n-3 polyunsaturated fatty acids (PUFAn-3), n-6 polyunsaturated fatty acids (PUFAn-6) and ERK activation in nuclear and neuronal plasma membranes of different brain regions of rhesus macaque (n=11)

	Total	Nuclear	Neuronal
	Prefrontal Cortex		
Lutein	-0.43	0.45	0.02
α-tocopherol	-0.06	-0.16	0.27
DHA	0.04	-0.09	0.02
PUFAn3	0.04	-0.07	0.01
AA	-0.25	0.02	0.06
PUFAn6	-0.15	-0.03	0.01
	Cerebellum		
Lutein	0.13	-0.12	0.28
α-tocopherol	0.01	-0.02	**0.60***
DHA	**-0.52***	0.16	0.45
PUFAn3	**-0.55***	0.13	0.44
AA	-0.50	0.27	0.39
PUFAn6	**-0.56***	0.18	0.22
	Striatum		
Lutein	**-0.51***	-0.39	-0.30
α-tocopherol	0.08	-0.41	0.37
DHA	0.13	-0.31	**0.55***
PUFAn3	0.08	-0.41	**0.55***
AA	-0.30	**-0.52***	**0.70****
PUFAn6	**-0.70****	**-0.56***	**0.67****

Partial correlations adjusted for age. Lutein was log transformed
**P ≤ 0.05, *P ≤ 0.1

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4.3 Differential Gene Expression Profiles in Rhesus Macaque Brain with High and Low Lutein Content: Exploratory Analysis of the Potential Role of Lutein in Brain Function and Cognition

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Funding source: This work was supported by a grant from Abbott Nutrition through the Center for Nutrition, Learning, and Memory at the University of Illinois, DSM Nutritional Products, and USDA (58-1950-0-014).

Abbreviations used: PFC, prefrontal cortex; CER, cerebellum; ST, striatum; MP, macular pigment; RNA-seq, RNA sequencing; HPLC, high performance liquid chromatography; DHA, docosahexaenoic acid; FPKM, fragments per kilobase of exon per million fragments; IPA, ingenuity pathway analysis; ROS, reactive oxygen species; NAA, N-acetylaspartic acid; 10-formylTHF, 10-formyltetrahydrofolate

Abstract

Lutein, a carotenoid with antioxidant and anti-inflammatory functions, accumulates in the primate retina and brain. Studies have shown that brain levels of lutein are related to better cognitive test scores in humans, and that supplementation with lutein can improve verbal fluency, memory scores, and rates of learning in older women. However, the mechanism by which lutein functions in cognition is unclear. The aim of this study was to determine the relationship between brain lutein concentrations and gene expression profiles in brain regions controlling cognition in adult rhesus macaque. Next generation RNA sequencing was used to identify differentially expressed genes in the prefrontal cortex (PFC), cerebellum (CER), and striatum (ST) of monkeys with high lutein content (n=3) versus low lutein content (n=3). Lutein concentrations were determined in these brain samples using high performance liquid chromatography. Differential gene expression analysis revealed differences in expression of genes related to the immune response, oxidative stress, and lipid metabolism between animals with high and low lutein content across all brain regions. Region-specific differences in gene expression between high and low lutein content were also observed. Down-regulation of pro-inflammatory stress-response and pain signaling pathways, as well as protein ubiquitination, were observed in prefrontal cortices with high lutein content compared to low, but not in the other regions. Conversely, differential expression of genes related to folate uptake and metabolism were observed in the cerebellum only, while genes related to glutamatergic neurotransmission were primarily down-regulated in the striatum. Results from this study provide the first steps towards understanding possible mechanisms underlying the beneficial effect of lutein on cognition.

Introduction

Lutein is a xanthophyll carotenoid found in a variety of colorful fruits and vegetables, as well as dark leafy greens and eggs. Primates cannot synthesize lutein, and therefore its presence in the body is a result of dietary consumption (1,2). Lutein, along with its isomer zeaxanthin, accumulates in the macula of the retina to form macular pigment (MP). As a component of MP, lutein functions to protect the eye from oxidative damage both by filtering harmful blue light and by its antioxidant activity (3,4). In addition to these roles, recent studies indicate lutein may exert protective effects in the eye by reducing the expression of inflammation-related genes (5). As a result of these functions, lutein (and zeaxanthin) have been investigated extensively for their potential role in reducing the risk and progression of age-related macular degeneration (3). More recently, however, it has been discovered that lutein also accumulates in the brain (6,7). Studies have shown that MP, as well as serum and brain lutein concentrations are all associated with higher cognitive function in humans (8–11). Furthermore, supplementation with lutein for four months improved cognitive performance in older women and neural processing speed in young healthy adults (12,13). Collectively, the current evidence indicates that lutein may have an important role in maintaining and improving brain function and cognition. However, its mechanism of action in the brain remains unknown.

The characteristics and functions of every cell that make up an organism are influenced by the genes within them. RNAs transcribed from genes under a certain condition (nutritional manipulation, environment, aging etc.) reflect the current state of a cell and can reveal pathological mechanisms underlying disease (14). A number of microarray studies comparing gene expression profiles in normal, cognitively impaired, and/or

demented brains have determined significantly different gene expression profiles across the three brain types (15). Genes related to synaptic signaling (such as vesicle trafficking and neurotransmitter receptors), energy production, and protein homeostasis are down-regulated in AD brains (16), but are up-regulated in mild cognitive impairment, suggesting both hypo- and hyperexpression of genes involved in synaptic activity and cell metabolism lead to poorer cognition (17). Furthermore, genes related to innate immunity are upregulated with both aging and cognitive impairment (18). Therefore, it appears that the dysregulation of genes alters brain cell function which likely contributes to the pathogenesis of cognitive impairment and dementia.

With the development of powerful sequencing methodologies, it has become easier to assess the genetic variables involved in a pathological state. RNA-sequencing (RNA-seq) uses next generation sequencing to characterize genome-wide RNA sequences and determine their abundance in a given sample with extremely high resolution. Due to its high sensitivity and broad scope, RNA-seq is quickly becoming the preferred method over microarrays for differential gene expression studies (14). Given that lutein has been shown to alter gene expression in the retina to exert protective effects (5), it is possible that it carries out a similar function in the brain to positively influence cognition. Therefore, the objective of this study was to determine the relationship between lutein content and gene expression profiles in rhesus macaque brain using RNA-seq in order to explore potential functions of lutein in the brain and begin to elucidate the mechanism underlying its postulated beneficial effect on cognition. Unlike other animal models, but like humans, rhesus monkeys have been shown to absorb and store lutein in the brain

(19,20). Furthermore, these animals are a well-accepted model for human brain physiology (21), making them an ideal model for this study.

Materials and Methods

Rhesus Macaque Brain and Total RNA Extraction

Post-mortem brain samples were obtained from the Oregon National Primate Research Center (ONPRC) at Oregon Health and Science University. All monkeys analyzed for this study consumed a corn-based chow diet containing lutein (~16 µmol/kg diet) along with seasonal fruits and vegetables throughout their life prior to termination. Brain regions were immediately frozen after dissection and shipped to Tufts University for analysis. Lutein content was previously determined in the prefrontal cortex (PFC), cerebellum (CER), and striatum (ST) of 11 adult rhesus macaque using high performance liquid chromatography (HPLC) (20). Six monkeys were selected for RNA-seq analysis based on the concentration of lutein in each region. Age, sex, brain content of vitamin E (also determined by HPLC) and docosahexaenoic acid (DHA) (determined by gas chromatography) (22) were controlled for when choosing monkeys since these factors may also influence changes in gene expression (**Table 1**).

Total RNA was isolated from PFC, CER, and ST of six rhesus monkeys using the RNeasy lipid tissue mini kit (Qiagen) per manufacturer's instructions. RNA purity and concentrations were determined using a NanoDrop ND-1000. All RNA samples had a 260/280 ratio greater than 2.05. One to 2 µg RNA was aliquoted for RNA-seq analysis. RNA integrity was determined to be satisfactory for sequencing (RIN range = 6-8) using an AATI Fragment Analyzer.

Library Preparation, Next-Generation Sequencing, and Processing of Reads

For library preparation, the TruSeq RNA library preparation kit (Illumina) was used according to the manufacturer's protocol. Single-end 50bp sequencing was performed on the HiSeq 2500, High Output v4 (8 lanes flow cell) system. For this analysis 6 samples were sequenced per lane (total of 3 lanes used). Quality control of the resulting reads was performed using the FastQC tool on the Tufts Galaxy server. **Table 2** shows the average number of reads and the mean quality score (PHRED format) for high and low lutein groups in each brain region.

Mapping of RNA-Seq Reads Using TopHat

Sequence reads were aligned to the Ensembl rhesus monkey reference genome (mmul1) using TopHat for Illumina (version 1.5.0) on the Tufts University Galaxy server. TopHat aligns reads to mammalian-sized genomes using the high-throughput short read aligner Bowtie and then analyzes the mapping results to identify splice junctions between exons (23). Default settings for TopHat were used.

Differential Expression Testing Using Cuffdiff

Using the resulting BAM files (.bam) containing mapped reads from TopHat and a reference *Macaca mulatta* iGenome GTF annotation file, transcripts were assembled and normalized to fragments per kilobase of exon per million fragments (FPKM) expression units to estimate the relative abundance of transcripts. Differential expression of FPKM estimates was determined using Cuffdiff (24). Differential gene expression is expressed as log2 fold change between groups with high versus low brain lutein content. Significant p-values were corrected for multiple testing using the Benjamini-Hochberg (false discovery rate) correction.

Output from Cuffdiff was uploaded to Qiagen Ingenuity Pathway Analysis (IPA) to determine biologically relevant differentially expressed genes and enrichment/suppression of pathways, and to compare and contrast differentially expressed genes across the three brain regions. IPA employs a database of finite functional gene classes, pathways, and biological interactions extracted from the literature, and has been previously used to analyze rhesus macaque data (25). It uses a Fisher's exact test to calculate probabilities of up-/down-regulation of functional classes of genes, pathways, or biological networks. All functional analyses in IPA described in this paper referenced to the human genome as the background. Canonical Pathway Analysis in IPA also uses z-scores to predict activation or inhibition of pathways based on the IPA Knowledge Base.

Results

Gene Expression is altered in Animals with High versus Low Brain Lutein Content

Cuffdiff analysis revealed a total of 406, 212, and 703 statistically significant differentially expressed genes between animals with high and low lutein concentrations in the PFC, CER, and ST, respectively. **Figure 1a-c** shows the distribution of up- and down-regulated genes in each region. For PFC and ST, higher lutein concentration resulted in a marked predominance of suppressed gene expression compared to low lutein concentration, with only 11% and 4% of genes being up-regulated, respectively. In CER, gene expression was also skewed towards down-regulation with higher brain lutein compared to low lutein concentration; however, there was a greater portion of up-regulated genes (38%) in this region compared to the others.

In an effort to put the Cuffdiff results into a biological context, data for each brain region was uploaded to IPA for further investigation. Of the 406, 212, and 703 differentially expressed genes for the PFC, CER, and ST, 317, 143, and 561 genes were successfully mapped to the background reference human genome used in IPA, respectively. A Venn diagram was generated in IPA to determine which differentially expressed genes were shared among the three brain regions. The IPA brain tissue filter was used in an effort to focus only on genes functionally relevant to the brain. **Figure 2** shows that there were 33 differentially expressed genes common in each brain region. Of the 33 genes, 85% were down-regulated in brains with high lutein content versus low lutein content (**Figure 1d**).

Differentially Expressed Genes Common Across the PFC, CER, and ST

Genes that had a log2 fold change less than -0.5 for down-regulated genes and greater than 0.5 for up-regulated genes were included in the subsequent analysis to focus exclusively on the most differentially expressed genes in the brain. This equates to a fold change cutoff of 1.5. Of the 33 genes differentially expressed in all three brain regions, 14 genes fit this cutoff criterion (**Table 3**). A full list of all differentially expressed genes can be found in the supplementary material. One of the most significant and consistently down-regulated genes for all regions was *NDUFA1*, which encodes the mitochondrial respiratory chain complex protein and reactive oxygen species (ROS)-generating NADH dehydrogenase (ubiquinone), with log2 fold changes of -5.1, -5.6, and -4.8 for the PFC, CER, and ST, respectively. Conversely, all three regions showed an up-regulation of *AKR1B10* (aldose reductase) with log2 fold changes of 0.9, 1.4, and 1.4 for the PFC, CER, and ST, respectively. This gene encodes an enzyme that converts glucose to sorbitol in the polyol pathway. It also functions to reduce toxic conjugated unsaturated

aldehydes formed from lipid peroxidation in tissues susceptible to build up of these products, such as the brain (26). In addition to differential expression of oxidation-related genes, three genes related to the pro-inflammatory immune response, *MMP21* (log2 fold change -2.5, -2.2, -3.0), *CD8A* (log2 fold change -1.0, -0.8, -1.5), *HLA-DRB1*(-0.9, -1.1, -1.8) for PFC, CER, and ST, respectively, as well as two genes related to energy metabolism/lipid metabolism, *AHI1* (log2 fold change -0.8, -0.9, -1.2) and *LPIN1* (log2 fold change -0.6, -0.6, -0.9) for PFC, CER, and ST, respectively, were all down-regulated with high versus low lutein content.

The PFC and ST showed more overlap with one another with regard to differentially expressed genes (107 genes) compared to either region with CER (11 genes in common between PFC and CER; 29 genes in common between CER and ST). Twenty-three of the 107, 7 of the 11, and 10 of the 29 genes differentially expressed between the PFC and ST, PFC and CER, and CER and ST, respectively met the log2 fold change cut off criterion (**Table S1-3**). Differentially expressed genes shared between the PFC and ST were all down-regulated in those with high lutein content. Down-regulated genes shared between these two regions include those related to pro-inflammatory immune response and stress response pathways, protein regulation/degradation, mitochondrial stress response/neuroprotection antagonists, lipid metabolism, as well as signal transduction implicated in cell death, proliferation, differentiation, and adhesion. Differentially expressed genes shared between the PFC and CER included those related to vitamin A transport, cell cycle regulation, blood brain barrier transporters and binding proteins. Differentially expressed genes shared between the CER and ST included those involved

in pro-inflammatory immune response, synaptic transmission, and transcriptional regulation, all of which were down-regulated in the brain high lutein content samples.

Differential Gene Expression Specific to PFC

To gain in-depth insight into region-specific gene regulation, IPA core analysis was run on the differentially expressed genes unique to each brain region. IPA species and tissue filters were used in an effort to focus only on genes functionally relevant to the particular brain region being analyzed. For each region the species filter was used to include human and exclude mouse and rat. The tissue filter was used to specifically focus on the brain region being analyzed. The filter used for the PFC was "cerebral cortex". Using this filter, 48 biologically relevant genes were identified. Of these genes, 18 (all of which are down-regulated with high lutein) fit the log2 fold change cutoff criterion (**Table 4**). A full list of all differentially expressed genes can be found in the supplementary material. The two most down-regulated genes in this region were *POMC* (-2.2 log2 fold change, $p=8.29x10^{-4}$) and *TRH* (-2.0 log2 fold change, $p=1.16x10^{-3}$). *POMC* encodes the POMC protein, which can be cleaved to numerous different peptides, and has multiple tissue-specific functions. One of these functions is as a component of inflammation and pain signaling (27). *TRH* encodes thyrotropin releasing hormone. This protein also has a diverse set functions; however, in the cerebral cortex, it plays a role in modulating neuronal resting membrane potential and excitatory postsynaptic potentials (28). A total of 6 genes related to immunity and pain signaling, including *POMC*, were found to be down-regulated with high lutein content (*P2RY12*, *B2M*, *SELPG*, *SPARC*, and *SLIT2*). A particular gene of interest in this functional group is *B2M*, which encodes the protein β2-microglobulin (-0.6 log2 fold change, $p = 4.41x10^{-4}$). β2-microglobulin is elevated with

aging and is thought to negatively regulate cognitive function (29). Seven genes related to neuronal excitability, plasticity, survival, and proliferation (including *TRH*) were also down-regulated with high lutein (*FOS*, *RNDS*, *EGR1*, *PTPRB*, *DUSP6*, and *BHLHE40*).

