Docosahexaenoic acid and the Brain– what is its role?

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Running title: DHA and the brain

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ABSTRACT

Docosahexaenoic acid is a 22-carbon omega 3 PUFA highly enriched in the neuronal cell membranes and rod outer segment membranes. When DHA is depleted from these cell membranes it is replaced nearly quantitatively by a 22-carbon omega 6 PUFA, docosapentaenoic acid, which has similar, but less potent, biophysical and physiological properties to DHA. It is speculated that omega 6-docosapentaenoic acid is a buffer to prevent the possible catastrophic effects of DHA depletion on brain and visual function. The primary insult from the loss of DHA from cell membrane glycerophospholipids, and replacement by omega 6-docosapentaenoic acid, is on the flexibility/compression of the membrane lipids which affects the optimal function of integral membrane proteins (receptors, voltage-gated ion channels and enzymes). This leads to effects on second messenger systems, and subsequently affects neurotransmitter concentrations due to 'weakened' signals from the initiating receptors. Remembering there are more than 80 billion neurones and many times more synaptic connections between neurons, a very small loss of "efficiency" in signal due to altered properties of membrane proteins would likely result in meaningful changes in brain and visual function. Additionally, impairment of neurotransmission could be due, in part, to sub-optimal brain energy metabolism (glucose entry into the brain), which is significantly reduced in omega 3 deficiency. Many studies report that dietary omega 3 deficiency results in changes in learning, coping with stress, behavioural changes, and responses in visual function. It is thus concluded that DHA is an essential fatty acid for optimal neuronal function.

Key Words: brain, DHA, PUFA, mechanisms, diet

Omega 3 fatty acids in the brain

The brain contains the second highest concentration of lipids in the body, after adipose tissue, with 36-60% of the nervous tissue being lipids.[1] The lipids in the brain are located entirely in the membranes of the billions of cells or in subcellular organelles such as mitochondria. These membrane lipids are polar lipids, and include glycerophospholipids (glycerol-PL) (phospholipids with glycerol backbone), sphingolipids (sphingomyelin and cerebrosides), gangliosides, and cholesterol, with little or no triglycerides and cholesterol esters.[1] This contrasts with other tissues, such as liver and muscle, which are rich in neutral lipids such as triglycerides and cholesterol esters, as well as polar membrane lipids. Interestingly, the cell membrane has a highly asymmetric distribution of lipids contributing to dynamic nanodomains in the membrane.[2] Phosphatidylserine (PS), phosphatidylethanolamine (PE) and

phosphatidylinositol are mostly confined to the inner leaflet of membranes and phosphatidylcholine (PC), sphingomyelin and glycolipids are mostly confined to the outer membrane leaflet. Glycero-PL contain two fatty acids, one of which is generally unsaturated and in the brain, more particularly, a PUFA. Another important class of glycero-PL in the brain are the ethanolamine plasmalogens.[3] These contain one fatty aldehyde (in position-1) and a PUFA in position-2 on the glycerol backbone.

In the mid-1960's, it was reported that there were three major PUFA present in the human brain-docosahexaenoic acid (DHA, C22:6ω3), arachidonic acid (AA, C20:4ω6) and docosatetraenoic acid (C22:4ω6), with negligible concentrations of linoleic acid (LA), alpha-linolenic acid (ALA) and EPA.[4,5] The ω3 or ω6 notation indicates whether the fatty acids belong to the omega 3 or omega 6 class of fatty acids; these omega notations reveal where the first double bond is in the carbon chain counting from the methyl end of the molecule (Figure 1). DHA has 6 cis-double bonds, and is designated as all-cis 4, 7, 10, 13, 16, 19-22:6. DHA is a product of synthesis from the essential fatty acid, ALA (all-cis 9, 12, 15-18:3). ALA cannot be synthesised de novo, so DHA must be synthesised in the liver from ALA consumed in the diet or consumed directly as DHA from the diet. Previously, before gas chromatography had been invented, Klenk and Bongard in 1952[6] were the first to describe the presence of a fatty acid with 22 carbon atoms and six double bonds (written as C22:6) in the human brain, identified by spectrophotometric procedures.

In 1972, it was reported that brain grey matter from more than 30 different mammalian species (from mouse to elephant) all had DHA and AA PUFA profiles similar to those reported for the human brain (Figure 2).[7-9] These workers also analysed muscle and liver fatty acids from this wide variety of species and reported very wide divergence in the proportions of DHA and AA between species in these two tissues, in contrast to the consistent proportions of AA and DHA in the brain.[9] In other words, this suggests that the brain filters the PUFA in the blood so that only selected PUFA such as DHA and AA are "allowed " to accumulate in neural cells, however research from Rapoport's and Bazinet's groups disputes this and suggests that other PUFA (EPA, LA) do enter the brain but are extensively beta-oxidised rather than being incorporated into glycero-PL.[10,11] These data strongly imply that the characteristic PUFA fingerprint of the brain, DHA and AA, play roles of fundamental importance in the brain. Since then, there have been many thousands of papers on DHA and the brain, but we are a still a long way short of knowing what the specific roles of DHA are in relation to "brain function".

The aim of this paper is to describe what is known about the role(s) of DHA and brain function, based on research in vitro and in vivo (mainly animal studies). Arachidonic acid is present in the brain in relatively high proportions, and clearly has important roles in the brain,[12-15] however, this paper will focus on DHA.

The brain

When people write about 'DHA and the brain', the words used are typically very vague, such as 'the brain is rich in DHA', or 'DHA is important for brain function'. These descriptions ignore the fact that "the brain" is an extremely complex structure and that it is more appropriate to refer to specific regions, aspects, attributes and/or functions of the brain.

