Poly-unsaturated Fatty Acids

Neural Function & Mental Health

Edited by
Ole G. Mouritsen & Michael A. Crawford

Biologiske Skrifter 56

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Poly-unsaturated Fatty Acids

Neural Function & Mental Health

Proceedings of an international and interdisciplinary symposium.
The Royal Danish Academy of Sciencesand Letters, August 9, 2007

Edited by
Ole G. Mouritsen & Michael A. Crawford

Biologiske Skrifter 56

Det Kongelige Danske Videnskabernes Selskab
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Abstract

Several lines of current research have strongly indicated that the poly-unsaturated omega-3 fatty acids are not only important for preventing coronary and heart diseases but also play a pivotal role for neural and brain development, mental health, and human behavior. The present volume is the written record of an international and interdisciplinary symposium entitled *Poly-unsaturated Fats, Neural Function & Mental Health* held under the aegis of The Royal Danish Academy of Sciences and Letters in Copenhagen on August 9, 2007. It contains topical contributions from scientists with different background, including biochemistry, physics, neurology, nutritional science, and psychiatry. The purpose of the symposium was to present and synthesize different viewpoints on the role of poly-unsaturated lipids for human brain development, neuronal functioning, vision, membrane properties, and human behavior. The symposium also addressed the dangers incurred by the severe unbalance in the modern Western diet towards omega-6 fatty acids.

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Preface

The seven contributions in the present volume constitute the Proceedings of an international and interdisciplinary symposium entitled *Poly-unsaturated Fats, Neural Function & Mental Health* held under the aegis of The Royal Danish Academy of Sciences and Letters on August 9, 2007, in Copenhagen at the Carlsberg Academy.

The idea behind the Symposium germinated with the organizers in early 2006 and was fostered by the conviction that time was ripe to create an interdisciplinary forum to discuss the importance of poly-unsaturated fatty acids on neural function and the implications for our well being. We were very fortunate that some of the internationally leading scholars within the field were able to contribute to the Symposium. They made it very easy for us to organize this Symposium because they all, without hesitation, promptly and with great enthusiasm accepted our invitation to speak at the Symposium and later contribute to the Proceedings.

In the first paper of these Proceedings, Michael A. Crawford describes the importance of poly-unsaturated fatty acids from an evolutionary perspective, highlighting the need of certain lipids for hominid brain development. The second paper by Joseph R. Hibbeln discusses the impact of poly-unsaturated fatty acids on mental health and human behavior, with particular focus on the importance of fatty acids for understanding and possibly treating psychiatric disorders. Robert E. Anderson and colleagues address in the third paper the question as to how the visual forefront of the brain, the retina in our eyes, adapts to oxidative stress. In the fourth paper, Lotte Lauritsen and collaborators investigate the importance of omega-3 fatty acids for the function of the brain and the retina in human infants. Harald S. Hansen describes in the fifth paper the relation between dietary fatty acids and bioactive lipids. In the sixth paper, Ole G. Mouritsen provides an overview of the physical effects of poly-unsaturated lipids on membranes and protein function. Finally, in the seventh paper Nicolas G. Bazan gives an account of neuroprotective substances derived from docosahexaenoic acid. These substances are crucial for maintaining the functioning of the brain and the retina.

The contributions to these Proceedings were submitted electronically and after editing they were organized, formatted, and typeset by Jonas Drotner Mouritsen. The financial funding for the Symposium was provided by generous contributions from The Royal Danish Academy of Sciences and Letters, The Carlsberg Foundation, The Danish National Committee for Biophysics, The Graduate School of Molecular Biophysics, and MEMPHYS-Center for Biomembrane Physics.

Ole G. Mouritsen
Odense in November 2007

Michael A. Crawford
London in November 2007

A role for lipids as determinants of evolution and hominid brain development

Michael A Crawford

Abstract

The thrust of this paper is that lipids played a major, as yet unrecognised, role as determinants in evolution. Life originated 2.5 billion years ago during which time there was ample opportunity for DNA modification. Yet there was little change in the life forms seen in the fossil record for the first 2.5 billion years. It was not until the oxygen tension rose to a point where oxygen utilising life forms became thermodynamically possible that change is seen. The sudden appearance of the 32 phyla in the Cambrian fossil record was associated with the rise in the oxygen tension and the appearance of intracellular detail and cell differentiation. That detail was provided by cell membranes in which the lipids were structural essentials. Docosahexaenoic acid (DHA) provided the basic membrane backbone of the novel photoreceptors that converted photons into electricity laying the foundation for the evolution of the nervous system and ultimately the brain. Although there are two closely related fatty acids with only one double bond different, DHA was not replaced despite some 600 million years of genomic change. The second obvious lipid involvement occurred during the Cretaceous. As flowering plants evolved their protected seeds they introduced into the land food web a novel, rich source of omega-6 fatty acids. Coincidentally mammals evolved using the omega-6 fatty acids for the synthesis of powerful adhesion molecules to capture the fertilised egg, and vascular development enabling placentation associated with the switch from egg-laying to mammalian reproduction. With the brain utilising omega-6 and omega-3 fatty acids in a ratio of 2:1 the injection of the omega-6 to an already omega-3 rich food web would have played a critical role in the advance of brain evolution and finally the cerebral expansion in human evolution. Lipids are still modifying the present evolutionary phase of our species with their contribution to a changing panorama of non communicable disease which includes the sharp rise in brain disorders.