Two genes related to increased oxidative stress, *TXNIP* and *GPD2*, were also down-regulated (both -0.5 log2 fold change) with high lutein content. *TXNIP* encodes thioredoxin interacting protein, which inhibits the antioxidant activity of thioredoxin, while *GPD2* encodes glycerol-3-phosphate dehydrogenase 2 (mitochondrial), which converts glycerol-3-phosphate to dihydroxyacetone phosphate, and is a known producer of reactive oxygen species (30).

In addition to analyzing individual genes, pathway enrichment/suppression analysis using IPA core analysis was also determined. **Figure 3** shows significantly modulated pathways in PFC. All pathways were suppressed with high lutein content compared to low lutein content. The top 3 differentially regulated pathways were the neuropathic pain signaling pathway (6 of 70 molecules), chemokine signaling (4 of 38 molecules), and protein ubiquitination (9 of 156 molecules). In addition to other immune-related and stress-response pathways, calcium signaling was also suppressed.

Differential Gene Expression in CER

The tissue filter used for differential gene expression analysis in CER was “cerebellum”. Using this filter, 29 biologically relevant genes were identified. Of these genes, 14 fit the log2 fold change cutoff criterion (**Table 5**). A full list of all differentially expressed genes can be found in the supplementary material. Unlike the PFC, the majority of these genes (10 of 14) were up-regulated with high lutein content. The top 3 up-regulated genes in

this region were *SEMA3A* (2.1 log2 fold change, $p = 2.09x10^{-4}$), *NDST4* (2.0 log2 fold change, $p = 1.58x10^{-8}$), and *PRLR* (2.0 log2 fold change, p = 0.0). *SEMA3A* encodes a protein with an immunoglobulin-like domain, a PSI domain, and a Sema domain that is known to inhibit axonal outgrowth and is necessary for normal neuronal pattern development (31). *NDST4* encodes the enzyme N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 4, which is required for heparan sulfate biosynthesis. *PRLR* encodes the prolactin receptor, which is known to be a regulator of the stress response and stimulator of neurogenesis in certain regions of the brain (32), although not much is known about its function in the CER.

Other noteworthy differentially expressed genes with high lutein include *KL* (1.9 log2 fold change, $p = 4.24x10^{-7}$), which encodes the protein klotho, a known antioxidant (33–35), and *PLA2R1* (-0.8 log2 fold change, $p = 3.2x10^{-5}$) , which encodes the phospholipase A2 receptor, which may function to clear phospholipase A2 from the cell, thus inhibiting its action. Phospholipase A2 is known to cleave fatty acids, such as the anti-inflammatory docosahexaenoic acid and pro-inflammatory arachidonic acid, from the sn2 position of membrane glycerophospholipids (36). Additionally, *FOLR1*, which encodes folate receptor 1, was found to be upregulated with high lutein content (1.8 log2 fold change, $p = 2.6x10^{-4}$), while *ALDH1L2*, which encodes aldehyde dehydrogenase 1, and is responsible for converting 10-formyltetrahydrofolate to tetrahydrofolate in the mitochondria (37), was down-regulated with high lutein (-0.6 log2 fold change, $p = 2.37x10^{-5}$). Differential expression of genes related to neurotransmission (*HTR2C* and *SLC1A1*) was also observed in this brain region.

Pathway enrichment/suppression analysis was also performed for the CER using IPA core analysis. However, no pathways were observed to be significantly activated or suppressed for this brain region.

Differential Gene Expression in ST

The tissue filter used for differential gene expression analysis in ST was "striatum". Using this filter, 45 biologically relevant genes were identified, all of which satisfied the log2 fold change cutoff criterion. Similarly to PFC, but in contrast to CER, all of these genes were down-regulated with high lutein content (**Table 6**). The top 3 down-regulated genes in this region were *CRHR2* (-6.1 log2 fold change, $p = 9.81x10^{-3}$), *HRH1* (-2.1 log2 fold change, $p = 3.85x10^{-4}$), and *NEUROD1* (-2.4 log 2 fold change, $p = 1.32x10^{-3}$). *CRHR2* encodes corticotropin releasing hormone receptor 2, which binds corticotropin releasing hormone and produces a pro-inflammatory response under stress (38). *HRH1* encodes histamine receptor H1, a potentiator of the pro-inflammatory allergic response (39,40). In addition to *CRHR2* and *HRH1*, 5 other pro-inflammatory immune response genes were down-regulated with high lutein in the S (*CXCL12*, *SHC3*, *PSRX7*, *TCF4*, and *ITSN2*). The second most down-regulated gene, *NEUROD1*, encodes neuronal differentiation 1 protein, a transcription factor required for neuronal differentiation, survival, and formation of connections (41,42). A number of other genes related to regulation of cell survival/apoptosis and MAP kinase signaling were down-regulated as well (*MEF2C*, *IGF2R*, *RGS5*, *TGFBR1*, and *HDAC7*).

Among other noteworthy down-regulated genes with high lutein content were 9 genes related to neurotransmission, including four involved in glutamate transmission -- *GRM2* (-1.5 log2 fold change, $p = 9.89x10^{-7}$) *GRM5* (-0.6 log2 fold change, $p = 1.85x10^{-3}$),

GRIN3A (-1.4 log2 fold change, $p = 2.26x10^{-6}$), and *SLC1A2* (-0.5 log2 fold change, $p = 6.92x10^{-4}$) -- and two involved in serotonin transmission, *HTR2A* (-1.5 log2 fold change, $p = 2.00x10^{-10}$) and *HTR4* (-0.6 log2 fold change, $p = 1.85x10^{-3}$). *GABRB3*, which encodes gamma-aminobutyric acid (GABA) A receptor, was also down-regulated (-0.5 log2 fold change, $p = 1.65x10^{-3}$) along with *CHRNA7*, which encodes a neuronal cholinergic receptor (-0.8 log2 fold change, $p = 4.66x10^{-5}$), and *STX1A*, which encodes syntaxin 1a, a protein which plays a role in docking synaptic vesicles to presynaptic membranes (-1.3 log2 fold change, $p = 3.42x10^{-5}$). Four genes involved in calcium signaling (*ADCY1*, *PDE4D*, *PTY-2B*, and *CAMK2D*), also an important component of neurotransmission, were similarly down-regulated with high lutein.

Finally, two other down-regulated genes in this region were *RTN4R* (-1.1 log2 fold change, $p = 1.46x10^{-3}$) and *LINGO1* (-1.0 log2 fold change, $p = 4.34x10^{-5}$). *RTN4R* encodes reticulon 4 receptor, which mediates inhibition of axonal regrowth (43) and *LINGO1* encodes a leucine-rich repeat and Ig domain-containing, NOGO receptor interacting protein, which inhibits oligodendrocyte differentiation and myelination, as well as neuronal survival and axonal regeneration (44).

Pathway enrichment/suppression analysis was also performed for the ST using IPA core analysis. However, similarly to CER, no pathways were observed to be significantly modulated in this region.

Discussion

Concentrations of lutein in serum and brain, as well as macular pigment density, are positively related to higher cognitive function (8–11). However, the mechanism

underlying this beneficial relationship remains unknown. Microarray studies have shown that gene expression profiles are altered with cognitive status (17), suggesting that regulation of genes influences brain cell function, likely contributing to the pathogenesis of cognitive impairment. Therefore, we sought to determine whether gene expression profiles would differ between brain samples with high versus low lutein content in the PFC, CER, and ST of rhesus monkeys fed a corn-based chow diet with fruits and vegetables throughout their lifespan. Six monkeys were chosen (out of 11) for this analysis based off of their lutein concentrations in the brain. To the best of our ability, we controlled for age, sex, and brain tissue content of DHA and α-tocopherol (by choosing monkeys that had similar concentrations of these two nutrients between the high and low lutein groups) since these factors may also influence changes in gene expression. Results from this exploratory study indicate that gene expression profiles differ between the two groups in each brain region. Differential gene expression analysis for each region indicates lutein may have common functions across brain regions, while also potentially possessing unique, region-specific functions.

Immune Function

Across all three brain regions, those with high lutein content showed down-regulation of genes involved in immunity compared to those with low lutein content. All regions showed down-regulation of *MMP21*, a gene required for lymphocyte survival and encoding a protein secreted by microglia; *CD8A*, a gene encoding a co-receptor on T-lymphocytes; and the *HLA-DRB1* gene, which encodes a membrane-anchored protein on antigen presenting cells (45). Furthermore, PFC and ST both showed down-regulation of genes related to early phase T-cell activation, microglial activation and action,

macrophage migration, and increased inflammation from pain response (*CD4*, *CD74*, *TMEM100*, and *HLA-F)* (45). Down-regulation of genes involved in anchoring proteins on antigen-presenting cells and macrophage cell-cell interactions were also observed in both CER and ST (*HLA-DPA1* and *SIGLEC1*) (45). Furthermore, both the PFC and ST had specific sets of immune-related genes (6 and 7 genes, respectively) that were down-regulated with high lutein. Collectively, the results indicate that lutein concentrations may be related to suppression of the immune response or that there is decreased inflammation in brain tissue with higher lutein concentrations.

These findings are generally consistent with current findings describing a role of lutein in immunomodulation. However, the direction of this modulation (either activation or suppression) is highly varied and depends on the tissue and animal model being studied. In fact, many studies in animals (mice, dogs, cats, birds) show enhanced immune responses with lutein supplementation (46). However, all of these studies focused on systemic immune function, with no current evidence to date on the relationship between lutein and immunity in the brain. Studies on the role of lutein in regulating the immune response in humans is extremely limited, with the research focus primarily being placed on β-carotene instead (47). However, research on age-related macular degeneration (AMD) in humans has provided evidence that the alternative complement pathway underlies the pathogenesis of AMD and this pathway can be suppressed by the xanthophylls (46,48,49).

Although acute inflammation from an immune response is critical for removing pathogens and preserving healthy neurons, chronic activation may cause more harm than good, and is implicated in neuroinflammation and degeneration, as is the case for the

retina and AMD (50). Given the brain's high susceptibility to inflammation and oxidation, regulating and containing the immune response to pathogens before acute inflammation becomes chronic is highly critical. Therefore, the function of lutein in the brain, specifically regarding its role in modulating immune function, may differ from its role in systemic immunity. By aiding in the suppression of the immune response, lutein may play a role in preventing chronic inflammation, thus preventing neuroinflammation and preserving brain cell function. A number of the genes down-regulated with high lutein are associated with neuroinflammation in some way, including *MMP21* (51), *CD4* and *CD74* (52), *SIGLEC* (53), *SELPLG* (54), *CXCL12* (55), and *B2M*, which is one of the more well-characterized as being related to impaired neurogenesis and cognitive function (29,56).

Several immune response and stress response pathways were significantly suppressed with high lutein content compared to low lutein content in the PFC, but not CER and ST. This observation may be unique to the PFC due to the fact that this region is particularly susceptible to stress and neuroinflammation, ultimately making it more vulnerable to dysfunction and impairment than the other regions (57–60). Animal and human studies alike have shown that pathways such as neuropathic pain signaling, corticotropin releasing hormone signaling, and calcium signaling are all elevated during the stress response (61–63). Elevated stress in turn, can lead to an increased inflammation-immune response (chemokine signaling, leukocyte signaling etc.). Suppression of these pathways with higher lutein content provides intriguing clues towards a potential mechanism by which lutein may protect the brain and maintain cognitive function.

Antioxidant Function

Across brain regions, differential expression of genes related to oxidative stress were observed between samples with high and low lutein content. Two genes differentially expressed by all three regions were *NDUFA1* and *AKR1B10* which encode NADH dehydrogenase and aldose reductase, respectively. Down-regulation of NADH dehydrogenase (a member of the electron transport chain and an ROS generator) and up-regulation of aldose-reductase (an enzyme which reduces toxic lipid peroxidation products) with high lutein content indicates lutein may play a role in reducing oxidative stress and damage (26,64). This result may be expected given that lutein is a well-established antioxidant. However, this also provides evidence of an anti-oxidant function of lutein outside its well-characterized role of direct ROS quenching. In the PFC, down-regulation with high lutein content of *TXNIP*, which encodes a thioredoxin inhibitor (45), and *GPD2*, which encodes the mitochondrial enzyme glycerol-3-phosphate (an ROS-generating component of energy metabolism) (30) provide further evidence for this function. Evidence for a protective, antioxidant function was also observed in the CER and ST. *KL*, a gene encoding the anti-oxidant, anti-aging klotho protein, was up-regulated with high lutein content in CER (33,35). In ST, genes related to mitochondrial stress response (*PINK1* and *PPM1K*) (65,66) and antagonists of neuroprotective proteins (*NUDT6* and *VSTM2L)* (67–69) were all down-regulated with high lutein.

Although some of these differentially expressed genes encode general antioxidants or, in the case of suppression, inhibitors of general antioxidants, several of the genes differentially expressed with lutein content are related to mitochondrial energy production (*NDUFA1*, *GPD2*) and mitochondrial health (*TXNIP*, *PINK1*, *PPM1K*).

Similar to the potential role of regulating immune function to limit inflammation, it is possible lutein modulates energy metabolism and mitochondrial integrity during times of stress to limit ROS production and release and thereby limit damage and maintain cell structure and function. Oxidative stress and inflammation are closely linked, and their roles in aging and cognitive impairment are well documented (70–75). Although results from this study cannot determine whether lutein directly regulates both processes, evidence for the differential expression of genes involved in both phenomena may partially explain why lutein has a stronger relationship with cognitive function than other antioxidants found in the brain.

Lipid Metabolism

Consistent with the expression patterns of oxidative stress/antioxidant related genes previously discussed, differential expression of two genes related to oxidative metabolism and lipid metabolism were down-regulated with high lutein content in PFC and ST. *AMACR* encodes a mitochondrial racemase involved in β-oxidation of branched chain fatty acids. Increased mitochondrial β-oxidation can increase oxidative stress, and a recent study discovered that inhibition of this process reduces production of nonenzymatic oxidative polyunsaturated fatty acid metabolites in the brain (76). Similarly, *ASPA* encodes aspartoacylase, and enzyme that cleaves acetate from N-acetylaspartic acid (NAA) for use in myelin synthesis (77). Accumulation of NAA is associated with Canavan disease, but low levels of NAA are associated with compromised neuronal integrity (77). Research has shown that NAA is an important contributor to both acetyl coenzyme A dependent fatty acid synthesis and oxidative phosphorylation (77). Therefore, a delicate balance of NAA (and thus aspartoacylase

expression/activity) is critical for brain health. It is possible lutein may play a role in monitoring or modulating this balance to avoid risk of oxidative stress.