a. The brain is the most complex organ in the body, comprising many different regions including the cerebrum, corpus callosum, thalamus, hypothalamus, hippocampus, cerebellum, medulla oblongata and the spinal cord, which have specialised functions. For example, the cerebrum is the largest part of the human brain, associated with higher brain function such as thought and action; the corpus callosum connects the left and right cerebral hemispheres, facilitating inter-hemispheric communication; the thalamus relays sensory and motor signals to the cerebral cortex, and regulates consciousness, sleep, and alertness; the hypothalamus controls body temperature, hunger, thirst, fatigue, sleep and circadian cycles; the hippocampus concerns the consolidation of information from short-term memory to long-term memory and spatial navigation, and the cerebellum is involved in motor control and learning to adjust to sensorimotor relationships.
b. There are different cell types within the brain, including neurons (containing axons, cell body and dendrites), astrocytes, glial cells, ependymal cells, oligodendrocytes, microglia, Schwann cells, and satellite cells.
c. It has been estimated that the adult human brain contains more than 80 billion cells[16] (neurons) and more than 8,000 billion synapses, the function of which is to pass information from one cell to another cell based on electrical and chemical signalling. The cells connect with one another at specialised junctions (structures known as synapses), via chemical messengers (called neurotransmitters). The neurons have branches known as neurites (dendrites) and the number of these increases with age, as does the myelination of neuronal fibres up to about 2 years of age.[17]
d. Although the types and shapes of neurons vary, the basic neuronal cell structure is shown in Figure 3. The neuron is a specialised cell capable of generating electrical signals, propagating these signals to the axon hillock to create an action potential which transmits

the electrical signal along the axon (of the neuron) to different, adjacent cells. This occurs at the synapse (axon terminal, bouton) which upon receiving the electrical signals releases neurotransmitter chemicals (glutamine, dopamine, serotonin, gamma-aminobutyric acid, acetylcholine) into the synaptic cleft; these are picked up by receptors on the post-synaptic dendrite of an adjacent neuron and lead to formation of electrical signals in these cells, and so on.

e. Cell Membranes. Because there are so many cells (and subcellular organelles) in the brain, the cell membranes play a particularly important role in their structure and function. Cell membranes are composed of polar lipids organised into a bimolecular leaflet and the membranes are impermeable to water, ions and macromolecules. Proteins embedded in the cell membranes (integral proteins) permit movement of polar and ionic species through voltage-gated ion channels which allow passage of Na^+, K^+ and Ca^{++} ions involved in the electrical signalling in the brain. Many of these integral proteins are G-protein coupled receptors. These typically have seven transmembrane-spanning alpha-helices, an extracellular N terminus, an intracellular C terminus and three interhelical loops on each side of the membrane. One of the principal signalling modalities of the central nervous system and peripheral nervous system is G-protein coupled signal transduction. G-protein coupled receptors are the largest family of membrane surface receptors and respond to a broad spectrum of extra-cellular stimuli, such as hormones, neurotransmitters, odour, taste molecules, light, and generate intra-cellular responses via coupling to G proteins.[18] The cell membranes of the neuronal cells play a fundamental role for the integral membrane proteins, since these proteins typically change their shape (conformation) during their activated state which requires compressibility of the cell membrane lipids, enriched in DHA, which surround the integral proteins. Therefore, the functions of these integral membrane proteins are highly dependent on the lipid bilayer of the cell membrane, with interactions between the cell membrane lipids and these proteins required for efficient opening and closing of the voltage-gated ion channels, optimal functioning of the Na^+/K^+ ATPase and receptor proteins such as rhodopsin and receptors for neurotransmitters at synapses. It has been estimated that there are between 14,000 to 70,000 ion channels on a single neuron.[19]

f. What is special about the cell membrane glycerol-PL in the brain and retina is that they are enriched in DHA, AA and C22:4ω6 compared with other tissues[8] (Figure 2). The proportion of DHA in the glycero-PL of brain grey matter is higher than the white matter,[4] with PE and PS containing the most DHA amongst all the glycero-PL.

g. The DHA content of the adult cerebral cortex is approximately 3% of the dry weight and 0.4% of the white matter.[1,4] In white matter glycero-PL there is a higher proportion of omega 6 than omega 3 PUFA.[4]

h. The highest proportions of DHA in membrane lipids are found in the disk membranes of the rod outer segments of photoreceptor cells in the retina.[20] The structural lipids of the photoreceptor outer segment membranes are 80-90% glycero-PL and 8-10% cholesterol and the proportion of DHA in the PE and PS fractions is up to 50 mol %.[18] In the retina, some glycerol-PL contain two DHA molecules per mol of glycero-PL[21] and other phospholipids (PC in particular) contain very long chain PUFA with 24 to 38 carbon atoms in position -1 of PC, with DHA in position-2 on the glycerol moiety.[22] The outermost retinal neurons, the photoreceptor cells, contain highly specialized membranes and are the site of the initiation of the process of vision (phototransduction), occurring via the retinal ganglion cells which convey visual information from the retina to the visual cortex.[22,23]

i. The large amounts of DHA in the photoreceptor membrane glycerol-PL results in a highly fluid membrane that permits efficient conformational changes to occur in rhodopsin and its associated G-protein (transducin) during phototransduction.[18]

j. One study in primates by Brenna's group analysed the DHA and AA concentrations in 26 regions of the brain from 40-week old baboons. The highest concentrations of DHA and AA were in grey matter such as basal ganglia. The % DHA (of total FA) ranged from 15.8 (globus pallidus) to 4.5 (optic nerve), while the % AA (of total FA) ranged from 13.7 (amygdala) to 6.8 (optic tract).[24]

k. The brain, unlike other tissues, has an almost absolute reliance on using glucose as a fuel for ATP production, and brain glucose uptake is highly dependent on glucose transporter 1, localised in the endothelial cells of the blood brain barrier and astrocytes. To give an indication of just how much ATP is required in the brain, it has been estimated that the brain, at rest, consumes as much as 20% of daily energy intake, despite the human brain accounting for only 2% of the body weight. The high rate of oxidative metabolism in the brain comes mainly from mitochondrial oxidative metabolism, with cytochrome oxidase being a key enzyme.[25] The oxygen consumption is highest in the retina.[23] In the rodent brain, it has been reported that 25% of the energy consumption is used in maintaining neurons and glia and the rest in sending and processing electrical signals, the bulk of which is energy consumed at synapses (pumping ions is very energy intensive).[26] The Na^+/K^+ ATPase is considered to be a major user of ATP in the brain and is responsible for maintenance of the Na^+/K^+ equilibrium through neuronal membranes and of ionic gradients

which are involved in nerve impulse propagation, neurotransmitter release and cation homeostasis. This enzyme activity is concentrated in synaptic nerve terminals.