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Key words: evolution, genomics, lipids, docosahexaenoic acid, arachidonic acid, omega-6, omega-3, brain, vascular development, cerebral expansion, Darwin

Introduction

This paper will review ideas about the conflict between Darwin's original concept of origin of species and the subsequent disassembly of his broad view into a narrow focus on natural selection. His concept of the conditions of existence as the higher law persists in his writing into the 6th and final edition was discarded by most evolutionary biologists. However, Darwin spent much of the latter part of his life searching for what he called "pangenes" which he felt would explain how the environment interacted with evolution. Darwin's pangenes are now in evidence in the nutritionally and environmentally dependent ligands for nuclear receptors which influence the expression of genetic information. The biological features that can lead to multigenerational changes in gene behaviour referred to as epigenetics is essentially the pangenomic behaviour for which Darwin was searching.

The origin of life and the Cambrian explosion

The stromatolite reef at Pilbara in Western Australia has been dated to 3,43 and 3,35 billion years old when it was submerged in a shallow sea (Allwood *et al.,* 2006). Although there is some debate as to whether or not these stromatolites represent the first signs of the biosphere, it is generally agreed that life had evolved by about 3 billion years ago on this planet. The relatively brief period before the appearance of life can be considered as the period of chemical evolution.

Conceptionally the appreciation of chemical evolution from the origins of matter in a super nova is important because it makes the point so often forgotten, that life is chemistry and physics. It was the appropriate conditions of chemistry that brought life into being. Without the correct conditions this would not have happened. The forces in action are Darwin's "*Conditions of Existence*". It follows that life emerged in an energy and chemically rich environment.

The chemical synthesis of reduced molecules of greater and greater complexity is what would be expected following the creation of elements gifted by a super nova. At the high temperatures of the nuclear furnace of a maturing star and its final super nova, the energy is simply too great for elements to combine. As energy levels subsided and gravity took over the expectation is of element combinations to occur. Oxygen being as ferocious a combiner as it is, the burning of silicone and the like would be predicted and of course that is what happened, otherwise there would be no crust to the earth, Mars or the moon. Similarly, high-energy phosphates, sulphates, oxides of hydrogen, carbonates and a plethora of other carbon compounds would also be predicted. Once oxygen was consumed by burning hydrogen and the many elements, the early atmosphere and aquatic environments were reducing. Given the condensation of such material into a solid planet which would include many if not all elements of the periodic table, seams of elements would form through normal crystallisation mechanisms, water soluble compounds would collect in the primitive lakes and oceans as the surface of the planet cooled. The intense solar radiation, volcanic and electrical storm hold all the necessary stimulants for the increasing complexity of natural chemical interactions until, as has been shown experimentally, the building blocks of life were formed. Viewed from the perspective of chemistry and the conditions of existence, self-replicating systems and then life is predictable and unsurprising (Oparin, 1924). It is indeed curious that the discovery of an amino acid or other biochemicals in outer space or on a meteorite raises such extraordinary headlines in the scientific press. Such molecules are expected to emerge as a consequence of interaction between elements and natural chemical evolution.