In all three brain regions, *LPIN1* expression was down-regulated with high lutein content. Similarly, *PPAPDC1A* and *CERS4* were down-regulated with high lutein in PFC and ST. Both *LPIN1* and *PPAPDC1A* encode phosphatidate phosphatases that convert phosphatidic acid (PA) to diacylglycerol (DAG), the former in a magnesium (Mg)-dependent manner, the latter in an Mg-independent manner (45,78,79). The regulation of this reaction has critical downstream effects on phospholipid synthesis and signal transduction, both of which influence membrane composition and cell function (78). Similarly, *CERS4*, which encodes ceramide synthase 4, was also down-regulated in PFC and ST. This enzyme is important for the synthesis of ceramide, an important intermediate in the synthesis of sphingolipids, a critical component of cell membranes, and a cell signaling molecule with effects on cell survival (80) as well as age-related inflammation (81). *CERS4* expression levels have been shown to be elevated in AD brains (82).

Collectively, these results indicate that lutein concentrations in the brain may be related to regulation of lipid metabolism at the transcription level. This result is consistent with previous findings that carotenoids modulate lipid membrane physical properties (83) and that lutein, specifically may be related to lipid metabolism in the brain. A previous exploratory metabolomics study in infant brain observed significant correlations between region-specific lutein concentrations and lysophospholipids, important metabolites of phospholipid synthesis (84). Researchers found these correlations to be strongest in the frontal cortex compared to occipital cortex and hippocampus. Interestingly, this study

also found significant correlations between lutein and energy metabolism molecules (84). For example, this study observed that lutein and NADH concentrations correlated with one another, a result that is consistent with the finding from this study that high lutein content is associated with down-regulation of NADH dehydrogenase.

Cerebellum-specific Folate Metabolism

Although many genes, or functional classes of genes, were similarly differentially expressed across brain regions as a function of lutein content, region-specific differential gene expression was also observed. In CER, up-regulation of the gene encoding folate receptor 1, and down-regulation of the gene encoding aldehyde dehydrogenase 1 (mitochondrial homolog of 10-formyltetrahydrofolate dehydrogenase) occurred with high lutein content. These results suggest an increase in uptake of folate and accumulation of 10-formyltetrahydrofolate (10-formylTHF) with high lutein content. 10-formylTHF is required for the formylation of methionine-tRNA, a process essential for translation in the mitochondria (37). Previous research has shown a relationship between folate deficiency and cerebellar degeneration and ataxia (85). Therefore, this region may be particularly sensitive to changes in folate concentration. Monkeys in this study consumed an adequate amount of folate throughout the course of their life (800µg-2mg/day). A potential interaction between lutein and folate content in the brain is a novel finding and warrants additional research to confirm this potential relationship.

Striatum-specific Neurotransmission

Although genes related to synaptic transmission were differentially expressed with lutein content in both CER and ST, differences in expression of glutamate-specific neurotransmission genes was most apparent in ST. Four different glutamate

receptor/transporters (*GRM2*, *GRM5*, *GRIN3A*, and *SLC1A2*) were down-regulated in this region with high lutein content. Recent research has shown that both acute and chronic stress influence glutamatergic transmission in PFC (57). ST is one of the main regions to receive glutamate signals from the PFC (86). This might explain why the relationship between lutein concentration and glutamate receptor expression is only observed in this region. These results indicate lutein may modulate the glutamatergic response to stress in a region-specific manner.

Limitations and Future Work

To our knowledge, this is the first study to explore the relationship between brain lutein concentrations and genome-wide gene expression profiles in different regions of the primate brain. Findings from this study indicate that lutein concentration may be related to regulation of immune function, inflammation, oxidative stress/energy metabolism, and lipid metabolism across brain regions. Lutein may also be related to regulation of folate uptake and metabolism in the CER, and glutamatergic transmission in the ST.

Although strong efforts were made to adjust for as many factors as possible in the experimental design (other nutrients, sex, age, etc.), it is possible that results from this study are driven by other potential factors not accounted for. Post design and analysis, information was provided to our group indicating that the monkeys analyzed in this study were of two different origins. Monkeys in the "high lutein" group were of Indian origin, while the "low lutein" animals were of Chinese origin.

The extent to which different origins influence gene expression is not well characterized. However, it is possible that the effect of origin might account for most or almost all of the

observed gene expression differences. Indian and Chinese-origin monkeys have been shown to differ in terms of behavior (monkeys of Chinese origin exhibit higher levels of aggression and diminished social comnpetence) as well as morphometrically (differences in weight and height). Furthermore, monkeys of Chinese origin were raised outside the ONPRC prior to adulthood. Therefore, their enivornment may contribute to differences in gene expression compared to the Indian-origin monkeys as well.

While the variable of origin confounds the results presented in this chapter, the discovery that monkeys of Indian origin tend to accumulate more lutein in the brain compared to Chinese-origin monkeys, despite consuming the same diet, is an important one. Studies have shown that genetic variations contribute to the inter-individual variability in serum carotenoid status and macular pigment density that is observed in humans (87–90). Therefore, it is important to investigate whether gene expression of these same variants play a role in the differing brain lutein concentrations observed in this study. Gene variants known to effect carotenoid absorption and metabolism in humans include, but are not limited to, those involved in lipid and lipoprotein transport and metabolism (*LPL*, *LIPC*, *NPC1L1*, *ABCG5*, *ABCA1*, *SCARB1*, *ELOVL2*, *FADS2*, and *ALDH3A2*) as well as carotenoid uptake, metabolism, and transport (CD36, *BCMO1*, *BCDO2*, *GSTP1*, and *RPE65*) (87–90). Since the RNAseq analysis described in this chapter provides data on the whole genome of each monkey, we will use this opportunity to investigate differences in expression of genes known to influence carotenoid status in humans between the two sub-species. Future studies validating the results of this analysis with PCR are warranted. Furthermore, assessment of differences in expression of the proteins that are encoded by these genes will be critical towards understanding why lutein concentrations differ with

origin. This future analysis will shed light on the role of differences in gene and protein expression on lutein accumulation in the primate brain.

Acknowledgements

Authors would like to acknowledge Emily E. Johnson and the ONPRC Veterinary Pathology Service for assistance with tissue collection, the Tufts University Genomics Core Facility (TUCF) for performing the cDNA library preparation and RNA sequencing for all samples, and Dr. Albert Tai of the TUCF for providing guidance on cleaning, mapping, and analyzing the raw sequencing data in Galaxy.

Author Contributions

Conceived and designed experiments: ESM EJJ JWE Jr. Performed the experiments: ESM. Analyzed the data: ESM. Contributed materials/tools: MK, MN. Intellectual contribution to manuscript: ESM, EJJ, JWE Jr. MN MK.

Table 1. Rhesus monkey characteristics and brain lutein, vitamin E, and docosahexaenoic acid content (mean ± SD) in brain regions controlling cognition.

	High Lutein (n=3)	Low Lutein (n=3)	P-value
Age, years	13 ± 4	11 ± 1	0.53
Sex, % female	67%	67%	1
Prefrontal Cortex			
Lutein Content, pg/mg wet weight	**34.53 ± 11.75**	**11.80 ± 5.69**	**0.04**
α-tocopherol Content, ng/mg wet weight	26.77 ± 5.95	21.91 ± 10.54	0.53
Docosahexaenoic Acid (DHA) Content, mole %	10.44 ± 0.82	8.54 ± 2.06	0.21
DHA Content, µg/mg protein	52.14 ± 5.86	48.41 ± 1.80	0.35
Cerebellum			
Lutein Content, pg/mg wet weight	**18.83 ± 4.78**	**8.47 ± 3.41**	**0.04**
α-tocopherol Content, ng/mg wet weight	16.87 ± 6.99	27.40 ± 6.49	0.13
DHA Content, mole%	9.26 ± 0.83	10.80 ± 1.01	0.11
DHA Content, µg/mg protein	39.54 ± 13.50	49.61 ± 2.90	0.28
Striatum			
Lutein Content, pg/mg wet weight	**81.59 ± 42.77**	**19.99 ± 7.2**	**0.07**
α-tocopherol Content, ng/mg wet weight	30.98 ± 3.11	28.50 ± 11.65	0.74
DHA Content, mole%	8.33 ± 1.28	8.86 ± 1.12	0.62
DHA Content, µg/mg protein	44.41 ± 4.17	50.50 ± 2.21	0.09

Table 2. Mean number of reads (± SD) and quality score (PHRED format ± SD) for high and low lutein content in each brain region

	Number of Reads	Mean Quality Score
Prefrontal Cortex		
High	41,761,479 ± 3,293,565	34.80 ± 0.00
Low	43,522,584 ± 588,885	34.82 ± 0.02
Cerebellum		
High	47,902,114 ± 4,730,013	34.85 ± 0.01
Low	50,180,395 ± 1,238,260	34.84 ± 0.01
Striatum		
High	33,834,270 ± 3,448,786	34.81 ± 0.02
Low	39,881,422 ± 3,718,910	34.81 ± 0.01

Table 3. Top differentially expressed genes observed in PFC, CER, and ST for high versus low lutein content

Symbol	Description	log2 (Fold Change) PFC	q-value	log2 (Fold Change) CER	q-value	log2 (Fold Change) ST	q-value	Function
NDUFA1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1	-5.1	3.77E-03	-5.6	1.36E-02	-4.8	3.51E-03	ROS generation or antioxidant function
AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	0.9	3.71E-02	1.4	9.33E-03	1.4	4.20E-04	
MMP21	matrix metallopeptidase 21	-2.5	3.38E-02	-2.2	3.08E-03	-3.0	4.91E-03	Pro-inflammatory immune response
CD8A	CD8a molecule	-1.0	7.19E-04	-0.8	3.74E-02	-1.5	2.60E-06	
HLA-DRB1	major histocompatibility complex, class II, DR beta	-0.9	1.17E-03	-1.1	7.19E-04	-1.8	3.93E-11	
DSCC1	DNA replication and sister chromatid cohesion	-4.6	7.85E-04	-6.3	1.97E-04	-4.9	4.01E-03	Cell cycle;S phase and mitosis
CENPJ	centromere protein J	1.9	3.15E-04	1.9	1.47E-07	1.5	3.89E-02	
CENPT	centromere protein T	2.6	4.49E-06	2.6	2.85E-07	2.3	2.04E-03	
PNLDC1	poly(A)-specific ribonuclease (PARN)-like domain containing 1	-2.5	2.52E-03	-2.5	1.26E-03	-2.2	8.99E-03	RNA degradation
METTL13	methyltransferase like 13	-0.6	2.21E-03	-0.6	3.53E-06	-0.9	4.82E-05	Methylation
AHI1	Abelson helper integration site 1	-0.8	4.80E-04	-0.9	6.23E-04	-1.2	1.44E-04	Energy metabolism; lipid metabolism
LPIN1	lipin 1	-0.6	7.62E-03	-0.6	7.24E-03	-0.9	1.24E-03	
SLC7A9	solute carrier family 7 (amino acid transporter light chain), member 9	1.3	2.06E-08	1.1	7.93E-03	1.3	3.94E-12	Blood Brain Barrier transporter
FGD3	FYVE, RhoGEF and PH domain containing 3	3.6	0.00E+0	3.0	0.00E+00	3.0	0.00E+00	cell motility

Differential expression calculated by taking log2 of the fold change (ratio of high to low lutein FPKM values as expressed by Cuffdiff

Table 4. Top differentially expressed genes unique to the PFC with high versus low lutein content

Symbol	Description	FPKM low lutein	FPKM high lutein	log2 (Fold Change)	q-value	Function
POMC	proopiomelanocortin	1.98	0.43	-2.2	3.64E-02	Pro-inflammatory immune response; pain signaling
P2RY12	purinergic receptor P2Y, G-protein coupled, 12	19.25	11.55	-0.7	4.08E-02	
B2M	beta-2-microglobulin	604.24	407.71	-0.6	2.24E-02	
SELPLG	selectin P ligand	12.39	8.49	-0.5	3.54E-02	
SPARC	secreted protein, acidic, cysteine-rich	209.30	143.60	-0.5	1.48E-02	
SLIT2	slit guidance ligand 2	10.47	7.59	-0.5	3.66E-04	
TXNIP	thioredoxin interacting protein	59.97	42.94	-0.5	3.91E-02	Oxidative stress
GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	14.18	10.23	-0.5	1.68E-02	
DNAJA4	DnaJ (Hsp40) homolog, subfamily A	44.60	31.65	-0.5	3.46E-02	Ubiquitination
TRH	thyrotropin-releasing hormone	2.94	0.73	-2.0	4.73E-02	Regulators of neuronal excitability, plasticity, morphology, survival, differentiation
FOS	FBJ murine osteosarcoma viral oncogene homolog	25.28	14.92	-0.8	1.12E-02	
RND2	Rho family GTPase 2	68.71	44.72	-0.6	1.53E-02	
EGR1	early growth response 1	55.99	36.15	-0.6	1.71E-03	
PTPRB	protein tyrosine phosphatase, receptor type, B	5.13	3.56	-0.5	1.19E-02	
DUSP6	dual specificity phosphatase 6	34.23	24.96	-0.5	0.00E+00	
BHLHE40	basic helix-loop-helix family, e40	82.27	59.18	-0.5	2.26E-02	
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1	49.68	35.67	-0.5	4.63E-02	Blood Brain Barrier transporter
BDP1	B double prime 1, subunit of RNA polymerase III transcription initiation	19.54	12.96	-0.6	2.26E-02	Transcriptional regulator

Differential expression calculated by taking log2 of the fold change (ratio high to low lutein FPKM expressed by Cuffdiff)

Table 5. Top differentially expressed genes specific to the CER in high versus low lutein content

Symbol	Description	FPKM low lutein	FPKM high lutein	log2 (Fold Change)	q-value	Function
SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	0.50	2.13	2.1	1.95E-02	Neuronal pattern development
NDST4	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 4	2.46	9.55	2.0	5.63E-06	Heparan sulfate biosynthesis
FHL2	four and a half LIM domain2	1.65	2.87	0.8	3.60E-02	Presenilin-2 signaling.
KL	klotho	1.37	4.99	1.9	1.10E-04	Antioxidant
PRLR	prolactin receptor	4.32	17.34	2.0	0.00E+00	Stress Response; Neurogenesis
IGFBP7	insulin-like growth factor binding protein 7	51.83	98.20	0.9	1.45E-03	Cell adhesion and migration
NOV	nephroblastoma overexpressed	2.51	7.07	1.5	1.40E-02	
GRB7	growth factor receptor-bound protein 7	0.64	1.77	1.5	7.79E-04	
FOLR1	folate receptor 1 (adult)	2.31	8.06	1.8	2.38E-02	Folate uptake; metabolism
ALDH1L2	aldehyde dehydrogenase 1, member 2	3.65	2.41	-0.6	3.12E-03	
HTR2C	5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled	2.74	9.72	1.8	1.48E-08	Neurotransmission
SLC1A1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter), member1	16.63	11.49	-0.5	5.40E-03	
PLA2R1	phospholipase A2 receptor 1, 180kDa	29.65	17.58	-0.8	4.11E-03	Lipid metabolism
POLR1B	polymerase (RNA) I polypeptide B, 128kDa	37.77	27.46	-0.5	2.54E-03	Transcription of rRNA genes

Differential expression calculated by taking log2 of the fold change (ratio of high to low lutein FPKM expressed by Cuffdiff)

Table 6. Top differentially expressed genes specific to the ST in high versus low lutein content