About DHA

a. Biochemistry – what is the effect of replacing DHA with DPAω6 in brain cell membranes? Omega 3 deficiency in the diet leads to a depletion of DHA in brain cell membranes, but this loss is invariably offset by increases in a substitute long chain 22- carbon atom omega 6 PUFA, DPAω6,[27,28] as shown in Figure 4. The formation of DPAω6 results from metabolism of AA by an elongase to C22:4ω6 and then a desaturase to insert another double bond to form C22:5ω6. This substitute fatty acid has similar but not identical biophysical properties in membrane function. Of note, while the difference between DHA and DPAω6 is only one double bond (Figure 1), there is a longer stretch of saturated carbon chain in DPAω6 which is thought to influence the biophysical properties of membranes containing this fatty acid. This topic has been especially studied in relation to visual function. Rhodopsin is the light receptor and transmits electrical signals to the brain via the retinal ganglion cells. Animals raised on omega 3 deficient diets exhibit deficits in visual function (reduced amplitude and delayed response of the a-wave), which is consistent with sub-optimal neural signalling.[18] Biophysical studies have revealed that rhodopsin function is sensitive to the whether DHA or DPA (C22:5ω6) is present in the retinal membrane glycerol-PL. These studies indicate that the omega 3 bond configuration uniquely optimises the early steps in signalling. Since the loss of one (specific) double bond out of six can be responsible for very observable functional losses, these studies highlight the sensitivity of G-protein coupled receptor signalling to membrane physical properties.[29]
b. Where does brain DHA come from? ALA is the precursor of DHA in mammals, with the liver being a major site of synthesis. The rates of liver DHA synthesis vary between rodents and humans (very low rates in humans, with female rates of conversion greater than those of men). One study in rodents showed that while the conversion rate from ALA to DHA in the liver is low, it remains at least 3-fold higher than brain uptake of DHA.[30] However, it is clear that ALA is widely distributed throughout the body, a substantial amount is subject to beta-oxidation for energy and only a small proportion is therefore available as DHA for uptake by the brain (Figure 5).[31]
c. How does DHA get into the brain – as ALA or as DHA? The evidence suggests that ALA and EPA are extensively degraded by beta-oxidation when they enter the brain by

comparison with the direct uptake of DHA, which is mainly esterified into glycero-PL rather than subjected to beta-oxidation.[32-34]

d. Lipid class preference for brain uptake of DHA. It has been assumed that DHA enters the brain as a free fatty acid (hydrolysed from glycero-PL).[3] Pan et al[35] reported that fatty acid binding protein 5 regulated endogenous brain DHA uptake at the blood brain barrier and had a subsequent impact on cognition. However, emerging evidence supports the view that 1-lyso-2-DHA-phosphatidylcholine in the plasma is an important and possibly the preferred carrier of DHA to the brain.[36-38] Recent work has shown a critical role for a transporter (Mfsd2a) expressed in the blood brain barrier as a major transporter of DHA as 1-lyso-2-DHA-phosphatidylcholine into the brain.[39] Furthermore, these authors described three families with a functional mutation in Mfsd2a that presented with severe microcephaly and intellectual disability.[40,41] In the retina, it has been reported that the adiponectin receptor is a key regulator of the uptake and retention of DHA.[42]

What research approaches have been used?

Animal studies

The most common approach has involved dietary studies in which are animals (rodents mainly) are fed diets with very low concentrations of omega 3 fatty acids, usually provided by vegetable oils with low ALA proportions (<1% total fatty acids), such as safflower oil or peanut oil. Often these oils are highly enriched in the omega 6 fatty acid LA. The control diets typically contain vegetable oils with modest to high ALA contents (canola oil or flaxseed oil), although some control diets have used fish oils containing EPA and DHA. Another approach used by Salem's group has involved hand-rearing pups on liquid formula diets with low LA concentrations and negligible ALA concentrations (<0.02% total FA).[43]

The dietary protocols have varied from studies for one generation where dams were fed the diets and offspring weaned onto same diets, to studies of several generations of animals on these diets. Most commonly studies have used rats, however mice, guinea pigs and primates have been used. These studies have shown reductions in the proportions of DHA in brain and retinal lipids, with the greatest reductions occurring when the studies have involved several generations on the omega 3 deficient diet.[44]

The studies have examined behavioural aspects of the animals, as well as characterising membrane fatty acid concentrations, and biochemical features of neuronal cells (enzyme activities, neurotransmitter concentrations, downstream effects on second messengers), and gene and protein expression in various regions and/or cells in the brain.

In vitro studies

Studies have been conducted on a variety of cell types (such as rat hippocampus embryonic primary cells, human neuroblastoma M17 cells, rat sympathetic neurons) where typically the cells are incubated with DHA or other PUFA to determine effects on specific aspects of cellular biochemical and physiological functions.[45-48] Studies have also been conducted using hippocampal slices,[13]and whole cell patch clamps.[49]

Reconstituted membrane studies

These studies have been conducted with isolated rod outer segment membranes and artificial cell membranes (lipid bilayers), incorporating specific lipids, fatty acids and rhodopsin, to determine functional aspects of membrane receptor proteins.[29,50-52]

What are the findings in human and animals?

Human studies guiding the research

Several studies in infants have examined the temporal accumulation of DHA and AA in the brain, from 22 weeks gestational age to 8 years of age.[53-55] Both DHA and AA are present in high proportions during gestation, however postnatally the proportion of DHA in the brain regions studied rises with time, relative to AA.

Following the publications on the accretion of fatty acids in the developing human brain, researchers started to look at the fatty acids present in human milk. It was found that both colostrum and mature milk contained long chain PUFA, including DHA and AA.[56,57] At this time, infant formulas were devoid of DHA and AA. It only in the past 20-25 years that long chain PUFA, such as DHA and AA, have been added to infant formulae.

Researchers have also studied visual function in infants fed infant formulas with low omega 3 contents. Makrides et al[58] reported that visual acuity in infants was positively correlated with the red blood cell DHA content, while Uauy et al[59] reported that in term infants the visual acuity was more mature at 4 months and at 3 years of age in breast-fed infants (breast milk containing DHA and AA) than in formula-fed infants (infant formula lacking DHA and AA).

Physiological effects in animals

Developmental. Animals raised on omega 3 deficient diets typically exhibit very little if any obvious changes in appearance. However, one study carefully documented very high rates of perinatal mortality of pups in omega 3 deficient animals across several generations.[60]

Subsequently, it was reported that 40% of the omega 3 deficient dams attacked their newborn pups or did not nurse them.[61]