Once life formed, the earth's life history is usu-

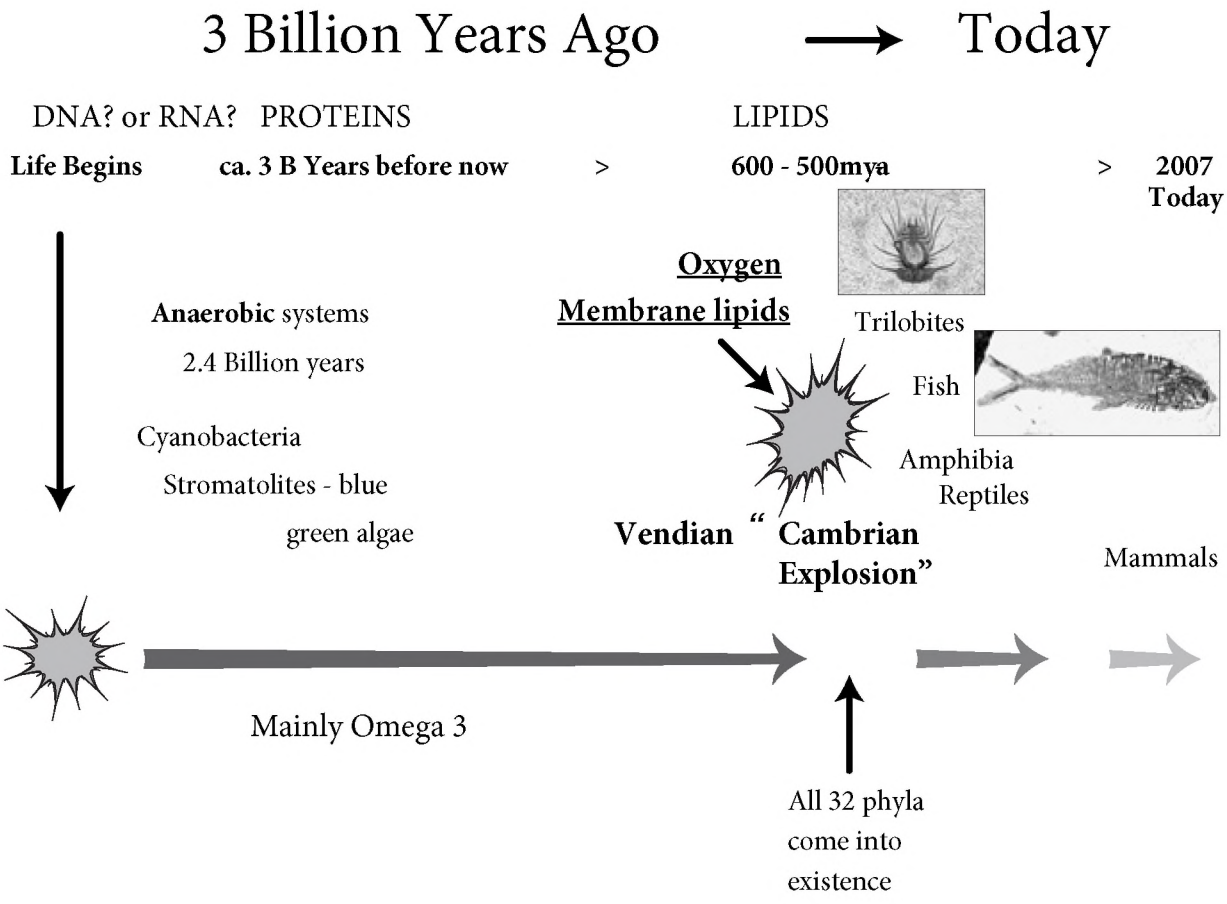

Diagram: A rough sketch of the time lines of the evolution of life

ally divided sharply into two parts, to which I will add a third. These three parts again reflect chemistry as a driving force:

1. During the first 2.5 billlion years, the only fossils found in abundance in the pre-Cambrian era are the stromatolites. This vast pre-Cambrian era was dominated by the Cyanophyta generally attributed to the blue-green algae. Notably, they show no intracellular detail. Their photosynthetic systems synthesize proteins, nucleic acids and other chemicals in an essentially reducing atmosphere. We can expect some polyenoic fatty acids to have been made and that these would likely to be of the omega-3 variety.

2. The second part is the Vendian followed by the Cambrian era of about 600 million years ago. It was then that the oxygen tension rose above the Pasteur point at which aerobic metabolism becomes thermodynamically possible (Fischer, 1965). The 32 phyla we know today suddenly explode into the fossil record in a short period of time. These fossils provide considerable intracellular detail.

3. We shall add a third part; the evolution of flowering plants and the new reproductive system of the mammals. This latter period laid a new chemistry making possible as will be asserted, the evolution of the human brain.

Each of the three phases is an example of a change in chemistry and in evolutionary direction and style. In the first instance chemistry became biochemistry. In the second, biochemistry shifted up through eight gears in efficiency to aerobic metabolism giving birth to 32 phyla. In the third, biochemistry made a lateral shift to a change in the reproduction system that would lead to mass extinctions of many species with the dominance of one.