Symbol	Description	FPKM low lutein	FPKM high lutein	log2 (Fold Change)	q-value	Function
CRHR2	corticotropin releasing hormone receptor 2	7.17	0.10	-6.1	2.74E-02	Pro-inflammatory immune response; stress response
HRH1	histamine receptor H1	3.57	0.85	-2.1	1.32E-02	
CXCL12	chemokine (C-X-C motif) ligand 12	9.76	3.03	-1.7	1.86E-02	
SHC3	SHC (Src homology 2 domain containing) transforming protein 3	14.28	7.26	-1.0	4.27E-02	
P2RX7	purinergic receptor P2X, ligand gated ion channel, 7	5.21	3.19	-0.7	6.98E-04	
TCF4	T-cell specific transcription factor 4	30.26	20.73	-0.5	0.00E+0	
ITSN2	intersectin 2	10.95	7.72	-0.5	3.33E-02	
GRM2	glutamate receptor, metabotropic 2	3.35	1.19	-1.5	1.04E-04	Neurotransmission
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	10.02	3.66	-1.5	5.76E-08	
GRIN3A	glutamate receptor, ionotropic, N-methyl-D-aspartate 3A	3.64	1.36	-1.4	1.98E-04	
STX1A	syntaxin 1A (brain)	58.37	24.48	-1.3	1.84E-03	
CHRNA7	cholinergic receptor, nicotinic, alpha 7 (neuronal)	3.11	1.75	-0.8	2.39E-03	
HTR4	5-hydroxytryptamine (serotonin) receptor 4	6.68	4.41	-0.6	4.43E-02	
GRM5	glutamate receptor, metabotropic 5	23.37	15.45	-0.6	2.90E-02	
GABRB3	gamma-aminobutyric acid (GABA) receptor β 3	68.08	47.94	-0.5	4.05E-02	
SLC1A2	solute carrier family 1 (glial high affinity glutamate transporter), member 2	163.61	117.23	-0.5	2.12E-02	
NEDD4L	neural precursor cell expressed, E3 ubiquitin protein ligase	45.55	31.49	-0.5	1.45E-02	Protein Regulation/Degradation
SYVN1	synovial apoptosis inhibitor 1, synoviolin	20.25	14.05	-0.5	8.19E-04	
ADCY1	adenylate cyclase 1 (brain)	25.23	12.52	-1.0	1.24E-04	Calcium Signaling
PDE4D	phosphodiesterase 4D, cAMP-specific	4.10	2.33	-0.8	1.07E-02	

PTK2B	protein tyrosine kinase 2 beta	44.46	27.30	-0.7	4.70E-04	
CAMK2D	calcium/calmodulin-dependent protein kinase II δ	24.42	15.38	-0.7	4.14E-13	
PVRL1	poliovirus receptor-related 1 (herpesvirus entry mediator C)	4.22	1.18	-1.8	1.98E-04	Cell adhesion; migration
PCDH8	protocadherin 8	24.05	12.41	-1.0	3.56E-03	
PODXL	podocalyxin-like	11.77	6.15	-0.9	2.00E-02	
L1CAM	L1 cell adhesion molecule	52.57	34.45	-0.6	7.40E-03	
CDH10	cadherin 10, type 2 (T2-cadherin)	11.31	7.31	-0.6	3.02E-03	
NCAM1	neural cell adhesion molecule 1	157.18	112.04	-0.5	1.87E-04	
NEUROD1	neuronal differentiation 1	1.06	0.20	-2.4	3.45E-02	Regulation of cell death and differentiation
MEF2C	myocyte enhancer factor 2C	29.46	10.10	-1.5	0.00E+00	
IGF2R	insulin-like growth factor 2 receptor	8.82	5.14	-0.8	9.04E-04	
RGS5	regulator of G-protein signaling 5	154.46	93.21	-0.7	3.41E-03	
TGFBR1	transforming growth factor, beta receptor 1	9.52	5.80	-0.7	7.01E-05	
HDAC7	histone deacetylase 7	4.92	3.09	-0.7	8.89E-03	
RTN4R	reticulon 4 receptor	13.88	6.34	-1.1	3.68E-02	Inhibitors of neurite extension, axonal regeneration and myelination
LINGO1	leucine rich repeat and Ig domain containing 1	40.83	20.99	-1.0	2.24E-03	
GDA	guanine deaminase	27.31	10.81	-1.3	1.97E-06	Modulate cytoskeleton: axon guidance, cell shape and motility
PLXNA1	plexin A1	7.11	3.59	-1.0	2.26E-02	
FGF1	fibroblast growth factor 1 (acidic)	155.57	89.97	-0.8	3.66E-02	
SPTBN1	spectrin, beta, non-erythrocytic 1	124.43	85.78	-0.5	1.90E-03	
SDC3	syndecan 3	57.17	40.28	-0.5	2.55E-02	
KALRN	kalirin, RhoGEF kinase	16.05	8.53	-0.9	7.73E-03	Interacts with huntingtin-associated protein 1
PRICKLE1	prickle homolog 1	8.34	4.54	-0.9	4.96E-04	Transcriptional regulator

Differential expression calculated by taking log2 of the fold change (ratio of high to low lutein FPKM values as expressed by Cuffdiff)

Figure 1. Proportion of up-regulated and down-regulated genes in different brain regions

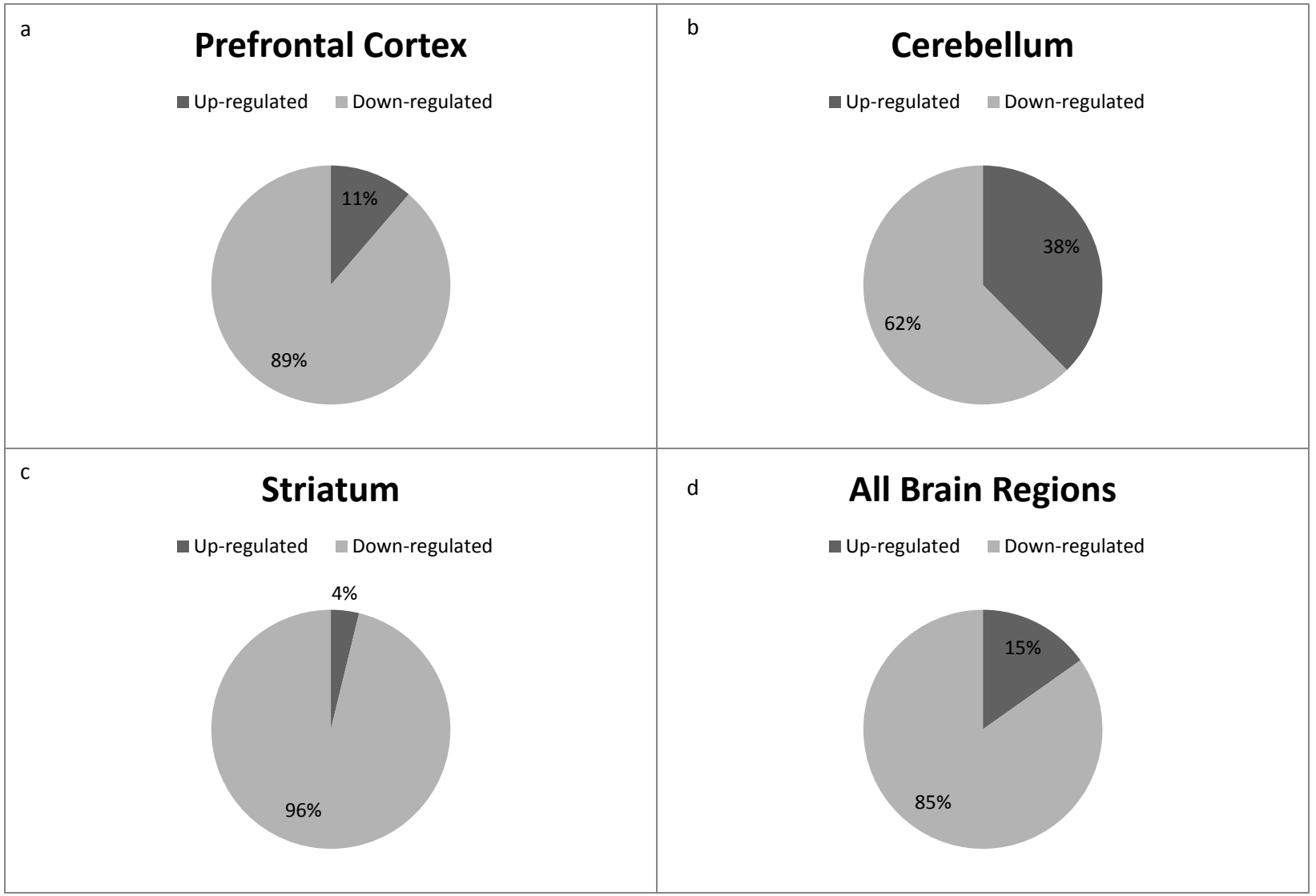


Figure 2. Venn diagram of differentially expressed genes among different brain regions in high versus low brain lutein content

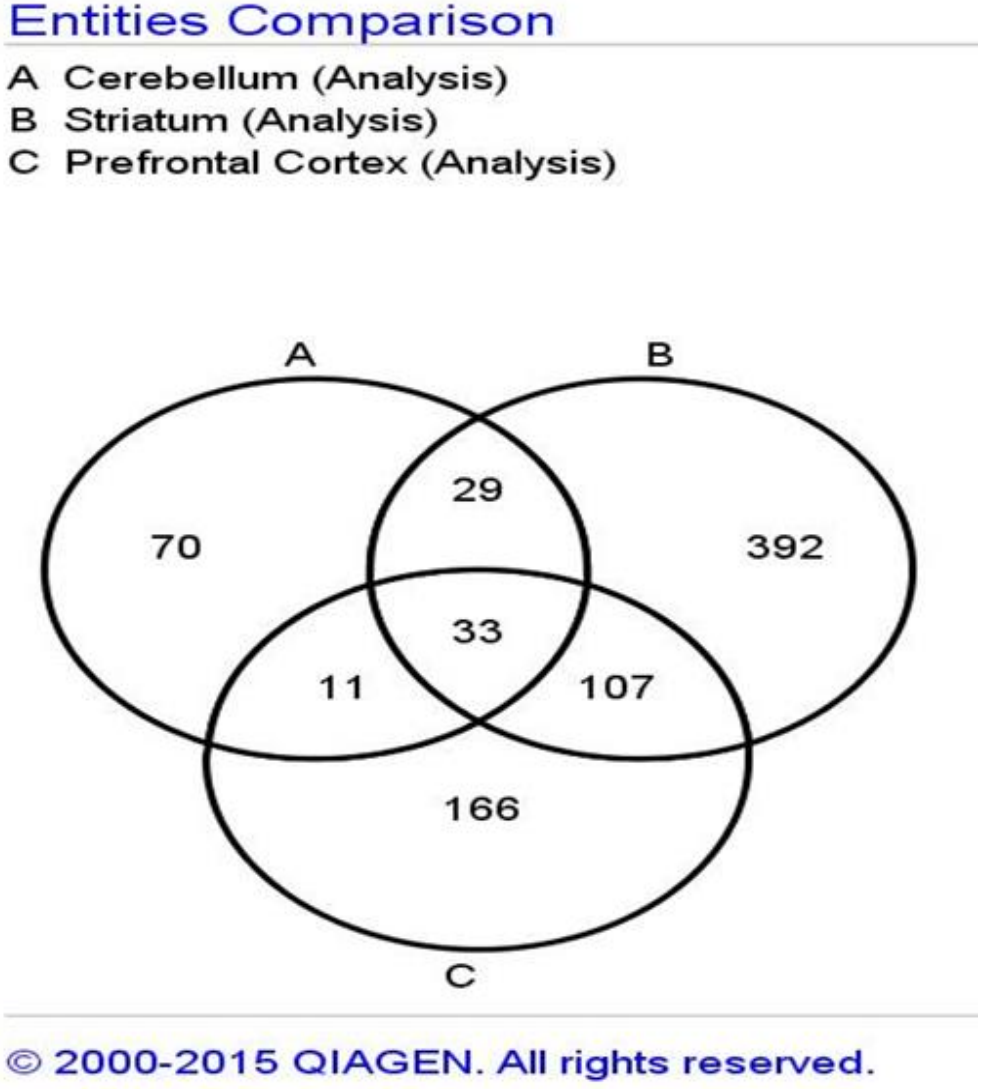
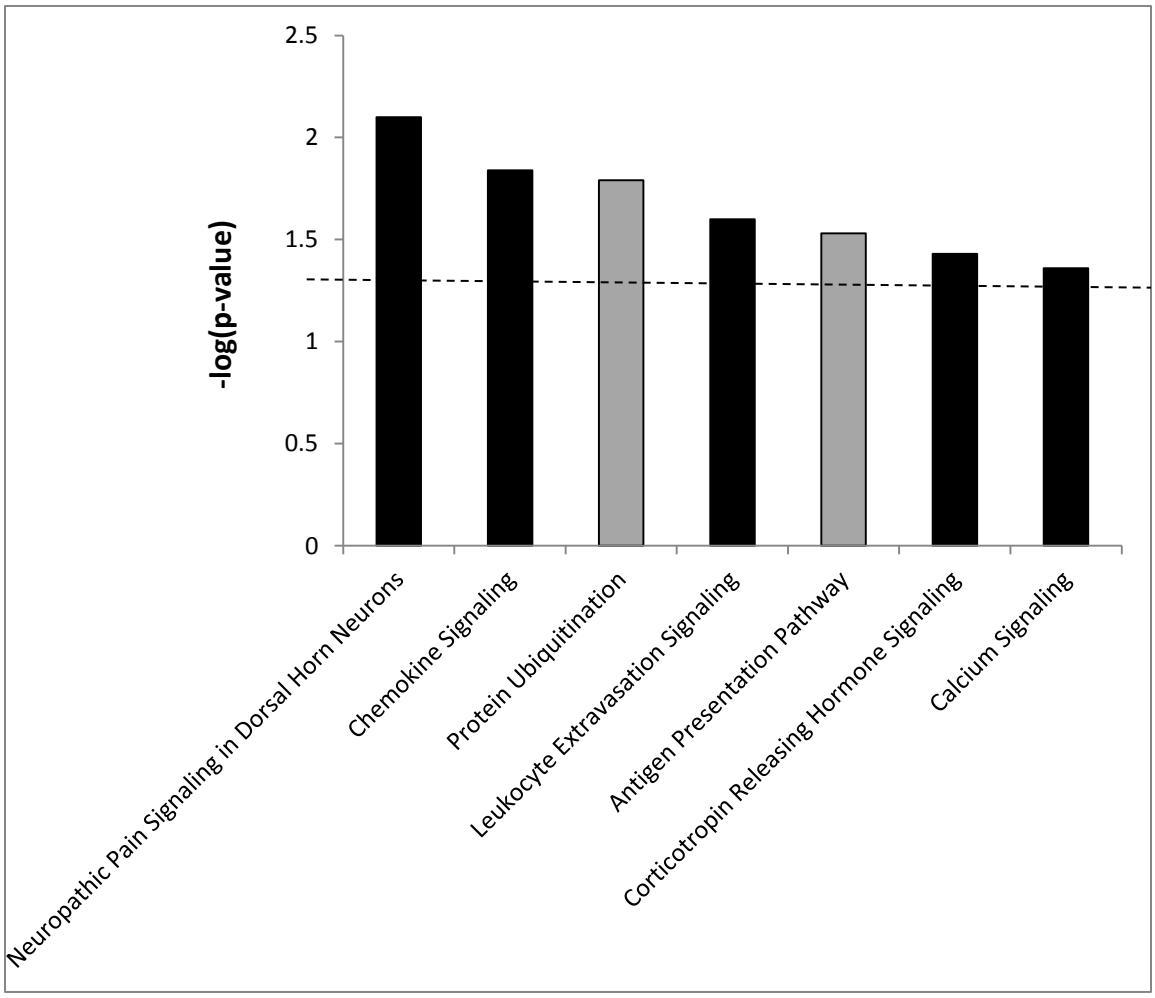