Visual function. The work on visual function was initiated because of the very high DHA content of rod outer segment membranes, rather than because omega 3 deficient animals had been observed to have visual abnormalities. Furthermore, it was relatively easy to test visual function using electroretinography on anaesthetised animals maintained in the dark for several hours. What has been consistently found is that omega 3 deficient animals have an altered response of the retina to light. This was first reported in adult rats fed fat-free diets (no ALA or LA) and then provided with the addition of pure ALA or LA into the fat-free diets for 40 days.[62] They showed that the electrical response of the photoreceptor cell was a relative function of dietary ALA and LA content, with the greatest response being to diets containing ALA. In other words, a selective functional role for omega 3 PUFA, presumably because of the very high DHA content of retina membrane glycero-PL (arising from liver metabolism of the ALA in the diet). Subsequent work reported that rats maintained on diets deficient in ALA (omega 3 PUFA) had reduced retinal responses to light compared with rats fed diets containing ALA.[63] Deficits in retinal function have also been reported in other species maintained on ALA deficient diets: in primates[64] and in guinea pigs.[65] Bush et al[66] reported that, when rhodopsin was detergent-extracted from retinas, from rats maintained on diets deficient in ALA for up to 24 weeks, the rhodopsin showed a reduced capacity for photon absorption which could play a role in lowering the retinal sensitivity to light. In all these studies, the diets deficient in ALA (and rich in in LA) were associated with significantly reduced concentrations of DHA in the glycero-PL in the retina and the occipital cortex (visual processing centre) and elevated DPAω6 concentrations (the omega 6 "DHA" substitute fatty acid). Studies on rhodopsin-containing vesicles with purified molecular species of phosphatidylcholine (18:0-DHA; and 18:0-DPAω6) and with rod outer segment membranes containing rhodopsin, transducin (Gt) and phosphodiesterase revealed that the DPAω6 preparations not only reduced rhodopsin activation but also the formation of the rhodopsin-Gt complex. In addition, there was a decrease in phosphodiesterase activity compared with the DHA containing preparations,[29] indicating the omega 3 bond configuration uniquely optimises the early steps in the signalling process. The findings reported here provide an explanation for the reduced amplitude and delayed response of the electroretinogram a-wave observed in omega 3 deficiency in rodents and non-human primates.[52]

Behaviour. Alterations in learning/memory tasks, in mood, in response to stress, and in aggression have been reported in rodents maintained on omega 3 (ALA) deficient diets.

Learning and memory tasks are the most commonly studied responses of animals on omega 3 deficient diets. The first report of behavioural effects in rats of omega 3 deficient diets was in 1976.[67] They reported significant differences in first generation adult rats in Y-maze tests between control and deficient rats. Brain DHA concentrations were reduced by more than 75% in these animals. Since then, there have been many reports of significant differences in learning ability in omega 3 (ALA) deficient rats compared with controls using different test procedures (Y-maze, Morris Water maze, Porsolt Forced swim test, Open field test, Locomotor activity, Elevated Maze, Barnes circular maze).[43,63,68-74] The findings have been consistent and a typical outcome is that the omega 3 (ALA) deficient rats exhibited a longer escape latency and poorer memory retention in the Morris water maze compared with control (omega 3 (ALA) fatty acid adequate) and dam-reared rats.[43] Behavioural differences have also been reported in rhesus monkeys maintained on omega 3 deficient diets for a long period (>3 years) compared with control monkeys, including more bouts of stereotyped cage behaviour and more frequent bouts of locomotion75. Behavioural changes have also been reported in capuchin monkeys maintained on diets with little ALA compared with diets containing omega 3 fatty acids.[76]

Behaviour and neurotransmitters. The effects of omega 3 deficiency on neurotransmitter concentrations and their receptors have been studied since the 1990's in an attempt to explain the behavioural changes in omega 3 (ALA) deficient rodents. Many of these studies have originated from French research groups and they have studied concentrations of neurotransmitters (dopamine, 5-HT, glutamate), endocannabinoid actions, receptors concentrations/expression, density of vesicles (by histological techniques and electron microscopy) and functional changes in response to tyramine stimulation in early development, adult and aged rodents.[77-83] The dopaminergic system has been studied most frequently, while other studies have looked at glutamatergic,[84] 5HT[78] and endocannabinoid systems.[85] In general, dopamine concentrations are lower, there is increased expression of dopamine receptors in different brain regions and a decrease in the number of dopamine vesicles in pre-synaptic terminals. Evaluation of the ontogeny of glutamatergic responses in hippocampus of female rats deficient in omega 3 PUFA showed decreased glutamatergic-related proteins postnatally, but not by weaning at day 21. Behavioural performance was diminished at day 60 compared with control animals.[84] Two ultrastructural studies have shown a decreased synaptic vesicle density and dopaminergic synapses in the hippocampus in omega 3 deficiency in the rat.[82,86] The findings have shown that differences in dopaminergic signalling do exist in response to omega 3 deficiency between young and adult rats[83,84] and between virgin and

parous rats.[81] The authors generally concur that the reported changes are consistent with poorer performances in cognitive tasks which have been observed in rodents fed diets deficient in omega 3 PUFA. Endocannabinoids are another class of compounds capable of regulating synaptic function.[87] Two compounds derived from AA fall into this category: 2-arachidonylglycerol and arachidonoylethanolamide. They suppress neurotransmitter release by acting as retrograde messengers at pre-synaptic cannabinoid receptors. Neural concentrations of the endocannabinoids can be altered by deficiency of omega 3 in the diet[88] or by the addition of long chain PUFA to the diet.[89] It has been reported that omega 3 deficiency in mice abolishes endocannabinoid neuronal functions in the pre-frontal cortex.[85,90] These studies showed that presynaptic CB1 receptors normally responding to endocannabinoids were uncoupled from their effector proteins. These changes were associated with an impairment in emotional behaviour. Subsequent studies have revealed the importance of the endocannabinoid system in mood regulation (in rodents).[91] The involvement of omega 3 PUFA on melatonin production has been studied in rats and Syrian hamsters. In the latter animal model, omega 3 deficiency was associated with a significantly increased locomotor activity (on a running wheel) in both dark and light phases.[92] This behaviour was associated with significantly reduced melatonin concentrations in the pineal gland, weakening the endogenous rhythms of the circadian clock. In addition, there were significantly increased dopamine (and metabolite) concentrations in the striatum, which is involved in the regulation of voluntary movement. Previous work in omega 3 deficient rats had reported increased 12-HETE, which is a lipoxygenase metabolite of AA, in the pineal gland of rats[93] and which has been reported to stimulate melatonin release. Lavaille et al[92] referred to studies implicating melatonin in dopaminergic pathways and suggested that alterations in melatonin production could be involved with nocturnal sleep disturbances.

Reversal of learning defect: role of metabolites of AA. A recent surprising finding is that learning defects in mice reared for 3 generations on omega 3 deficient diets could be reversed not only by the addition of omega 3 fatty acids (ALA) to the diet, but also by a COX-2 inhibitor.[94] This data might be explained by results which reported that omega 3 deficiency significantly increased the activity, protein concentration and mRNA expression of the AA regulatory phospholipase A2 (PLA2) (calcium-dependent cPLA2 and secretory sPLA2), and COX-2 in rat frontal cortex[95] and the expression of cPLA2, COX-2 and PGE2 in the rat hypothalamus[96] thus presumably increasing the availability of free AA for metabolism to PGE2. These data suggest that exaggerated metabolism of AA to bioactive inflammatory lipid mediators might be contributing to the learning defect observed in omega 3 PUFA deficiency.