The evolution of complex life forms

In the first phase of prokaryote life, the biochemistry of nucleic acids and proteins held sway and there was little change on the design of the life forms despite the 2.5 billion year time period offering ample opportunity for the rate of mutational change to provide for substantial modification. There is of course evidence of variation but it is limited. There is however no evidence of such events as witnessed in the Cambrian explosion. This 2.5 billion year stasis is powerful evidence for Darwin's view that there were two forces in evolution; "natural selection and conditions of existence". Of the two, he wrote, the latter was the most powerful. The absence of an appropriate oxygen tension was a condition of existence which did not permit the advance to more complex life. When the Pasteur point was breached, all the 32 phyla we have today appear in the fossil record with a remarkable suddenness leading to this phase being described as the Cambrian explosion. This is, again, clear evidence of the importance of the "Conditions of Existence".

Of special interest in the eukaryotic evolution were the appearance of intracellular structures in the fossils and the emergence of differentiated cells. The intracellular detail is made largely of membrane lipid bilayers and embedded proteins. The organisation of cellular structures was made possible by membrane lipids. With the 32 phyla emerging in the Cambrian explosion it seems likely that it was not only the rise in the oxygen tension that was important but also the cell structural complexity in which the lipids would have played an important role in the genesis of specialisation and then speciation.

Was increased complexity of lipids responsible for cell structures and specialisation amongst the eukaryotes?

The nucleic acids and the proteins derived from them would have been in abundance during the first 2.5 billion years. The state of the lipids can only be a topic of conjecture. Were there highly unsaturated fatty acids available prior to the Cambrian explosion? Fresh water algae operate more anaerobically than sea water algae and synthesise little of the long-chain super-unsaturated, poly-unsaturated fatty acids which have 20 and 22 carbons or more, with 3-6 double bonds (SUFA). Moreover, anaerobic systems, as in the gut flora or rumen, use unsaturated fatty acids as hydrogen donors suggesting that in the anaerobic phase of life, the highly unsaturated fatty acids might have been rare.

Although one can only speculate, the complexity of the lipids for structures is likely to have been greatly enhanced by aerobic metabolism. The synthesis of the double bonds in DHA requires 6 oxygens without including the energy requirement for the chain elongations. These lipids are today used for the organisation of complex cellular structures, as in the reticulo-endothelium, electron transport systems, nuclear envelope and plasma membranes which accommodate receptors, transporters, signaling systems and antioxidant enzymes. Oxidative metabolism and with it the possibility of desaturating fatty acids and producing the SUFA in quantity would have provided a great and novel wealth of architectural possibilities to engage in sophisticated organisation and function not previously possible. A largely anaerobic system would be unlikely to produce SUFA in quantity. Hence one has to consider that sophisticated membrane lipids rich in SUFA was also a feature of the Cambrian explosion making possible sophisticated membrane systems.

The peroxidation paradox and Alzheimer's disease

Another way of considering the transition from the prokaryotes to eukaryotes is that the chemistry of the former was based on the robust chemistry of the nucleic acids. The proteins, which were less robust, possessed a new dimension of complexity with their chemistry being dependent on arrangement of the nucleotides of the DNA. The eight gear shift of energy efficiency with aerobic metabolism brought lipid chemistry into play, introducing a new order of complexity of molecules. The paradox was synthesis of reduced molecules in the pre-Cambrian era was challenged by the novel use of oxygen as the dynamic driver of the new aerobic life forms. Whilst being favourable to the eukaryotes, oxygen was none the less toxic to the reduced systems.

The eukaryotes embraced the reduced cellular interior using the many reduced molecules such as ascorbic acid, carotenoids and tocophopherols for protection which are now referred to as antioxidants. The puzzle still lies with the super-unsaturated fatty acids, DHA in particular. Despite its susceptibility to peroxidation it was actually used in the cell membranes where there is the greatest use of oxygen as in the photoreceptor, brain and mitochondria where hydrogen peroxide is a routine end product. A plausible explanation for such a paradox is that these highly unsaturated fatty acids stimulate the expression of antioxidant enzymes (Phylactos *et al.*, 1994) and indeed their own protective molecules (Lukiw *et al* ., 2005).

Although it can be argued that the reduced life forms never truly died, eukaryote evolution introduced the clear concept of death. Despite 600 million years of evolution no solution to death was discovered. A plausible reason is that the substance responsible for this new evolutionary life style was also responsible for death. The many new research centres for ageing usually with a focus on Alzheimer's disease consider this principle

as there is already evidence of peroxidative damage as a component of cause and conversely the protective nature of seafood and omega-3 DHA in alleged cause and prevention (Bazan, 2007; Butterfield and Sultana, 2007; Moreira *et al.*, 2007).