Figure 3. Top differentially regulated canonical pathways in the PFC with high lutein content



Bars in black are significantly down-regulated (z-score ≤ -2.00); bars in grey do not have prediction available

Supplementary Data

Table S1. Top differentially expressed genes observed in PFC and CER in high versus low lutein content

Symbol	Description	log2(Fold Change) PFC	q-value	log2(Fold Change) CER	q-value	Function
OBP2A	odorant binding protein 2A	5.008	3.43E-04	1.80E+254	7.47E-10	Binds toxic odorants
SLC13A4	solute carrier family 13 (sodium/sulfate symporter), member 4	0.958	6.74E-07	1.35	0.00E+00	Blood Brain Barrier transporter
ZNF177	zinc finger protein 177	0.678	3.39E-02	0.97	3.55E-03	Transcription regulation
CDC6	cell division cycle 6	1.541	3.46E-02	2.80	3.87E-04	Cell cycle
CHTF18	chromosome transmission fidelity factor 18	-0.954	2.50E-03	-0.70	4.42E-06	
TTR	transthyretin	1.836	3.46E-04	1.69	0.00E+00	Vitamin A transport
RBP1	retinol binding protein 1, cellular	-0.743	5.99E-03	-1.07	1.83E-03	

Differential gene expression was calculated by taking log2 of the fold change (ratio of high to low lutein FPKM values as expressed by Cuffdiff)

Table S2. Top differentially expressed genes observed in PFC and ST in high versus low lutein content

Symbol	Description	log2 (Fold Change) PFC	q-value	log2 (Fold Change) ST	q-value	Function
CD4	CD4 molecule	-1.9	2.48E-05	-1.8	6.88E-05	Pro-inflammatory immune response; stress response
TMEM100	transmembrane protein 100	-1.4	2.48E-05	-0.9	2.52E-02	
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	-1.1	7.07E-03	-1.3	3.46E-04	
HLA-F	major histocompatibility complex, class I, F	-0.6	4.38E-02	-0.7	2.95E-02	
RNF43	ring finger protein 43	-0.8	7.01E-03	-1.0	4.13E-03	Protein regulation; degradation
USP15	ubiquitin specific peptidase 15	-0.6	2.73E-11	-0.5	3.04E-03	
PINK1	PTEN induced putative kinase 1	-0.7	2.73E-05	-0.9	8.63E-04	Mitochondrial stress response; neurprotection antagonists
NUDT6	nudix (nucleoside diphosphate linked moiety X)-type motif 6	-0.6	4.52E-03	-0.6	6.49E-06	
PPM1K	protein phosphatase, Mg2+/Mn2+ dependent, 1K	-0.5	3.09E-02	-0.8	1.44E-03	
VSTM2L	V-set and transmembrane domain containing 2 like	-0.5	3.82E-03	-0.7	9.79E-03	
ASPA	aspartoacylase	-0.9	7.21E-04	-0.9	4.91E-03	Lipid Metabolism

PPAPDC1A	phosphatidic acid phosphatase type 2 domain containing 1A	-0.8	3.70E-04	-0.7	7.36E-03	
AMACR	alpha-methylacyl-CoA racemase	-0.6	0.00E+00	-0.6	0.00E+00	
CERS4	ceramide synthase 4	-0.6	1.91E-04	-0.7	1.31E-06	
WDR60	WD repeat domain 60	-0.9	1.33E-02	-1.1	1.59E-02	Cell death, proliferation, growth
RAB11FIP1	RAB11 family interacting protein 1 (class I)	-0.9	1.31E-02	-1.3	5.79E-05	
COL24A1	collagen, type XXIV, alpha 1	-0.7	3.36E-03	-1.6	1.43E-03	
DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	-1.3	4.95E-02	-1.5	6.88E-05	
FREM3	FRAS1 related extracellular matrix 3	-0.8	4.28E-02	-2.0	2.93E-02	Multifunctional; role in cell adhesion
CHL1	cell adhesion molecule L1-like	-0.5	6.35E-03	-0.8	1.57E-02	
BEST3	bestrophin 3	-4.3	3.39E-02	-4.6	2.58E-02	Anion channel
NT5DC3	5'-nucleotidase domain containing 3	-1.4	5.73E-03	-2.2	3.05E-03	Hydrolase
OPN3	opsin 3	-0.5	8.18E-13	-0.5	3.67E-02	Vitamin A transport; metabolism

Differential gene expression was calculated by taking log2 of the fold change (ratio of high to low lutein FPKM values as expressed by Cuffdiff)

Table S3. Top differentially expressed genes observed in CER and ST in high versus low lutein content

Symbol	Description	log2 (Fold Change) CER	q-value	log2 (Fold Change) ST	q-value	Function
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	-3.0	0.00E00	-1.9	6.34E-08	Pro-inflammatory immune response
SIGLEC1	sialic acid binding Ig-like lectin 1, sialoadhesin	-1.9	8.92E-10	-1.4	1.00E-03	
SYT13	synaptotagmin XIII	-0.9	8.45E-03	-1.2	7.36E-03	Synaptic transmission
AFAP1L1	actin filament associated protein 1-like 1	-0.9	1.10E-02	-0.8	2.95E-02	
CACNA1F	calcium channel, voltage-dependent, L type, alpha 1F subunit	-0.7	1.11E-02	-0.8	3.57E-02	
TCF19	transcription factor 19	-0.7	7.05E-03	-1.0	1.75E-02	Transcriptional Regulation
ALS2CL	ALS2 C-terminal like	-0.7	0.00E00	-1.7	9.34E-04	
MTMR11	myotubularin related protein 11	-0.5	2.54E-04	-0.9	9.49E-03	
CIART	circadian associated repressor of transcription	-0.7	2.76E-02	-0.7	4.05E-02	circadian clock
TBC1D16	TBC1 domain family, member 16	-0.6	1.57E-04	-0.6	3.35E-02	Rab4A GTPase activator; receptor recycling, EGF receptor signaling

Differential gene expression was calculated by taking log2 of the fold change (ratio of high to low lutein FPKM values as expressed by Cuffdiff)

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5. Summary and Discussion

5.1 Research summary

Lutein, a xanthophyll with antioxidant and anti-inflammatory properties, is selectively deposited into the retina of primates where it serves as a blue light filter, free radical scavenger, and a regulator of neural cell survival (1). Emerging evidence indicates that lutein also preferentially accumulates in the primate brain at different life stages (2–4) and is positively associated with better cognition in the elderly (3,5). Furthermore, supplementation with lutein has been shown to improve cognitive performance in adults (6–8). Lutein accumulates in membranes due to its amphipathic structure (9) and both cross-sectional and intervention studies suggest it may function with the n-3 polyunsaturated fatty acid (PUFAn-3), docosahexaenoic acid (DHA), to influence cognition (3,7). However, the mechanism underlying the beneficial effect of lutein on cognitive function, and its relationship to nutrients important for brain health (ex. DHA and vitamin E), remain unclear. The goal of this thesis was to determine the relationship between lutein and membrane fatty acid composition as well as cell viability in regions of the brain known to control different domains of cognition. The central hypothesis is that lutein content in isolated nuclear, mitochondrial, myelin, and neuronal plasma membranes from different brain regions is related to cell health and viability as measured by markers of oxidative stress, cell signaling, and gene expression.

To meet this objective and test our hypothesis, we first determined not only the regional distribution of lutein in the brain, but also its subcellular distribution among membranes in each region (specific aim 1a). The rationale behind this work was that accumulation of lutein in a particular membrane may be indicative of its function in the cell. By reporting

on the membrane distribution of lutein in brain regions involved in cognition, we provided the first information on how lutein relates to specific cellular processes in brain cells. By also characterizing fatty acid profiles of brain regions and membranes, we were able to elucidate how lutein accumulation was related to fatty acid content in brain regions and membranes. Of particular interest was how membrane concentrations of lutein and DHA were associated (specific aim 2).

Lutein concentrations (ng/mg protein) were highest among all carotenoids in the brain. Specifically lutein was highest in the hippocampus, followed by striatum and prefrontal cortex, with cerebellum having the lowest concentrations. Total PUFA content, driven primarily by PUFAn-6, was also highest in hippocampus, followed by striatum and prefrontal cortex, with cerebellum having the lowest amount. Therefore, lutein appears to accumulate in PUFA-rich brain regions compared to those that are not. Within regions, lutein was detected in all subcellular membranes, accumulated in similar amounts in each, and was highly variable within each membrane. This differs from α-tocopherol (μg/mg protein), which tended to be highest in nuclear membranes and lowest in mitochondrial membranes. Membrane PUFAn-6 concentrations (μg/mg protein) followed a similar distribution pattern to α-tocopherol. Membrane PUFAn-3 concentrations were also lowest in mitochondrial membranes, but tended to be higher in neuronal plasma membrane and myelin. However, the relative amount of PUFAs in membranes (mole percent of total fatty acids) was actually highest in mitochondrial membranes compared to the others.

When associations between lutein/α-tocopherol and membrane PUFA content were evaluated, α-tocopherol and PUFA concentrations were strongly and consistently related

in membranes across all brain regions, while associations between lutein and PUFAs were weaker and only significant in some striatal membranes. Although total PUFA content was not strongly associated with membrane lutein content, lutein and DHA content were related in a membrane-specific manner. Lutein and DHA tended to be inversely associated in prefrontal cortex, cerebellum, and striatum myelin and neuronal plasma membranes, but positively associated in hippocampal mitochondrial membranes. The observation that lutein may have a specific association with DHA was confirmed when results showed that mitochondrial lutein was more strongly and consistently inversely associated with DHA oxidation compared to arachidonic acid (AA) oxidation. Again, this differed from α-tocopherol, which was similarly inversely related with both DHA and AA oxidation, with associations and trends being observed for total α-tocopherol only, not membrane-specific concentrations.

Given that lutein also accumulated in nuclear and neuronal membranes, we investigated whether concentrations of lutein in these membranes were related to cell viability markers. Specifically, we assessed the relationship between lutein and DNA damage, gene expression, and ERK activation (specific aim 1b). Surprisingly, we observed that membrane concentrations of lutein were positively related to DNA damage. However, upon closer inspection, we determined that this relationship may be influenced by nuclear membrane α-tocopherol concentrations, which were strongly inversely associated with both DNA damage and membrane lutein content. While protecting DNA from damage is one potential function of compounds accumulating in the nuclear membrane, another function may be gene regulation. Given evidence that lutein may modulate gene expression of inflammation-related molecules in the eye (10,11), we sought to determine

whether brain concentrations of lutein were related to differences in gene expression in rhesus macaque brain using next generation RNA sequencing. Results from this analysis showed that brains with higher lutein content had lower expression of genes related to the pro-inflammatory immune response and the stress response, as well as energy metabolism and lipid metabolism, but had higher expression of antioxidant-related genes. However, this study was completed using monkeys of two different origins, for which differences in gene expression are not well characterized.

Given the importance of the plasma membrane in signal transduction (12,13), we hypothesized that lutein accumulated in the neuronal plasma membrane may play a role in modulating cell signaling associated with cell viability. Since *in vitro* and *in vivo* studies investigating the function of lutein in the eye demonstrate an effect of lutein on ERK activation (14), this marker was chosen to determine if lutein carried out a similar function in the brain. Lutein concentrations, however, were not related to ERK activation in the brain. PUFAn-6 concentrations in the neuronal plasma membrane, on the other hand, were associated with ERK activation in the striatum.

5.2 Discussion

Collectively, the results from this thesis suggest that lutein may be related to multiple cellular processes involved in dictating cell viability and therefore, may influence cognitive function through several mechanisms. Cognitive impairment is a multi-factorial condition (15–17), therefore, the ability of lutein to influence a number of underlying processes may explain why it is more strongly and consistently related to cognitive function, compared to other antioxidants, like α-tocopherol (3). Given that α-tocopherol functions as a potent antioxidant, it is expected that it would preferentially accumulate in

PUFA-rich membranes. The observation that lutein concentrations were similar across membranes despite fatty acid composition being different, as well as being inconsistently related to membrane PUFA concentrations, suggests that PUFA content may not influence the distribution of lutein into membranes as much as it does for α-tocopherol. Therefore, we can hypothesize that lutein may not function exclusively as a direct scavenger of lipid radicals in the brain. Since α-tocopherol concentrations are so much greater than lutein in membranes, it follows that α-tocopherol would have a more prominent role, and thus a more significant impact on quenching reactive oxygen species than lutein. Results showing the inverse relationship between region concentrations of α-tocopherol and PUFA oxidation, as well as nuclear concentrations of α-tocopherol and oxidative DNA damage, lend supporting evidence to its postulated function as an antioxidant in the brain. However, given that brain lutein concentrations are more consistently related to better cognition than α-tocopherol, it follows that lutein must be functioning through other mechanism(s) to influence brain viability.

Findings from this thesis provide evidence that analyzing total concentrations of lutein in the brain may be too simplistic of an approach to understanding its function. That is, the benefits of lutein may have less to do with the total amount of lutein and more to do with how that total is divided and deposited to different locations in the brain and within cells. Given that subcellular deposition of lutein is not likely driven by PUFA concentrations, it is possible that factors such as membrane polarity, presence of binding proteins, sex, and individual genetics drive its accumulation into membranes.

Although results from this thesis work lend support to the hypothesis that lutein may possess multiple functions, it does not answer what those functions are. However, this

study was designed with the goal of generating hypotheses involving the role of lutein in the brain, which was successfully accomplished. One hypothesis regarding the function of lutein in the brain may be through a membrane-specific interaction with DHA. Our findings suggest that lutein and DHA co-accumulate in mitochondrial membranes but have opposing accumulation in myelin and neuronal plasma membranes. Although it is not clear from this thesis why lutein and DHA accumulate differently in different membranes, there are several possible explanations that warrant further investigation. Mitochondrial lutein may function to protect DHA from damage. Support for this hypothesis was observed when results from our neuroprostanes analysis showed an inverse association between lutein and DHA oxidation in mitochondrial membranes only. This is fitting given that this organelle is particularly susceptible to oxidative damage from free radicals produced from energy metabolism (18,19). The nature of the relationship between lutein and DHA in myelin and neuronal plasma membranes, however, may be different. Inverse accumulation in these membranes may indicate that lutein is related to the release of DHA for subsequent metabolism into its downstream anti-inflammatory mediators, protectins and resolvins (20). Since PUFAn-3s and PUFAn-6s are released and metabolized by the same enzymes, increased release of DHA, which makes up the overwhelming majority of PUFAn-3 in membranes (92-93%), would simultaneously lead to more PUFAn-6 staying in the membrane (21). Unlike PUFAn-3s, PUFAn-6s are metabolized into pro-inflammatory molecules when released from membranes (21). Although testing this goes beyond the scope of this thesis, additional evidence from our analysis showing a positive association between lutein and the

PUFAn-6/n-3 ratio in the same membranes in which lutein and DHA are inversely associated lends some support to this hypothesis.