Alternatively, it is possible that in omega 3 deficiency, reductions in DHA lipid mediators such as resolvins (RvD1 and RvD2) might play a role in the learning defects.[97]

Olfactory. Several studies have reported olfactory discrimination defects in omega 3 (ALA) deficient rats. For example, second generation omega 3 deficient rats made significantly more errors in olfactory-cued, 2-odour discrimination tasks associated with 83% less DHA in the olfactory bulb compared with control rats.[98] In another study by the same group, second generation omega 3 deprived rats were deficient in the acquisition of a 20-problem olfactory learning set. The authors suggested this appeared to represent a deficit in higher order learning.[99] In another study, second generation omega 3 deficient rats showed mild olfactory learning impairment as these rats required more days to master the olfactory task compared with control rats. The olfactory learning impairment was associated with DHA depletion, of approx. 80%, in brain regions processing olfactory cues.[100] Contributing to the understanding of the effects of DHA on olfactory discrimination, it has been shown that olfactory transduction in olfactory receptor neurons, particularly voltage-gated K^+ channels, are inhibited strongly by DHA (and AA).[49]

Auditory. Several studies have reported effects of omega 3 (ALA) deficiency on auditory responses in rats. It has been found that the central auditory nervous system ages faster or earlier in third generation omega 3 deficient rats compared with control rats, based on wave III amplitudes and latencies in older rats.[101,102]

What are the research findings on down-stream effects (within neurons)?

Release of DHA from membrane glycero-PL. The initiation of many downstream effects are mediated by DHA-specific Ca^{++}-independent phospholipase A2 (i-PLA2) to release DHA from glycero-PL in the cell membrane. Subsequently, the DHA would be bound to an intracellular transport protein or converted to DHA-CoA for further metabolism. In omega 3 deficient rat frontal cortex, the activity, protein concentrations and mRNA expression of i-PLA2 was significantly decreased.[95] Presumably, this has an impact on the downstream effects of intracellular DHA.

DHA-derived lipid mediators and inflammation. DHA released from cell membrane glycero-PL by i-phospholipase A2 can be metabolised to bioactive lipid mediators, known as docosanoids, which have been shown to regulate a number of cellular processes within the brain, in particular the resolution of inflammation.[103] The wide diversity of docosanoids are produced via several different LOX enzymes, or COX-2, or cytochrome P450; these lipid mediators include neuroprotectin D1 (NPD1), resolvins (RvD1 and 2) and aspirin-triggered

hydroxy-DHA derivatives.[3] NPD1 has been identified for its potent neural protective effects (against brain injury) and anti-inflammatory effects.[104] Studies in mice maintained on diets containing LA and either lacking or containing EPA and DHA have shown that a wide variety of lipid mediators of both the omega 6 and omega 3 type can be identified in the hippocampus after 2 months on the diets.[103] The omega 3 rich diet significantly raised the omega 3-derived lipid mediators and lowered those from omega 6 PUFA. DHA-derived lipid mediators have been identified as being involved in behaviour and cognition. Resolvins D1 and D2 have been shown to have anti-depressive effects in rodent models.[97,105] The potential importance of lipid mediators in neural function and recovery from brain injury was highlighted by Thau-Zuchman et al[106] who reported that a single injection of DHA following a traumatic brain injury (in rats) was associated with a significant reduction in lesion size and an upregulation of DHA lipid mediators, resolvins and protectins. Recently, it has been reported that dihydroxy derivatives of the very long chain PUFA (C32:6n-3 and C34:6n-3), termed elovanoids, are synthesised in neural cells and can counteract neuroinflammatory responses to injury.[107] The bioactivities of docosanoids and elovanoids have important implications in understanding how neural and retinal cells might modulate inflammatory responses following injury.[104]

Gene expression. Fatty acids are known for their effects on nuclear receptor transcription factors (such as PPARs and RXR's),[108] with resulting effects on the modulation of gene expression. In particular, studies have revealed effects on brain gene transcription in rodents both when omega 3 PUFA are added to[109, 110] or deleted from the diet.[96,111,112] The presumption is that fatty acids entering the cell bound to fatty acid binding proteins or released from cell membrane glycero-PL, by phospholipases, act on these nuclear receptors. In 2000, DHA was shown to be a ligand for the nuclear receptor RXR, which is a receptor for retinoic acid.[109] Subsequently, it was reported that DHA potentiated the effect of retinoic acid and improved cognitive symptoms in rodents.[113] This and other studies highlighted the importance of this receptor and its ligands in emotional behavior.[114] In the study by Kitajka et al,[110] rats were fed diets containing either a vegetable oil (rich in ALA) or a fish oil (rich in EPA and DHA). There was an increase in the brain DHA proportions in both test groups associated with highly significant alterations in the expression of more than 100 genes in the brain (approximately equal number over- and under-expressed).The control rats were fed a diet rich in LA (omega 6). Of interest was the fact that the ATP-generating machinery of the brain responded to the dietary omega 3 PUFA most intensively. The brain is known to exhibit a high metabolic rate and a high proportion of this is used to maintain Na^+/K^+ ATPase activity,

which regulates ion flow resulting from nerve transmission. Genes participating in signal transduction were also overexpressed by both the omega 3-rich diets. Other studies in omega 3 deficient rodents have reported effects on selected gene sets involved in brain glucose metabolism[112] (omega 3 deficiency repressed GLUT 1 gene expression in frontal cortex by 25-30% in both basal and neuronal activation), neural regulation of blood pressure[96] (dietary omega 3 deficiency was associated with changes both in the expression of key genes involved in central blood pressure regulation and in blood pressure) and on brain zinc metabolism[115] (perinatal omega 3 fatty acid deficiency associated with overexpression of zinc transporter T3 in the brain).