The protein-lipid interface

The cooperation between lipids and protein in the organisation as the cell membrane bilayer introduced the ability to specialise in a manner not seen if the previous 2.5 billion years or so. Specialisation of function of the plasma membrane was accompanied by the proliferation of unstable molecules with increasing complexity. The language of DNA has 4 words (nucleotides), that of proteins 20 words (amino acids). There are 64 main lipid words (molecular species) and several hundred minor words which some say are more than 1,000 in number. One can argue that this higher degree of structural diversity was essential to accommodate the stereochemistry of the more sophisticated proteins and in particular offered a supporting medium for the lipophilic proteins thus expanding the biological repertoire. The nucleic acid protein interplay in the largely aqueous medium of the prokaryotes would have been less versatile than in the eukaryote system with lipid systems to accommodate lipid protein interactions.

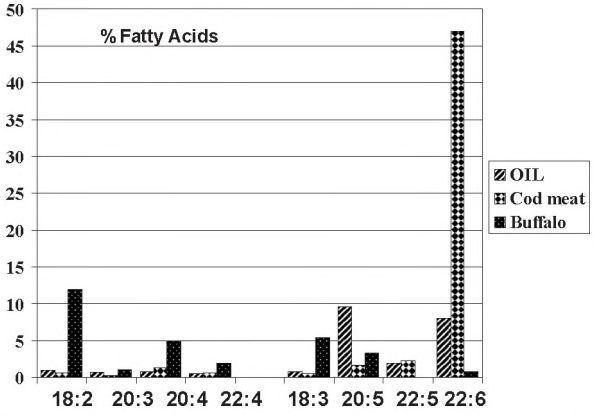

Fig. 1. Fatty-acid composition of cod muscle compared with cod liver oil and buffalo meat.

The cell membrane is the first point of contact between the cell and its external environment. The DNA determines the nature of the protein which cannot change in composition to the diet. However, we know cell behaviour responds to diet, temperature and pressure. In animals, the variable in the membrane is the lipid.

So it is the lipids that will respond to dietary influences and talk to the proteins which in turn influence the cell nucleus. Thus the variety of ways in which Nature could experiment with the design of organism would be greatly enhanced, particularly in view of the wide variety of temperatures and pressures as well as local chemical, energetic, and hence nutritional differences associated with the changing behaviour of the sun and radiation penetration, volcanic activity and as some claim greater intensity of meteorite bombardment. The latter two factors would have been much more active in the pre-Cambrian so again one comes back to the innovation of oxygen and lipids as the novelty opening the wider possibilities of gene environment interaction seen in the Cambrian explosion.

Vision as a trigger for neural development

Relevant to the species that ultimately led to human evolution was vision. The more efficient energy system of the aerobic metabolism led to the DHA/mitochondrial rich photoreceptor. For the previous 2.5 billion years, photon reception had converted photon energy to carbohydrates, proteins etc. In switching to the conversion of photons to "electricity", neural transmission was born, a nervous system evolved and then finally the brain.

The key structures of the neural signaling systems are lipid rich (60% of dry matter). The lipids involved are enriched with long-chain fatty acids

(20 carbon chain length and longer). Of special interest are the super-unsaturated fatty acids, particularly all-*cis*-docosa-4,7,10,13,16,19-hexaenoic acid (DHA). Although vision as we know it today is thought of in the context of eyes many other invertebrates that do not have obvious eyes have photo-sensitive systems. Figure 1 compares the composition of fish oil with cod muscle lipid and that of a land-based animal, the buffalo (*Syncerus caffer*). The cod muscle phospholipid content of DHA closely approximates the proportions used in the photoreceptor and synapse.

Lipid and cell function

The plasma membrane, long considered as a simple barrier between the extracellular and intracellular compartments, is being slowly recognised as playing a pivotal role in many physiological processes that respond to the communications from the environment to the cells. Lipids are closely associated with membrane proteins. Alteration of their composition alters protein function. Moreover, influencing their function alters signaling as well as shifting the dynamics of the individual fatty acids acting as ligands for nuclear receptors (Chawla *et al.,* 2001). The synthesis of lipid-derived second messengers with "activation of protein phosphorylation cascades has emerged as one of the fundamental mechanisms of signal transduction in animal cells" (Hindenes *et al.,* 2000, Underhaug Gjerde *et al.,* 2004, Rozengurt *et al.,* 2005).

On one hand changes in membrane structure contribute to the transcellular transfer of biological information. On the other hand the plasma membrane directly participates in intracellular signaling to the nucleus. The major actors implicated in these responses are in the variety of the polar phosphoglycerides, their composition and relationship to and balance with the non-polar lipids that constitute the rafts and caveolae and the domains around the lipophilic proteins. Evidence now exists for functional roles for individual molecular species of the phospholipids. Phosphatidylserine (PS) exposure on the cell surface accounts for the alteration of activities of several membrane proteins, including P2X7 as well as Ca^{2+} and Na^{+} transport through the P2X7 channel. Moreover, highly specific molecular species and their derivatives have now been identified in signaling as with arachidonyl-stearoyl diacyl glycerol derived from phosphatidylinositol (PI) activating protein kinase C (Hindenes *et al.*, 2000).