Results from this thesis also suggest that lutein may play a different role in the brain compared to the eye. Lutein has been shown to accumulate in PUFA-rich membranes of the primate retina (22). Furthermore, this accumulation was driven by PUFAn-3 as opposed to PUFA n-6, with the former being found in relatively higher amounts compared to the latter (22), the opposite of what we observed in rhesus macaque brain. Similarly, both *in vitro* and *in vivo* studies have shown that lutein can modulate expression of ERK activation in the eye (14,23). However, *in vivo* studies were performed in mouse models who were fed lutein in extremely high doses (14), while monkeys analyzed in this thesis were fed lutein in amounts that more closely reflect what is typically consumed in the diet (~1-3 mg/day) (24). Similarly, a study in which lutein was able to enhance neuronal survival through ERK modulation was performed in mice that were injected with lutein (25). Since lutein was injected rather than consumed, it is treated as a foreign substance in the bodies of these animals as opposed to a dietary compound. Therefore, it is possible that the role of lutein in ERK regulation occurs at pharmacological concentrations that are larger than that typically observed in the brain through the diet. Furthermore, mouse eye and brain physiology is different than that of primates (26), thus the function of lutein in these tissues may differ between these models. However, given that lutein does accumulate in neuronal plasma membrane, it is still possible that lutein plays a role in cell signaling by modulating pathways other than MAPK/ERK.

To my knowledge, our RNA sequencing experiment is the first application of next-generation sequencing to study the relationship between lutein content and gene expression in the brain. Genome-wide association studies in human brain have observed differences in gene expression profiles among individuals with differing cognitive status (17,27). These studies lend support to the hypothesis that alterations in gene regulation are one underlying mechanism for the pathogenesis of cognitive impairment and dementia. While our results indicate that lutein may be related to differences in gene expression, it is impossible to interpret these findings at present due to confounding. Replication of this study using animals that were supplemented with lutein versus those on a chow diet (and of the same origin, age, sex, and vitamin E and DHA brain content) would answer whether lutein plays a role in gene regulation. Additionally, given that monkeys of Indian origin tended to have higher levels of lutein in the brain compared to Chinese-origin monkeys despite eating the same diet, future studies using the existing data described in this thesis can be used to determine differences in expression of genes related to carotenoid absorption, transport, and metabolism. Differences observed in the RNAseq data can then be validated using RT-PCR.

In summary, results from this thesis confirm previous findings that lutein consumed in the diet is taken up into brain tissue. Furthermore, our results demonstrate for the first time that lutein is incorporated into membranes, along with vitamin E and DHA which are both important nutrients for brain function, and is related to membrane fatty acid composition and DHA oxidation. Evidence from this thesis also suggests that, although lutein may function as an antioxidant in the brain, it may be related to functions beyond that of a free radical scavenger. Lutein and DHA may also interact in a membrane-

specific manner. The implications of these results regarding cognition are unclear. However, results from this thesis indicate that the mechanism(s) underlying the beneficial effect of lutein on cognition may be dependent on its subcellular localization in brain cells. These findings provide a critical first step towards understanding lutein's function in the brain.

5.3 Limitations and strengths

The first limitation of this thesis is the cross-sectional study design. Therefore, none of the results provide information on the causative effect of lutein on membrane fatty acid composition or brain cell health. Another limitation is the use of protein to express carotenoid and fatty acid membrane data. Protein concentrations varied among different membranes. Therefore, this denominator may have slightly skewed the data when comparing concentrations across membranes. However, it is currently the most feasible option with regard to accuracy of measurement as well as consistency with the literature for this type of study. Another limitation is the small sample size, which prevented us from performing multivariate regression analyses that would have provided additional information on the independent contributions of individual fatty acids to lutein concentrations in membranes, as well as the postulated interactions between subcellular deposition of lutein, α-tocopherol, and PUFAs and their relationship to cell viability. Furthermore, the unequal distribution of males and females prevented us evaluating sex differences regarding lutein and fatty acid region and membrane accumulation. Given the number of trends observed regarding correlations between membrane compounds and cell viability markers, this sample size may not have been sufficient to assess these relationships. However, this exploratory analysis was performed to gain insight into

whether lutein plays a role in cell viability, which was successfully accomplished. Therefore, the results from this study should be replicated with a larger sample size to confirm, and potentially strengthen, findings from this thesis.

While a number of studies have investigated the effect of lutein on cognitive function, this is one of the first to evaluate potential functions of lutein underlying its beneficial effects. One of the primary strengths of this thesis is the novelty of the work that was performed. This study developed and adapted new approaches in an effort to provide clues on the function of lutein in the brain. To my knowledge, this is the first study to measure subcellular concentrations of lutein in the primate brain. Furthermore, while a number of studies have measured neuroprostanes (NPs) and isoprostanes (IsoPs) in plasma and cerebrospinal fluid (28,29), ours is one of only a few to successfully measure NP and IsoP concentrations in primate brain tissue. NP and IsoP provides a high amount of specificity that allows for the evaluation of lutein's relationship specifically with DHA and AA, as opposed to more general markers of lipid peroxidation, such as malondialdehyde. Finally, our study is also the first to use RNA sequencing, the emerging gold standard for genome-wide differential expression analysis, to evaluate the relationship between lutein and gene expression in the brain. The use of non-human primates over rodent models is also a major strength of this thesis work. Since their brain physiology and absorption/deposition of lutein so closely reflect humans (30,31), these results can more easily be related to humans than small animal models can.

5.4 Future work and conclusions

In conclusion, results from this thesis complete the very first steps on a long road towards determining how lutein improves cognition in humans. Our findings relating membrane-

specific lutein levels with membrane fatty acid composition and cell health were able to generate a number of new, more focused hypotheses regarding the influence of lutein on cell processes related to brain viability and its interaction with DHA. Specifically, lutein may possess functions in addition to that of an ROS quencher and the nature of its relationship to DHA may be membrane-specific. Future studies investigating the effect of lutein on membrane DHA accumulation, oxidation, and metabolism are needed to determine the exact mechanism by which to the two compounds interact. Additionally, studies exploring the relationship between FADS gene expression (which differed with origin), carotenoid status, and DHA concentrations in brain tissue may shed light on mechanisms by which lutein potentially influences DHA accumulation and synthesis. Furthermore, future studies looking at the effect of lutein on gene expression profiles in the brain and validating these results with more targeted assays of gene expression are warranted. Future studies looking at the relationship between myelin lutein and myelin thickness and integrity would shed light on the function of lutein in this particular membrane, something we could not perform in this thesis. Replication of our membrane distribution findings in human brain would also confirm that membrane lutein accumulates in a similar manner in humans. Finally, lutein intervention studies in non-human primates where cognitive performance is evaluated, in addition to brain lutein analysis and markers of cell viability, would not only provide insight into the effect of lutein on brain health, but also whether those changes relate to changes in cognitive function. This type of study could also be useful in determining how much lutein is needed to observe changes in both brain health and cognitive performance.

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6. Appendices

6.1 Methods

6.1.1 Isolation of nuclear, myelin, mitochondrial, and neuronal plasma membranes from brain regions of rhesus monkeys using differential centrifugation with a Ficoll density gradient

Objective: To isolate subcellular membranes from brain regions of rhesus monkeys for later determination of carotenoid, fatty acid, and protein content of each membrane (11 monkeys, 4 brain regions; total n=44)

Sample Preparation:

1) Pulverize brain tissue
    a. Take brain sample out of -80°C freezer and place in mortar. Add liquid nitrogen. Stir and crush with pestle until sample is a fine powder. Keep adding liquid nitrogen to keep frozen. Aliquot to pre-weighed tubes and weigh. Record.
2) Store aliquots at -80°C.

Preparation of Buffers:

1) To make 10x Buffer 1
    a. Measure ~0.9 L dH_2O into 2L beaker
    b. Add 23.8 g HEPES to dH_2O and dissolve
    c. Add 1.01 g EDTAdiNa to dH_2O and dissolve
    d. Measure pH using pH meter
    e. Adjust pH to 7.2 using 1M NaOH. Bring volume up to 1L using dH_2O
2) Prepare 1 L Buffer 2
    a. Add 109.536 g sucrose to flask
    b. Take 1x Buffer 1 (diluted from 10x) and add to new flask up to 1L.

3) Prepare Ficoll 14%
    a. Add 70 g Ficoll to flask
    b. Take 1x Buffer 1 and add to new flask up to 500mL.
4) Prepare Ficoll 7%
    a. Add 35 g Ficoll to flask and dissolve
    b. Take 1x Buffer 1 and add to new flask up to 500mL.

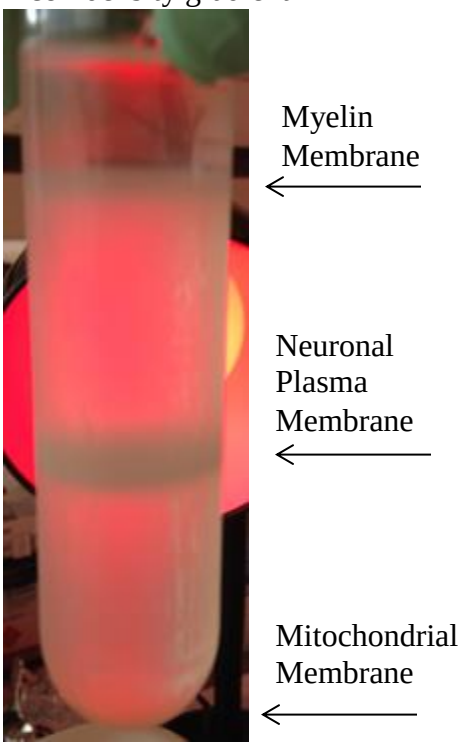
Isolation of Membranes:

1) Add protease inhibitors (Roche cOmplete protease inhibitor cocktail) to Buffers 2 and 1, respectively
    a. Use 1 tablet protease inhibitor/ 50 mL buffer
2) Weigh out 2g of pulverized brain tissue (for prefrontal cortex and cerebellum) or 1-1.5g (for striatum and hippocampus) and add to tubes
    a. Add buffer 2 to weighed out sample such that sample to buffer is in ratio of 1:10 (eg 10 mL of buffer per gram of tissue)
3) Homogenize sample on ice for 30 seconds. Use pipet to wash down sides of homogenizer into tube using Buffer 2
4) Equilibrate tubes using Buffer 2 (Mettler PC 4400 balance)
5) Centrifuge samples (SORVAL rotor SA 600, cod 04) at 1000 x g (3,000 rpm) for 10 min at 4°C.
6) Collect supernatant into new tubes. Place on ice.
    a. When pulling out supernatant, make sure to not disturb pellet. Leave some liquid in tube.

7) Add ~10-15 mL Buffer 2 to pellet. Re-homogenize for 30 seconds. Wash homogenizer between each sample.
8) Repeat steps 4 and 5.
9) Collect supernatant and add to first supernatant in tube from step 6.
    a. When pulling out supernatant with pipet, make sure to not disturb pellet.
10) Keep remaining pellet (crude nuclear membrane) at 4°C
11) Fill supernatant tubes with Buffer 2 and equilibrate using Buffer 2 (PR1203 Mettler Toledo balance)
12) Centrifuge to precipitate crude membrane pellet (CMP; myelin, mitochondria, neuronal plasma membrane) (SORVAL rotor SA 600, cod 04) at 17,000 x g (16,500 rpm) for 30 min at 4°C
13) **During step 12,** begin preparing Ficoll density gradient. Using repeater pipet, pipet 15 mL of 14% Ficoll solution into tube. Pipet 15 ml 7% Ficoll solution into separate beaker.
14) Using skinny tipped 1.5 mL plastic pipet, carefully layer 7% Ficoll on top of 14% Ficoll in tubes. Do this 5-6 times. Use larger skinny tip pipet to layer remaining 7% Ficoll into tube. Keep on ice. *Note: Tilt tube and layer 7% on side of tube*
15) **Once step 12 is complete,** discard resulting supernatant. Add 2 mL of Buffer 1 to CMP. Mix by scraping plastic pipet along bottom of tube to dislodge pellet and transfer to different tube.
16) Add 2 mL Buffer 1 and homogenize at low speed (to avoid foaming) until completely homogenous (no particles should be visible). Rinse homogenizer with 1 mL Buffer 1 and add to sample.

17) Apply sample to gradient using skinny tipped 1.5 mL plastic pipet for first layer. Once a clear layer is on top, using 5.8 mL pipet to apply rest of sample
18) Equilibrate tubes to the 1000th decimal place. Make sure equilibrate tubes in high speed centrifugation rotor tubes.
19) Centrifuge (Beckman SW28) at 23,000 x g (23,000 rpm) for 75 min at 4°C
20) **During step 19,** add Buffer 1 to nuclear pellet from step 10 and homogenize. Then fill tubes with Buffer 1 and equilibrate
21) Centrifuge (SORVAL rotor SA 600, cod 04) at 17,000 x g (16,500 rpm) for 30 min at 4°C.
22) Discard supernatant by dumping out. Put nuclear pellet in refrigerator (4°C)
23) **When step 19 is done**, remove tubes from centrifuge. Collect membrane fractions into MC tubes:
    a. Remove top waste layer
    b. Collect myelin fraction
    c. Remove middle waste layer
    d. Collect neuronal plasma membrane fraction
    e. Dump out remaining supernatant
    f. Collect mitochondria fraction by adding Buffer 1, scraping, and pipetting into new tube

Figure 1. Membrane separation on ficoll density gradient



24) Add Buffer 1 to each tube to fill. Equilibrate tubes using Buffer 1
25) Centrifuge (SORVAL rotor SA 600, cod 04) at 17,000 x g (16,500 rpm) for 30 min at 4°C.
26) **During step 25,** label and weigh empty 1.5 mL eppendorf tubes. Record.
27) **When step 25 is done**, discard supernatant by pulling of liquid with pipet.
28) Add 500 uL Buffer 1 (without protease inhibitors) to each membrane fraction pellet (including nuclear pellet from step 22) and mix. Immediately pour into Eppendorf tube. Add 500 uL buffer 1 (without inhibitors) to tube to wash and add to eppendorf
29) Microcentrifuge to precipitate pellet at maximum speed (16,000 rpm) for 20 minutes at 4°C.
30) Remove supernatant and discard.
31) Weigh eppendorfs with sample to get membrane wet weight.
32) Store samples in -80^{0}C.

6.1.2 Quantification of lutein and α-tocopherol in brain regions and membranes of rhesus monkeys by HPLC

Objective: To determine concentrations of lutein and α-tocopherol in subcellular membranes from different brain regions in rhesus monkeys (11 monkeys, 4 brain regions, 4 membranes per brain region; total n=176)

Sample Preparation:

1) Sample storage of membranes and brain regions at -80°C.

Preparation of Buffers:

1) Prepare the following solutions
    a. 25% sodium ascorbate (make fresh before every run)
    b. 5% NaOH in water
    c. 0.9% saline
    d. 1:1 ethanol: *tert*-methyl butyl ether

Extraction of Carotenoids and Tocopherols from Brain Membranes and Brain Regions:

1) Accurately weigh out samples into 40 mL screw-cap tubes
    a. Nuclear, neuronal, myelin membranes: ~300 mg
    b. Mitochondrial membrane: ~50-100 mg
    c. Brain region: 150-200 mg
2) Add 0.3 mL 0.9% saline to tubes
3) Homogenize samples for 30 seconds or until sample solution looks homogeneous
    a. Rinse homogenizer with solution of 0.3 mL 0.9% saline and 0.5 mL ethanol and add to homogenate
    b. Clean homogenizer with ethanol between each sample
4) To homogenate: add 50 µL internal standard (Echinenone in ethanol) and 2 mL absolute ethanol. When adding ethanol, pipet down sides of tube to wash down any sample.
5) Vortex thoroughly for 2 minutes. Ensure that level of solution does not exceed ¾ length of tube. Scrape samples down sides of tube. If tissue clumps, break apart with spatula.