Voltage-gated ion channels. The brain is an excitable tissue and the generation of electrical currents is required to pass information from one neuron to other neurons. This happens by the activation and inactivation of voltage-gated Na^+, K^+ and Ca^{++} channels, allowing ions to move across the cell membrane (Na+ moving out of the cell and K^+ moving into the cell against the resting ionic gradients). A considerable body of research has shown that free PUFA can have significant physiological and pharmacological effects on these membrane potentials through the voltage-gated ion channels (these are integral membrane proteins). Common features of the effects of the fatty acids include at least two cis-double bonds and a charged carboxyl group.[46,48] Five different sites have been identified where the PUFA might act on the ion channel, with the one most studied being the interface between the extracellular leaflet of the lipid bilayer and the voltage sensor domain. DHA has been extensively studied and presumably requires release from the membrane glycero-PL by i-PLA2. The binding to this site is by electrostatic interaction between the negatively charged PUFA to the positively charged sensor, leading to an opening of the ion channel. Depending on the specific site of the PUFA binding to voltage-gated ion channel, determines the effect on the ion channel. In the case of two other binding sites, the effect is to reduce the current; another two sites can either increase or decrease the open probability of the ion channel, and finally binding to the fifth site can regulate the slow inactivation of the ion channel. These studies are ongoing and to summarise the above, the overall effect of the PUFA would be determined by the relative contributions of the PUFA effects on the five sites. Leaf et al[46,116] found marked effects of free omega 3 PUFA on cardiomyocytes, where EPA and DHA inhibited fast, voltage-dependent sodium currents.

Cell size (neurite and axonal outgrowth). Several cell culture studies have reported that embryonic hippocampal neuron cultures treated with DHA showed an increased population of neurons with longer lengths per neuron and a higher number of branches.[45,117] In addition,

DHA promoted synaptogenesis and glutamatergic synaptic function. Subsequently, this group has shown that the effects of DHA described above in the embryonic hippocampal neurons are almost entirely due to a novel lipid mediator derived from DHA, N-docosahexaenoylethanolamide (named synaptomide).[118] The amount of synaptomide in mouse brain increases when the diet is supplemented with DHA.[119] In subsequent work, it was shown that an orphan G-protein coupled receptor (GPR110) was the functional synaptomide receptor mediating synaptomide-induced neurite growth and synaptogenesis in cortical neurons via cAMP-dependent signal transduction.[120] In vivo studies in omega 3 deficient neonatal rats reported that neurite growth and synaptogenesis were impaired,[45] cell proliferation was significantly decreased[121] and the cell body size of CA1 pyramidal neurons was significantly smaller in omega 3 deficient rats.[122] Furthermore, long term potentiation in young mouse hippocampi was significantly impaired in omega 3 deficient mice.[117]

The unique case of neural phosphatidylserine in omega 3 deficiency. Phosphatidylserine (PS) plays a unique role in neuronal cells in the brain and this is exemplified by changes which take place in omega 3 deficiency. PS is localised on the cytoplasmic side of the plasma cell membrane in the cell body and dendrites of neurons.[123] Activation of signalling proteins which support neuronal survival, neurite growth and synaptogenesis, such as Akt, Raf-12 and protein kinase C, requires interactions of these proteins with PS.[123] Neurotransmitter release by exocytosis and a number of synaptic receptors and proteins are modulated by PS. DHA promotes PS synthesis and leads to expansion of the PS pool in neuronal membranes, thereby influencing PS-dependent signalling and protein function. PS is synthesised from either PC or PE, in exclusively Ca^{++}-dependent reactions, where the headgroup of substrate glycero-PL is replaced by serine. The biosynthesis is catalysed by PS synthetases (Pss1 and Pss2),[124] and deletion of these enzymes causes embryonic mortality in mice, indicating PS is essential for metabolic function.[125] The major molecular species of PS in neural tissue is 18:0-DHA, representing 45-65% of total PS.[126] Dietary omega 3 deficiency decreases neural DHA content, but more specifically, there is 30-35% reduction in total PS content in rat brain cortex, mitochondria and olfactory bulb. This occurs without replacement of DHA-containing species with species such as 18:0-C22:5ω6, since this molecular species is not a good substrate for PS synthesis.[126] Therefore, unlike other glycero-PL, not only is there a decrease in DHA proportion in PS, there is a 30-35% reduction in PS concentration. Hippocampal neurons from DHA depleted animals showed reduced amounts of PS, and these were more susceptible to cell death.[127] In summary, the neuroprotective effect of DHA is linked to an increase in PS content of neuronal cells, which promotes activation of the Akt and Raf-21 signalling

pathways and thereby enhances neuronal survival. It should be noted that the DHA-induced PS increase and facilitation of PS-dependent signalling pathways as well as synaptomide production might both be contributing to these DHA effects.

Altered neural glucose metabolism and energy metabolism. Glucose entry into the brain and subsequent metabolism to produce ATP is a significant area which impacts on the ability of neural cells to generate energy to drive the "machinery" for the maintenance of the membrane potential, thus the ability to propagate the impulses along the axon. It has been estimated that about 50% of brain ATP is used by the Na^+/K^+-ATPase after an action potential.[128] There have been quite a number of publications on the impact of omega 3 PUFA on uptake of glucose by the brain (in vivo) and by isolated neural cells. These have largely come from the work of French groups since the early 1990's period.[129] The initial work was in rodents, however recent papers have used cell culture[130] and the mouse lemur (a non-human primate).[131] Initially, it was demonstrated that there was a 40% reduction in Na^+/K^+ ATPase in nerve terminals in omega 3 deficient rats[129] and later confirmed by Bowen and Clandinin.[132] Subsequently, it was shown by autoradiography that there was a 50% lower glucose utilisation in the cerebral cortex and hippocampus, and a 20-30% lower rate of oxidative phosphorylation in omega 3 deficient rats.[133] Brain glucose uptake is dependent on glucose transporter activity, especially GLUT 1 localised in endothelial cells of the blood brain barrier and astrocytes. Rats maintained on omega 3 deficient diets have lower expression of GLUT 1.[112,134] Altogether these data suggest that impairment of neurotransmission could be due in part to sub-optimal brain energy metabolism. In recent work in the mouse lemur, it was shown that 12 months supplementation with a DHA-rich fish oil to young animals resulted in a higher brain glucose uptake and cerebral metabolic rate of glucose compared with control animals on a low omega 3 diet. The omega 3 supplemented group also exhibited reduced anxiety.[131] The mechanism of action of DHA on the GLUT 1 receptor has not been established, however Pifferi et al[134] have suggested that post-translational regulation of GLUT 1 synthesis was likely since the GLUT 1 mRNA expression in rat brain cerebral cortex was not affected in omega 3 deficiency.

What has been overlooked and/or understudied?