Recent work has identified the topology of almost all the inner membrane proteins in *Escherichia coli* and advances in nuclear magnetic resonance spectroscopy now allow the determination of alpha-helical membrane protein structures at high resolution. There is a view that these transmembrane proteins in eukaryotes stride across the membrane without any direct connection with the lipids of the bilayer. The role of the lipid is explained as exerting lateral pressure on the protein with the degree of desaturation determining the liquidity of the system. However, one has to ask why would a protein sit in the bilayer and not anywhere else? Two plausible explanations come to mind. First, one end of the protein has an affinity to the extracellular or outer membrane environment whilst the other has an affinity for the opposite, inner membrane environment. These opposites could be in response to hydrogen ion, electrochemical gradients or differences in K^{+} and Na^{+} concentrations.

Trans-membrane proteins are commonly depicted as ribbons or cylinders. They are of course not like that. They are an arrangement of peptide groups, aromatic and heterocyclic structures all of which are polarisable. The likelihood is that the lattice network of the lipids, with their twisting and writhing unsaturated acyl groups will provide opportunities for a three dimensional marriage between the lipid and the protein. It is almost a certainty that an optimised relationship will be in

place in the bilayer. This is not a lock and key situation but then nor is it a stick in the sand. There has to be a thermodynamic relationship between the protein and the surrounding lipid which will be arranged to conform to the lowest energy in relation to the protein three-dimensional structure. The possibility of hydrogen bonding cannot be excluded.

Generally speaking, proteins can be separated from the membrane with mild conditions. By contrast, lipophilic proteins are difficult to crystallise and few structural determinations have been made, testifying to a strong protein-lipid interaction. So in effect there is a strong and weak force operating. One reason why the membrane protein lipid interaction is so poorly understood is because artificial membranes and reconstitution studies are frequently done with unreal membrane lipids e.g. studies of the hydrophilic loops in LacY from *E. coli* reconstituted in liposomes of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). The former does not naturally occur in mammalian phosphoglycerides.

The significance of microdomains influencing specific directions of signaling is also being recognised. Trans-membrane signaling requires modular interactions between signaling proteins, phosphorylation or dephosphorylation of the interacting protein partners and temporary elaboration of supramolecular structures, to convey the molecular information from the cell surface to the nucleus. Raft-based signaling pathways in T-lymphocytes have led to the suggestion of specific signaling compartments in raft microdomains. Moreover it has now become possible to visualize membrane proteins and lipids with atomic force microscopy (AFM) in living cells which describes a quite clear domain geography in the plasma membrane (Mouritsen, 2005).