6) Incubate mixture in 70^{0}C shaking water bath for 2 minutes
7) Add 25% sodium ascorbate (0.5 mL) and 5% NaOH (1 mL) to samples
8) Incubate mixture in 60^{0}C shaking water bath for 20 minutes
9) Add 0.5 mL deionized water to samples and cool for 5 minutes. Then add 5 mL hexane
10) Vortex vigorously for 2 minutes (ensuring level of solution does not exceed ¾ length of tube).
11) Centrifuge at 1000 x *g* for 10 minutes at 4^{0}C.
12) Collect upper hexane layer and evaporate under nitrogen gas in 40^{0}C water bath.
13) Add 5 mL hexane to remaining lower layer.
14) Repeat steps 10-11.
15) Collect upper hexane layer and add to first extract. Dry under nitrogen gas in 40^{0}C water bath.
16) Wash down sides of tube with 0.5 mL hexane to ensure all dried extract is concentrated at bottom of tube.
17) Dry under nitrogen gas in 40^{0}C water bath.
18) To dried extract, add:
    a. For membranes: 75 µL 1:1 EtOH:MTBE solution
    b. For brain tissue: 125 µL EtOH:MTBE solution
19) Vortex for 1 minute at maximum speed
20) Sonicate for 30 seconds

21) Transfer entire extract to HPLC inserts and centrifuge using eppendorf microcentrifuge (Eppendorf 5415D) at 3000 rpm for 2 minutes to remove any precipitate from extract
22) Transfer clear extract to new HPLC inserts. Do not disturb pellet.
23) Inject 25 µL into HPLC

HPLC system and method

1) Instrument: Alliance 2695 Waters
2) Column: Reverse-phase semibore C30 column; YMC carotenoid 3µm, 150 mm x 3.0 mm
3) Mobile phase:
    a. Solvent A: 85% methanol, 12% tert-butyl-methyl ether, 3% water (1.5% ammonium acetate)
    b. Solvent B: 90% tert-butyl-methyl ether, 8% methanol, 2% water (1.0% ammonium acetate)
    c. Solvent Gradient Method (total run time: 48 minutes)

Table 1. Solvent gradient for carotenoid and tocopherol separation

Time (min)	Flow (ml/min)	%A	%B
0	0.40	100	0
21	0.40	45	55
22	0.40	45	55
33	0.40	5	95
37	0.40	5	95
39	0.40	100	0

4) Detector: Photodiode array Waters 2475
5) Carotenoids detected at 445 nm (lutein, zeaxanthin, cryptoxanthin) and 455 nm (β-carotene, α-carotene, lycopene). Tocopherols detected at 292 nm.

6.1.3 Quantification of 8OHdG and 2dG in brain regions of rhesus monkeys by UPLC-MS/MS

Objective: To quantify levels of DNA damage as determined by 8OHdG per 2dG in brain regions of rhesus monkeys (11 monkeys, 2 brain regions; total n=22)

Sample Preparation:

1) Sample storage of brain tissue at -80^{0}C.
2) DNA extracted using the Wako DNA Extractor Tis kit per manufacturer's instructions
3) DNA hydrolysis performed using Wako 8-OHdG Assay Preparation Reagent Set kit per manufacturer's instructions
4) Hydrolyzed DNA extract was filtered through 0.22 µm filters prior to injection in UPLC-MS/MS system

UPLC system and method

1) Instrument: Agilent 1290 Infinity
2) Column: Reverse Phase Zorbax Eclipse Plus C18 column, RRHD; Agilent 1.8 µm, 50 mm x 2.1 mm
3) Mobile phase:
    a. Solvent A: Water with 0.1% formic acid
    b. Solvent B: Acetonitrile with 0.1% formic acid
4) Solvent Method:
    a. Total run time: 3 minutes
    b. Flow rate: 0.35 mL/min
    c. Isocratic method: 95% solvent A; 5% solvent B

MS system

1) Instrument: MDS Analytical Technologies AB SCIEX QTRAP 5500
2) Ion Source: Positive Turbo Spray
3) Selected Mass Filters (Q1 and Q3)
    a. 8OHdG: Q1 284.00 Da; Q3 168.00 Da
    b. $^{15}N_5$-8OHdG: Q1 289.00 Da; Q3 173.00 Da
    c. 2dG: Q1 268.00 Da; Q3 152.00 Da
    d. $^{15}N_5$-2dG: Q1 273.00 Da; Q3 157.00 Da

6.1.4 Quantification of fatty acids and protein in brain regions and membranes of rhesus monkeys by GC

Objective: To determine concentrations of fatty acids, protein, and cholesterol in subcellular membranes in brain regions of rhesus monkeys (11 monkeys, 4 brain regions, 4 membranes per region; total n=176)

Sample Preparation:

1) Sample storage of brain membranes at -80°C.

Preparation of Reagents:

1) Fatty acid internal standard: 2:1 C17:0, Heptadecanoic acid, unmethylated form in hexane. Store at 4°C
2) Folch Solution: Make in a 2:1:0.1 (v:v:w) ratio of chloroform:methanol:BHT.
3) 0.5 N methalonic Sodium Hydroxide (NaOH). Prepare before every run
4) 1:1 methanol/deionized water. Prepare every two weeks
5) 0.88% Potassium Chloride in water. Prepare every two weeks
6) 1 N Sodium Hydroxide Solution in water.

Extraction of Fatty Acids from Brain Membranes and Brain Regions (Note: Asterisk* delineates differences between membrane and region protocols)

1) Add 6 mL Folch solution into "A" tube for each sample and cap.
2) Retrieve brain membranes from -80°C freezer and quick thaw them in the 37°C water bath*.
    a. *Brain tissue removed from -80°C freezer and placed in -20°C. One sample at a time, remove from -20°C and allow sample to soften for cutting purposes (~30 seconds)
3) Add 50 mg of each membrane. Record weight. Cap immediately and store temporarily at -20°C. Place back in -80°C when done with protocol*.
    a. *Open brain tissue cryovial and pick up piece of brain region and place on tared aluminum foil on scale. Record weight (target = 15 mg). Add sample to "A" tubes. Homogenize samples briefly. Clean homogenizer between samples.
4) Flush samples with nitrogen (~10 seconds per sample) and cap tightly.
5) Place tubes on multi-tube vortexer for 10 minutes at level 1. After vortexing, shake tubes to dislodge any sample from top or sides of tube.
6) Leave tubes in 4°C refrigerator overnight.
7) The next day, let samples and fatty acid and cholesterol internal standards sit on bench top for 15-20 min to reach ambient temperature and to let the pellet settle. Do not disrupt pellet.
8) Add 50 µL 2:1 17:0 ISTD and 3 mL Folch solution to "FA-B" tube

9) If necessary, place “A” tubes in beaker with warm water in order for pellet to settle to the bottom.
10) Pipette off supernatant from “A” tubes into corresponding set “AA” tubes. If tissue particles are still floating in supernatant, set tubes in beaker containing warm water and allow to sit until pellet settles completely (~10 minutes). If pellet comes unsettled while pipetting, allow tube to sit in warm water until pellet resettles*.
    a. *For brain regions, if pellet does not look settled, centrifuge at 25°C at 2500 x g for 5 min. If particles are still floating, set tubes in beaker containing warm water and allow pellet to sit until it settles completely ~10 min.
11) Using a 3 mL volumetric glass pipette, add 3 mL of supernatant from each sample set “AA” tubes into the corresponding sample tube from set “FA-B” containing 17:0 ISTD + 3mL Folch solution.
12) Set “A” tubes with pellet aside in the hood uncovered to allow for evaporation (will need them at the end of the fatty acid extraction for protein determination).
13) To “FA-B” tubes, add 1.5 mL of 0.88% KCl with the repeater pipette and cap.
14) Shake vigorously by hand for 30 seconds (solution will appear milky white upon shaking) and let the layers settle for 5 minutes (top layer should now be clear)*.
    a. *For brain regions, if top layer is not clear, allow tubes to sit for an additional 10 minutes. If still not completely separated samples can be placed into 37°C water bath for up to 1 hour to allow layers to completely separate.

15) Pipet the upper layer into a waste tube. Transfer the lower layer into tube "FA-C" avoiding any remaining upper layer. Repeat for all samples.
16) Add 1 mL of methanol: DiH_2O to "FA-C" tubes and cap.
17) Shake "FA-C" tubes vigorously by hand for 30 seconds (solution will be milky white upon shaking) and let the layers settle for 15 minutes (top layer should now be clear)*.
    a. *For brain regions, if separation has still not completed, samples be placed into 37°C water bath for up to 1 hour to allow layers to completely separate.
18) Pipette the upper layer into a waste tube. Using a new pipette, transfer the lower layer into the clean tube "FA-D" avoiding any remaining upper layer. Repeat for all samples.
19) Evaporate the lower layer ("FA-D" tubes) under nitrogen.
20) To the dried down lipid extract in "FA-D", add 2 mL of 0.5 N methanolic NaOH. Cap tightly to avoid evaporation during incubation.
21) Vortex "FA-D" tubes and incubate in a dry heat bath at 90-95°C (do not exceed 100°C) for 15 minutes. (Samples should be pink-tinted after reaction has occurred.)
22) Cool the tubes on ice for 5 minutes.
23) Add 2 mL of 14% BF_3-MeOH into each "FA-D" tube.
24) Flush tubes with nitrogen (~10 seconds per sample) and cap tubes tightly.
25) Vortex samples and incubate in a dry heat bath at 90-95 °C (do not let temperature exceed 100°C) for 1 hour. (Flush BF3-MeOH bottle with nitrogen,

screw cap back onto bottle, and wrap Teflon tape around the cap, and return to 4°C refrigerator.)

26) Cool the tubes on ice for 5 minutes.

27) To the cooled tubes, add 2 mL of hexane.

28) Add 1 mL of DiH_2O to the tubes.

29) Place tubes on multi-tube vortexer for 2 minutes

30) Centrifuge tubes at 1500 x g (2500 rpm) for 5 minutes at 4˚C.

31) Transfer the supernatant containing the FAMEs (fatty acid methyl esters) into tube "E" and dry down under nitrogen (**do not over-dry**) on the N-EVAP. Be careful to avoid any bottom layer. Repeat for all samples.

32) Add 100 µL of hexane to the tubes.

33) Rinse sides of tubes and transfer samples (very carefully) into labeled amber GC vials with inserts*.

    a. *For brain regions, repeat steps 34-35 three times

34) Set GC vials in home-made GC vial holders and dry down GC vials under nitrogen (**Do not over-dry; this should take 1-2 minutes**).

35) Re-suspend each with desired amount of hexane (100 µL).

36) Cap GC vials with aluminum seal caps and crimper, and vortex. Store vials in -20˚C freezer until ready to inject into GC

Protein Determination for Brain Membranes and Brain Regions

1) Dry down pellet in "A" tubes under nitrogen

2) Once dry, add 0.5 mL 1N NaOH solution to each tube, flush with nitrogen, cap tightly, vortex, and allow to sit on bench top at ambient temperature.

3) Use Pierce BCA kit (PI-23227) assay to measure protein concentration (after 5 days for monkey brain membranes, after 8 days for monkey brain regions).

GC system and method

1) Instrument: CLARUS 650 gas chromatograph (Perkin Elmer, Boston MA)
2) Column: 100 m x 0.25 mm i.d. (0.25 µm film thickness) capillary column (SP-2560, Supelco)
3) Carrier gas: helium
    a. Flow rate: 2.5 mL/min
4) Split mode- ratio 14:1
5) Injector temperature 250°C
6) Flame ionization temperature: 260°C
7) GC temperature program:
    a. 80°C held for 16 minutes
    b. Increased to 180°C at 5°C/min and held for 10 minutes
    c. Increased to 192°C at 0.5°C/min and held for 4 minutes
    d. 250°C final temperature reached at 405°C/min and held for 15 minutes

6.1.5 Quantification of total neuroprostanes and isoprostanes in brain regions of rhesus monkeys using HPLC-GC/MS

Objective: To determine neuroprostane and isoprostane concentrations in brain regions of rhesus monkeys (11 monkeys, 3 brain regions; total n=33)

Sample Preparation:

2) Sample storage of brain regions at -80°C.

Preparation of Reagents:

2) Prepare the following solutions:
    1. Folch soln (2:1 v/v anhydrous chloroform: methanol w/0.05% BHT)
    2. 30% KOH in water
    3. Anhydrous hexane + 0.01% BHT
    4. Anhydrous methanol + 0.005% BHT
    5. 10% DIPEA (diisopropylethylamine) (make every 2 weeks)
    6. PFB-Br (pentafluorobenzyl bromide), Stock soln (good for 1.5 months)
        i. Add 5x acetonitrile of the amount of PFBBr. (ex. 1g material, 5mL ACN)
    7. PFB-Br (pentafluorobenzyl bromide), 30% (make every 2 weeks)
    8. BHT, 10 mM in methanol
    9. 1% BHT ethanol

Extraction and Derivitization of Neuroprostanes and Isoprostanes from Brain Tissue

1. Weigh out ~100 mg pulverized brain tissue into 40 ml glass screw-topped centrifuge tube containing 5 ml ice-cold Folch solution.
2. Homogenize tissue for approximately 30 seconds. May need to scrape tissue down the sides if it sticks above solvent level in tube.
3. Wash homogenizer with 5 ml ice-cold Folch solution and add to the homogenate.
4. Allow the mixture to stand sealed under nitrogen at room temperature for 1 hour. Vortex occasionally.
5. Add 2 mL of 0.9% saline to the mixture, cap, and vortex for 2 minutes

6. Centrifuge for 10 minutes (3000 rpm, 1000 x *g*) at 4°C.
7. Discard top layer by glass pipette.
8. Remove organic portion into new screw-topped tube.
9. Dry organic portion under nitrogen gas in 40^0C water bath
10. Add 1 ml methanol (containing 0.005% BHT) and 1 ml of 30% KOH
11. Cap and incubate at 37°C for 30 min in shaking water bath.
12. Add 5 ml hexane (w/ 0.01% BHT) and vortex for 2 minutes
13. Discard upper layer with glass pipette
14. Add another 5 ml hexane (w 0.01% BHT) and vortex for 2 minutes.
15. Discard upper layer
16. Add 2 ml water
17. Adjust pH of solution to 3.0 w/2.5 M & 6 M HCl
18. Add 100 µl of 0.01 µg/ml (1000 pg/ml) of internal std, [$^2H_4$] 15-F_{2t}-IsoP
19. Add 5 ml ethyl acetate and vortex for 1 min
20. Remove top clear portion to new test tube
21. Add the other 5 ml ethyl acetate to the tube in **step 19** and vortex for 1 min
22. Remove top clear portion to the tube in **step 21**
23. Add a little sodium sulfate if any water is visible in sample tube
24. Pour ethyl acetate into the new test tub w/ cap (can store in the refrigerator overnight, add 10 µl of 1% BHT made with ethanol)
25. Dry ethyl acetate under nitrogen (in 40^0C water bath). Once dry, wash down sides of tube with 0.5 mL ethyl acetate to concentrate residue at bottom of tube. Evaporate until dry.