Few, if any, studies have used imaging techniques in the brain to determine the extent and localisation of changes in omega 3 fatty acid deficiency. However, one study using immunohistochemistry reported that an omega 3-containing diet, during pregnancy and lactation, in mice was associated with promotion of growth and maturation of neurons,

astrocytes and myelin.[135] In a cross-sectional study in infants, researchers studied the relationship between breastfeeding or formula feeding in healthy children from 10 months through to 4 years of age, who were either exclusively breastfed a minimum of 3 months; exclusively formula-fed; or received a mixture of breast milk and formula.[136] They reported that breast-fed children exhibited increased white matter development in later maturing association brain regions. There have been very few studies in animals on the effect of omega 3 deficiency on myelination despite the fact that myelin does contain DHA, albeit in lower proportions than in grey matter.[137] In another study, the impact of AA and DHA supplementation in pregnancy on newborn infants' brain volumes found that male infants born to the supplemented mothers had significantly larger total brain volumes, total gray matter, corpus callosum and cortical volumes compared with the placebo group.[138]

Few studies have used brain "omics" to explore the consequences of omega 3 fatty acid deficiency. Typically, the understanding of molecular mechanisms underlying the effects of DHA in the brain have been limited by studies which have focussed on isolated molecular events. Now, by using systems nutrigenomics, it is becoming possible to study multidimensional molecular interactions which are under dietary modulation.[139] In a recent study, it was reported that DHA reverses biological pathways and gene networks perturbed by fructose via transcriptional regulators and essential network regulators.[140]

Very few studies have considered the importance and role of AA in brain function and the relationship between AA-derived lipid mediators and those formed from DHA. This is exemplified in a study by Hennebelle et al[15] who reported that the majority of oxylipins in the neonatal rat pup brain were LA-derived metabolites, despite low proportions of the LA precursor in the brain. This observation is in contrast with the low abundance of these LA oxylipins in the adult brain. Preliminary studies in primary cortical neurons suggested a role for these oxylipins in neuronal morphogenesis. The example cited previously that a COX-2 inhibitor could reverse learning defects in omega 3 deficient animals[94] highlights the importance of looking at both major PUFA in the brain (DHA and AA), rather than focussing on only one of them.

Interactions have been described between zinc and DHA *in vitro*, based on initial observations in rats deficient in omega 3 fatty acids. Omega 3 deficient rats were found to have an over-expression of zinc transporter 3 in the brain.[115] This transporter is a transmembrane protein involved in zinc transport into synaptic vesicles. Subsequently, studies using a human neuroblastoma M17 cell revealed that the expression concentrations of histone H3 and H4 were down-regulated by zinc and upregulated by DHA.[141] The same group,

established that zinc and DHA altered the post-translational epigenetic modification of histones, with the effects of DHA opposing those of zinc.[47] This research is in its infancy and requires a systematic approach to explore the relationship between zinc and DHA, perhaps using a systems nutrigenomics approach. Since both zinc and DHA are required for optimal neural function, further work is required to establish the significance of these findings *in vivo*.

Conclusions

In conclusion, it is clear that DHA plays a critical role in the function of G-coupled protein receptors and ion channels in the brain. These are of fundamental importance in the transmission of signals from one cell to another. When animals are placed on diets lacking omega 3 fatty acids, the major consistent biochemical finding is that DHA concentrations are significantly reduced in the membrane glycero-PL and that most of the DHA loss is replaced by an omega 6 PUFA (DPAω6). This fatty acid is chemically very similar to DHA but has one less double bond than DHA. Biophysical studies with reconstituted membranes have shown that DPAω6 functions less efficiently in the phototransduction process, but it none-the-less still allows rhodopsin to be converted to meta-rhodopsin and thus allows a signal to flow, so the effect of DHA depletion in the retinal cell membranes is not an all or none effect. There is no known situation in these studies where there is no replacement of the lost DHA by another 22-carbon PUFA. Thus, one could speculate that DPAω6 is a buffer to prevent the possible catastrophic effects of DHA depletion on brain and visual function. Consistent with these reported effects, the electrophysiological properties of the retina are significantly altered in omega 3 deficient animals (significantly decreased a-wave and b-wave amplitudes). If we look at other aspects of neuronal function in animals maintained on omega 3 deficient diets, we see reductions in neurotransmission specifically dopamine (responsible for some aspects of learning) and also significant reductions in the activity of the Na^+/K^+ ATPase (essential for maintaining the ionic balance between the inside and outside of the cell, a fundamental aspect of electrical signalling). Associated with these changes, there are clear and consistent effects on the behaviour of omega 3 deficient animals. These include effects on ability to learn, increased aggression, suggestions of a more depressed-like state, inability to distinguish between complex sets of smells and alterations in auditory responses.

Therefore, it is tempting to posit that the principal function of DHA in the brain is to allow optimal functioning of integral proteins in cell membranes (which are rich in DHA, in particular in PE and PS), which in turn allows the optimal function of G-coupled membrane proteins such as receptors and voltage-gated ion channels. In other words, DHA in neural cell

membrane glycero-PL is playing an important role in the optimal functioning of neural cell membrane proteins (receptors, voltage gated ion channels, enzymes) of which there are many thousands on each of the more than 80 billion neural cells. One of the downstream effects that could also contribute to an impairment of neurotransmission could be due in part to sub-optimal brain energy metabolism (facilitation of glucose entry into the brain), which is significantly reduced in omega 3 deficiency.

Future studies into the role of DHA-related lipid mediators (including NPD1, resolvins RvD2 & RvD3) in the brain will shed light on their importance in behaviour and cognition, and in the regulation of neuro-inflammation.

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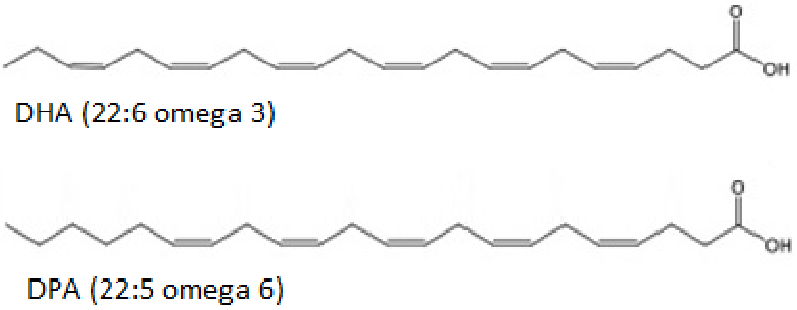

Figure 1. Structures of docosahexaenoic acid (DHA, C22:6 3) and docosapentaenoic acid-omega 6 (DPA-omega 6; C22:5 6). The major PUFA in mammalian brains is DHA. In omega 3 deficiency, DHA is partially replaced by DPA-omega 6.