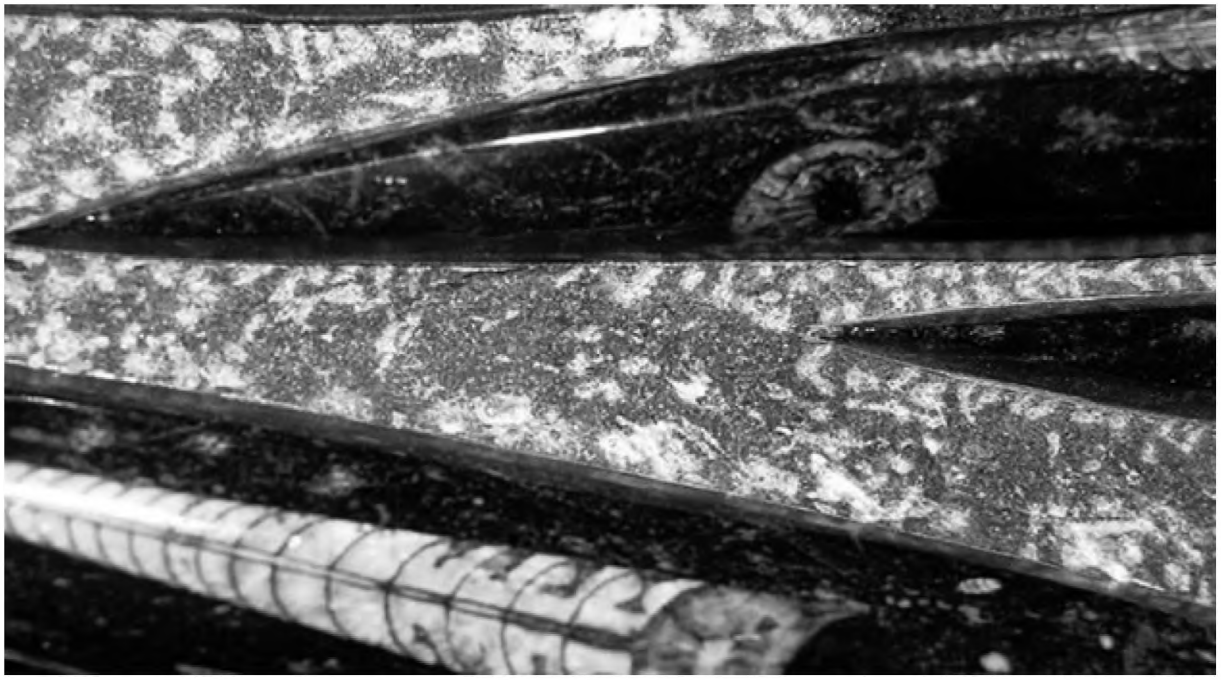

Fig. 2. The eye of orthocerus looking out from about 400 million years ago.

The language of DNA, proteins and lipids

There is a paucity of systematic information on lipids that are proximal and distal to membrane proteins. This is because of the difficulty of identifying the positions and measuring the relative effects of the lipids. There is a large number of lipid molecular species (words). A single double bond makes the difference to be or not to be a signaling molecule. This is not a liquidity issue as the difference made between say 4, 5, or 6 double bonds is minimal and insufficient to explain the striking conservation of molecules like DHA in signaling systems (Bloom *et al.*, 1999). The wealth of lipid molecular species is not unlike verbal language where several words can have similar meaning although a difference in subtle nuance. At the same time alteration of a single word in a sentence can change its function, for example replace "no" by "yes" or docosapentaenoic acid (DPA) by docosahexaenoic acid (DHA). Similarly the number of words in the language is an important determinant of the quality of expression and meaning. In that sense, the quality of the lipid language is undoubtedly of the highest biological order. The seductive robustness of the DNA is testified by the attempts to analyse the DNA of dinosaurs and mammoths which died out several thousand if not million years ago. By contrast the lipids most concentrated in signaling sites contain molecules most susceptible to peroxidative destruction.

Despite the wide genomic changes over the last 600 million years, DHA has been used in the photoreceptor and synaptic structures in the dynoglagelates, cephalpods, fish, amphibia, reptiles, birds and mammals, cf. Fig. 2. This extreme conservation is despite the fact that its immediate precursor all-*cis*-docosa-7,10,13,16,19-pentaenoic acid (ω3DPA - C22:5ω3), cf. Fig. 3, would have been more abundant, more readily synthesised and less susceptible to peroxidation (Bloom *et al.*, 1999). The omega-6 DPA would have also had the potential to replace DHA; however, it is seldom seen in natural products. It is the omega-6 product that appears in experimental omega-3 deficiencies. By contrast the omega-3 DPA is the major omega-3 in the cell lipids of the large herbivorous mammals (Crawford *et al.*, 1969) yet in none was the DHA replaced in the brain.

One could argue that for 600 million years whilst the genomes changed, the gene for DHA remained fixed; if so why? However, there are genes for enzymes involved in fatty acid synthesis but no known "gene for DHA". Conversely, as DHA stimulates the expression of over 107 genes involved in neural development, function and metabolism (Kitajka *et al.*, 2004), was the story the other way around? Was it the unique properties of DHA which determined the neural gene performance so rigidly for 600 million years? Nor can one neglect the role of rhodopsin which has similarly remained at the focal point of photoreception with its single molecule retinal from the lipid soluble vitamin A as the actual photon receptor. This remarkable consistency down some 600 million years during which genomics was in a fluid state of change, will have some, as yet unrecognised meaning for the manner in which cellular design was articulated during evolution of the species. At one conceptual level the protein could have been a determinant of the rigidity of structure and function.

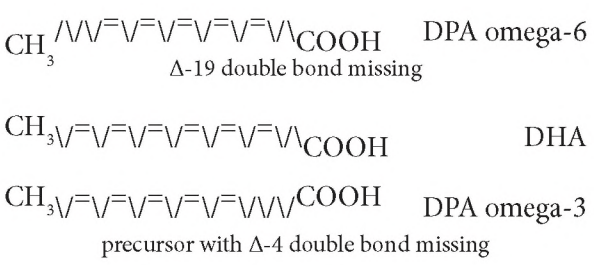

Fig. 3. DHA C22:6ω3 compared to two C22:5 fatty acids with only one double bond different, yet neither replaced DHA in 600 million years of evolution.