26. Add 20 µl of 10% DIPEA and 40 µl of 30% PFBBr to the residue
27. Cap the tube and incubate at room temperature for 30 minutes
28. Dry off under nitrogen air (no water bath). Blast under nitrogen for 15 min after it looks completely dry.
29. Resuspend residue in 150 µl of anhydrous hexane/isopropanol mixture
30. Remove the solution into microcentrifuge tube and microcentrifuge (15 minutes at speed 14).
31. Inject 100 µl into HPLC. Collect neuroprostanes and isoprostanes from 7-13.5 minutes.
32. Transfer eluent to conical tubes. Add 10 µl of 10 mM BHT (can save at –80°C for a month)
33. Dry under nitrogen (can do in water bath, except for last 15 minutes)
34. Add 20 µl of DIPEA (diisopropylethylamine) and 10 µl of BSTFA (N,O-bis(tri-methylsilyl)trifluoroactamide) to the residue
35. Incubate the mixture under 60°C for 1 hour
36. Dry under nitrogen (no water bath)
37. Dissolve the residue in 30 µl undecane
38. 3 µl sample injected into GC-MS

HPLC system and method

1) Instrument: Agilent 1050 HPLC
2) Column: Amino-bonded normal phase column; Phenomenex 3µm, 150 mm x 4.6 mm
3) Mobile phase:

a. Solvent A: 100% anhydrous hexane

b. Solvent B: 100% anhydrous isopropanol

c. Solvent Gradient Method (total run time: 18 minutes)

Table 2. Solvent gradient for neuroprostane/isoprostane separation

Time (min)	Flow (ml/min)	%A	%B
0	1.25	98	2
10	1.25	74	26
10.5	1.25	70	30
13	1.25	70	30
13.5	1.25	98	2
17	1.25	98	2
17.5	1.25	98	2

4) Fraction Collector: Gilson FC 203B

a. Collect 6.50 min per tube (7-13.5 minutes during run)

GC/MS system and method

8) GC system

a. Instrument: Agilent 7683 Series Injector; Agilent 6890 Series GC system

b. Column: 30 m x 0.25 mm DB-1701 column (J & W Scientific), 0.25 µm film thickness

c. Carrier gas: helium (5-psi column head pressure)

d. Standard injection port, splitless mode

e. Injector temperature: 250°C

f. GC temperature program:

i. 190-280°C at 20°C/min

ii. Column held at 280°C for 20 min

9) MS system

a. Instrument : Agilent 5973 *Network* Mass Selective Detector

b. Ion source: Negative chemical ionization mode

    i. Temperature: 250°C

c. Reagent gas: methane

d. Selected ion monitoring mass-to-charge ratios (*m/z*):

    i. Neuroprostanes: *m/z* 593

    ii. [$^{2}H_{4}$] 15-F_{2t}-IsoP internal standard: *m/z* 573

    iii. Isoprostanes: *m/z* 569

6.2 Abstracts

6.2.1 Differential Expression of Genes Involved in Inflammatory Immune Response and Protein Ubiquitination in the Prefrontal Cortex of Rhesus Macaque with High and Low Lutein Content

Emily S. Mohn, John W. Erdman Jr., Martha Neuringer, Matthew J. Kuchan, Elizabeth J. Johnson

Experimental Biology 2016

Lutein, a carotenoid with antioxidant and anti-inflammatory functions, accumulates in the primate brain. Studies have shown that brain levels of lutein are more consistently related to better cognitive test scores in humans than other antioxidants found in higher concentrations in the brain, such as vitamin E. Supplementation with lutein can improve verbal fluency, memory scores, and rates of learning in older adults. However, the mechanism by which lutein functions in cognition is unclear. The aim of this study was to determine the relationship between lutein concentration and gene expression profiles in rhesus macaque brain. Brain tissue was dissected from monkeys fed a chow diet containing lutein (~16 µmol/kg diet). Next generation RNA sequencing was used to determine differentially expressed genes in the prefrontal cortex of monkeys (11-13y) with high lutein content (mean=34.5 pg/mg wet weight, n=3) versus those with low lutein content (mean=11.8 pg/mg wet weight, n=3). Lutein concentrations were determined in these brain samples using HPLC. A total of 415 genes were differentially expressed between the two groups. Ingenuity Pathway Analysis (IPA) revealed the up-regulation of pro-inflammatory immune response pathways, such as chemokine signaling, antigen presentation, leukocyte extravasation, and neuropathic pain signaling, in brains with low

lutein content. A total of 16 genes were upregulated in this pro-inflammatory network, with specific genes of interest including beta-2-microglobulin and p38 MAPK (1.5 fold change for each), which are both associated with age-related cognitive impairment and neurodegeneration. Additionally, up-regulation of the protein ubiquitination pathway (9 genes), a hallmark of Alzheimer's disease, was observed in brains with low lutein content. These results indicate that lutein may play a role in suppressing neuroinflammation at the transcription level, and provide the first steps towards understanding the mechanisms underlying the beneficial effect of lutein on cognition.

This work was supported by grants from Abbott Nutrition through the Center for Nutrition, Learning, and Memory at the University of Illinois, USDA (58-1950-4-003), and NIH grant P51OD011092 to the Oregon National Primate Research Center

6.2.2 Lutein and DHA Co-localize in Cell Membranes of Brain Regions Controlling Cognition in the Rhesus Macaque

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Experimental Biology 2016

Lutein, a xanthophyll with antioxidant properties, preferentially accumulates in primate brain. Brain concentrations of lutein are associated with higher cognitive test scores and supplementation with lutein improves cognitive performance in older adults. The mechanism underlying this beneficial effect, however, remains unknown. Recent studies suggest the association between brain lutein and cognition may be dependent on brain docosahexaenoic acid (DHA) levels, which are also related to better cognitive function. Furthermore, supplementation with both lutein and DHA has been shown to provide

additional cognitive benefits in older adults compared to supplementation with either alone. Therefore, lutein and DHA may function together to influence cognition. Given that both compounds accumulate in membranes, the objective of this study was to determine the co-localization of lutein and DHA in membranes of brain regions controlling different domains of cognitive function in adult rhesus monkeys (age 7-20 y; n=11). Nuclear, myelin, mitochondrial, and neuronal plasma membranes were isolated from prefrontal cortex, cerebellum and striatum tissue using differential centrifugation with a Ficoll density gradient. Lutein and DHA were measured using high performance liquid chromatography and gas chromatography, respectively. Results document that both lutein and DHA were differentially distributed among nuclear, myelin, mitochondrial, and neuronal plasma membranes, with levels being the lowest in mitochondrial membranes (one-way ANOVA with Tukey HSD, $P<0.05$). Both followed a similar distribution pattern in the prefrontal cortex and striatum, but not cerebellum. In the prefrontal cortex, age adjusted lutein and DHA levels were inversely associated in myelin membranes only ($r = -0.73$, $P<0.03$), while in the striatum, lutein and DHA were positively related in nuclear ($r = 0.77$, $P<0.02$) and mitochondrial membranes ($r = 0.88$, $P<0.004$). Co-localization of lutein and DHA in some membranes and regions, but not others, may be indicative of varied, and specific, functions of lutein in different brain regions. Results from this study provide the first steps toward understanding lutein's mechanism of action in the brain and its relationship to DHA. *This work was supported by grants from Abbott Nutrition through the Center for Nutrition, Learning, and Memory at the University of Illinois, DSM Nutritional Products, and USDA (58-1950-4-003), and NIH grant P51OD011092 to the Oregon National Primate Research Center*

6.2.3 Distribution of Lutein in Membranes of Rhesus Macaque Brain

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Experimental Biology 2015

Lutein, a carotenoid with antioxidant and anti-inflammatory properties, selectively accumulates in primate brain and may be beneficial for cognition. Lutein incorporates into membranes. However, its subcellular distribution in the brain and its interactions with other nutrients important for cognition, like docosahexaenoic acid (DHA), is unknown. Determining lutein's location in the brain may elucidate its function. The study aim was to determine the distribution of lutein in neuronal, myelin, mitochondrial and nuclear membranes in the frontal cortex of adult *rhesus macaque* (n=3) fed a standard chow with fruits and vegetables. Membranes were isolated using differential centrifugation with a Ficoll density gradient. Carotenoids and fatty acids were measured using high performance liquid chromatography and gas chromatography, respectively. Lutein was the only carotenoid to accumulate in all membranes, despite detection of other carotenoids in whole brain, and followed a similar distribution pattern as DHA. Lutein concentration was significantly higher in myelin compared to mitochondrial membrane (14.3 and 5.2 pmol/mg protein, respectively) ($p<0.01$), and neuronal and nuclear membranes (7.3 and 6.7 pmol/mg protein, respectively) ($p<0.05$). These results provide insight into lutein's role in the brain. *This work was supported by a grant from Abbott Nutrition through the Center for Nutrition, Learning, and Memory at the University of Illinois, DSM Nutritional Products, and USDA (58-1950-0-014).*

6.2.4 Avocado Consumption Increases Lutein Status

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Experimental Biology 2015

Lutein, a dietary carotenoid, is selectively taken up into primate neural tissue such as the macula and brain. Lutein levels in serum, macula (macular pigment, MP) and brain are related to better cognition. MP is a biomarker of lutein levels in brain. Avocados are a highly bioavailable source of lutein. This 6 mo randomized, controlled trial tested the effects of daily intake of 1 medium avocado (AV, n=19) vs 1 medium potato or 1 cup of chickpeas (C, n=20) on serum lutein levels, MP density and cognition in healthy older adults (>50 yrs). Serum carotenoids were measured by HPLC. MP density, using heterochromatic flicker photometry, was used as a measure of lutein in neural tissue. A computerized cognitive assessment battery was used for cognition measures (CANTAB). At baseline there were no significant differences between the AV and C in any of the study measures. At 6 mo, serum lutein levels in the AV group significantly increased from baseline by 20% (34.7+11.2 to 41.8+14.3 pmol/dL, $p < 0.0005$) whereas the C group increased by 7% (34.8+17.3 to 37.1+16.3 pmol/dL, $p < 0.03$). At 6 mo there was a significant increase in MP density in the AV group (0.396+0.141 to 0.481+0.123 OD, $p < 0.001$). MP density did not change from baseline in the C group (0.385+0.166 vs 0.424+0.145 OD). In the AV group the change in MP density was significantly related to an improved spatial working memory ($p < 0.009$) and problem approaching efficiency ($p < 0.036$). No significant changes in cognitive function were observed in the C group. These data suggest that an intervention with avocados to increase neural lutein is an

effective dietary strategy for cognitive health. This study was supported by the Hass Avocado Board and USDA 58-1950-0-014

6.2.5 Preferential Accumulation of Lutein, Alpha-tocopherol, and Docosahexaenoic Acid in Subcellular Membranes of Primate Brain.

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New York Academy of Sciences: Developing Brain Conference 2014

Variation in brain membrane composition influences physical properties, thereby affecting specific functions relating to brain function. The objective of this research is to determine the concentrations of fat-soluble nutrients in subcellular membranes (neuronal, myelin, mitochondrial, nuclear) from non-human primate brain regions controlling cognition (striatum, hippocampus, and prefrontal cortex). Isolation of subcellular membranes from the striatum (n=3), hippocampus (n=3), and prefrontal cortex (n=3) from three monkeys used differential centrifugation with a Ficoll density gradient. Protein concentrations were determined using the BCA assay. Carotenoids and tocopherols were extracted from isolated membrane fractions using hexane and were analyzed using high performance liquid chromatography. Fatty acids were extracted using the Folch method and were analyzed using gas chromatography. Compared to matched serum and whole brain analyses and within each of their nutrient classes (carotenoids, tocopherols, fatty acids), lutein, alpha-tocopherol and docosahexaenoic acid (DHA) preferentially accumulated in all subcellular membranes and brain regions. The positive relationships observed in human studies between lutein, alpha-tocopherol and DHA status and cognitive function may be related to preferential accumulation of these

fat-soluble nutrients into subcellular membranes, thereby influencing brain function. Our future studies will relate fat-soluble nutrient concentrations in subcellular membranes and measures of cell health and survival.

6.2.6 Association between Serum and Brain Fatty Acid Profiles in Centenarians.

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Experimental Biology 2014

Profiles of brain fatty acids (FA) may be important indicators of age-related cognitive decline. There is limited data on brain FA profiles in centenarians. Serum FA may be related to brain FA profiles. The goal of this study was to test this hypothesis in centenarians. Tissue from the frontal, occipital, temporal cortices, and cerebellum were collected from decedents (n=40) in the Georgia Centenarian Study with available banked serum. Brain and serum FA were measured using gas chromatography. FA profiles were different between serum and brain ($P<0.0001$). On average, saturated FA (SFA) were predominant in the brain (47 mol%), followed by monounsaturated FA (MUFA) (23 mol%), n-3 polyunsaturated (n-3) FA (14 mol%, with 91% being docosahexaenoic acid), n-6 polyunsaturated (n-6) FA (12 mol%), and *trans* FA (0.7 mol%). N-6 FA were highest in serum (38 mol%), followed by SFA (30 mol%), MUFA (25 mol%), n-3 FA (3 mol %), and *trans* FA (3 mol%). Serum n-3 FA were related to brain levels in the frontal ($r=0.43$, $P<0.01$) and temporal ($r=0.35$, $P<0.05$) cortices and cerebellum ($r=0.41$, $P<0.05$). Serum n-3/n-6 was related to brain n-3/n-6 in all regions (frontal: $r=0.41$, occipital: $r=0.38$,

P<0.05; cerebellum: r=0.47, temporal: r=0.55, P<0.01). No other FAs were related between serum and brain. The data suggest that both n-3 FA and n-3/n-6 FA can be used as markers of brain fatty acid profiles. *Supported by: USDA (58-1950-0-014), NIH (1P01-AG17553), and Abbott Laboratories*

6.2.7 The Relationship of Lutein and DHA in Cognitive Function.

Emily S. Mohn, Rohini Vishwanathan, Alice H. Lichtenstein, Nirupa R. Matthan, Dorothy B Hausman, John L. Woodard, Adam Davey, Mary Ann Johnson, Leonard W. Poon, Robert C. Green, Marla Gearing, Elizabeth J Johnson. The Relationship of Lutein and DHA in Cognitive Function

Experimental Biology 2013

Alzheimer's disease (AD) likely stems from many mechanisms, two of which are oxidative stress and inflammation. Lutein, an antioxidant, and DHA, an anti-inflammatory fatty acid, are preferentially stored in neural tissue and are positively associated with cognition. While DHA decreases neuroinflammation, lutein prevents oxidation of DHA. DHA may also increase lutein in the brain by modulating levels of HDL, a main transporter of lutein. Therefore, the aim of this study was to determine if a synergistic relationship exists between brain levels of lutein and DHA and cognition. Sixteen cognitive tests were completed pre-mortem by subjects from the Georgia Centenarian Study. Brain tissues from 48 decedents were analyzed for carotenoids and fatty acids using HPLC and GC, respectively. Regression analysis revealed a significant, non-synergistic lutein/DHA interaction for 5 cognitive tests, 4 of which are from the Consortium to Establish a Registry for AD test battery. Eight significant interactions were found between the temporal and frontal cortices (P<.05). The results indicate that

the association of lutein or DHA with cognition is dependent on the other; however, their combined effect is less than expected if added together. Mechanistic studies on lutein and DHA are needed to further characterize the relationship between these two nutrients.

Support: NIH 1PO1AG1755, DSM, Abbott Nutrition, USDA (5819500014).