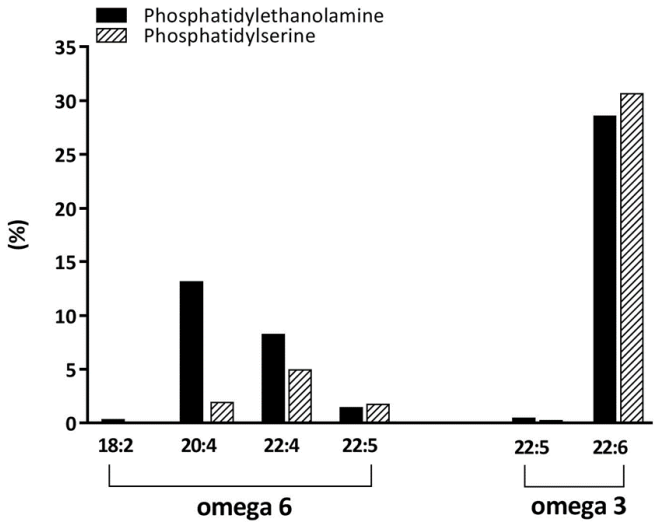

Figure 1. The proportions of omega 3 and omega 6 PUFA in human brain grey matter (cortex) phosphatidylethanolamine (filled bars) and phosphatidylserine (shaded bars). The data is expressed as the percentage of total fatty acids in each phospholipid fraction. Details from Svennerholm (1968).[4]

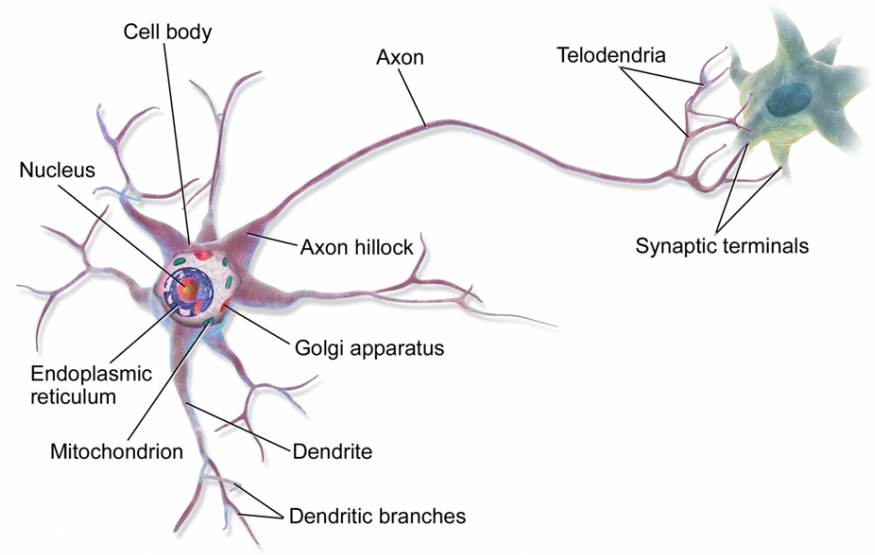

Figure 3. Structure of neuron. Attribution: By Bruce Blaus - Own work, CC BY 3.0, https://commons.wikimedia.org/w/index.php?curid=28761830

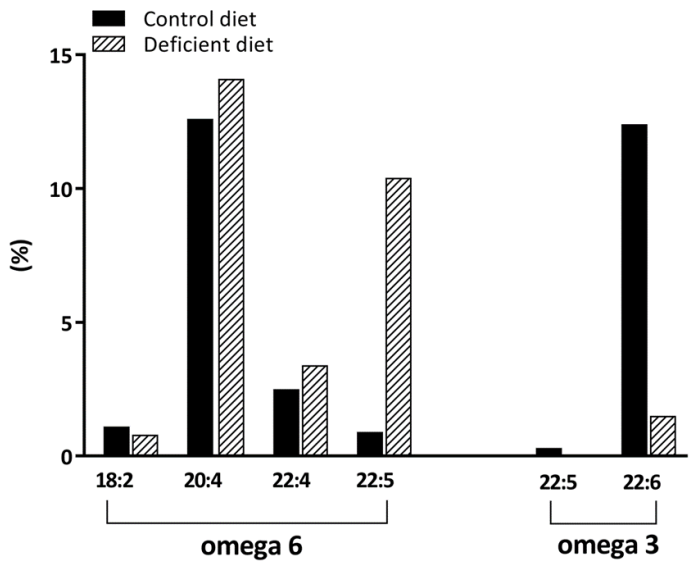

Figure 4. The proportions of omega 6 and omega 3 PUFA in rat brain (cortex) from rats fed on omega 3 sufficient, control diet (filled bars) and from those animals fed on an omega 3 deficient diet (shaded bars). The data is expressed as the percentage of total brain fatty acids from Greiner et al (2003).[28]

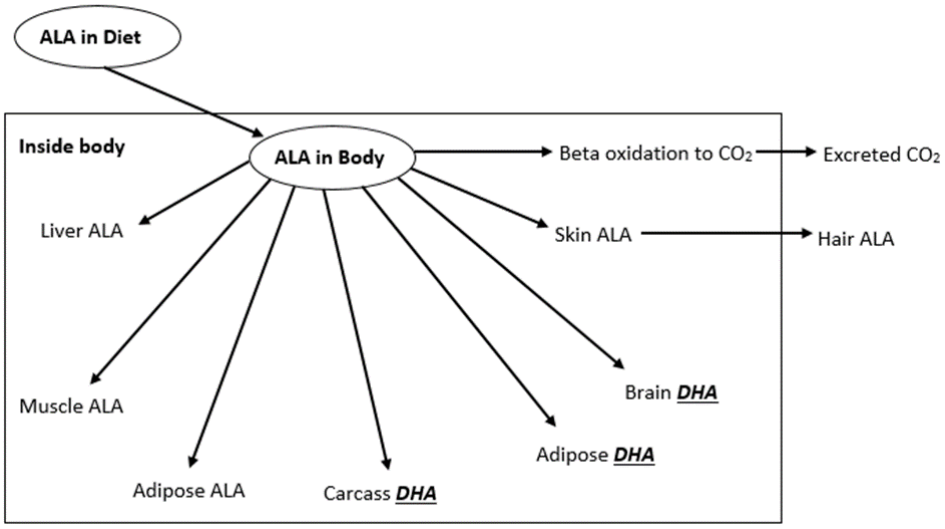

Figure 5. Pathways of ALA metabolism in mammals. ALA is widely distributed throughout the body in tissues (liver, muscle, adipose, heart, kidneys), a substantial amount is subject to beta-oxidation for energy and only a small proportion is metabolised to DHA and therefore available as DHA for uptake by the brain.[31]