However there are two arguments against the protein determinacy. Firstly, the expectation of evolutionary change through measurements made on DNA modifications works in some but not all situations. Rhodopsin is not known for its variability throughout different species.

Secondly, analysis of the 450 million year puffer fish genome and that of other species describes a remarkable consistency for conserved gene families which, as an example, encodes trans-membrane proteins with fibronectin, immunoglobulin, leucine rich repeat domains (FIGLER) and the plasma clotting factors of 38 to 99% overall amino acid identity with their human counterpart (Munfus *et al.,* 2007). Hence some factor other than the standard rate of DNA mutation was operating to conserve certain functions. The 600 million year track record of DHA in visual and other signaling structures provides compelling evidence that the lipids were determinants of evolution.

The end of the Cretaceous and the evolution of mammals

The collapse of the dinosaurs is attributed to volcanism or a meteorite impact which invokes dramatic catastrophe as a sudden explanatory event. There could have been a simpler, Malthusian answer at work. A 120 ton dinosaur would consume 1,146,531 calories per day, on the assumption it was not far off current estimates. This calculation might be in error by several 10,000s or more but the giant dinosaurs would still have exercised a phenomenal rate of food consumption. The likelihood that the vegetation could keep up with this rate of consumption in face of a population explosion as suggested by the large number of fossils is questionable.

There are arguments that the giant carnivorous species should have kept the population in check but two interesting facts remain that are relevant to the subsequent phase of evolution. First, many of the plant systems escaped extinction. The late Early Silurian is associated with fossils of one of the earliest known fossil of vascular plant on land Cooksonia. That is about 425 million years ago. It was a small plant of only a few centimetres high. Propagation was by spores dispersed by the wind. The Triassic Period, 248-208 million years ago was connected with the evolution of the giant ginkgos, ferns and their allies and the giant reptiles. Ginkgos grow in many places and line the peripheral road that surrounds the Emperor's Palace in Tokyo for example. However, they are "bonsai" ginkgos compared to what they were 100 million years ago. The same applies to the ferns and their allies, except when you see them on the slopes of mountains whose trace elements have been replenished by volcanism as for example the higher slopes of the Ruwenzori or indeed in parts of New Zealand. This principle is well illustrated with for example the dramatic eruption of Mount St. Helens in 1980. Having destroyed much plant life even in the proudly well kept gardens of Portland, Oregon, the following years saw spectacular blossoms in Portland gardens and astonishing regeneration of the mountain slopes.

Secondly, the converse is the fact that the age of the dinosaurs was the age of the giant plants which lived in a moist, fertile soil. The dinosaurs when eating them would urinate and defecate into the swamps with the elements being lost in the run off into the rivers and oceans. Whether or not the carnivores could keep the population in check, the prodigious amount of vegetation eaten and the excretion of the trace elements would surely have depleted the soil so that the plant life would loose its giantness. Hence two natural forces were operating in opposite directions, the body size of the reptiles and the fertility of the soil. When demand outstripped the supply, collapse of the dinosaurs would have been inevitable. It may be that volcanism or meteorite impact was involved in the final demise. However, it may not have been necessary.

Interestingly, the depletion of the soil fertility would have added fertility to the oceans. Although the planet has lost its giants on land, we still have giants in the oceans: the blue whale. The nutrition of the whales provides another link to the determinacy of the lipids which will be returned to later.

This portrayal of the pre-history is somewhat simplistic. Nonetheless, a key issue of the collapse of the dinosaurs is that the gentler flowering plants, which in order to survive in the more depleted soil systems, developed well protected seeds. Seed bearing plants had been evolving for some time and so also was the co-evolution of the mammals and pollinating insects.

The Cretaceous, omega-6 fatty acids and mammals

The evolution of the mammals towards the end of the Cretaceous period resulted in a leap in relative brain size compared to any living system in the previous 500 million years. The cod may lay up to a million eggs at a time, whilst the land based reptiles would lay 12 or more. In each case the offspring get one dose of brain-specific lipid nutrients. The biological breakthrough for the evolution of larger brains came with the evolution of the placenta which perfuses the new off-spring with brain specific nutrients (Crawford, 2000). A model of the switch to the placenta can be constructed from the involvement of the arachidonic acid derived adhesion molecules attaching the fertilized egg to the uterine wall. The process of placentation begins with implantation of the blastocyst beneath the uterine epithelium and differentiation of trophoblast cell lineage into embryonic and extra-embryonic structures of conceptus (Cross, 1998). This invasive behaviour follows a precise chronology of vascular events during the first weeks of gestation (Kingdom *et al.*, 2000a; 2000b). These events involve placental tissue angiogenesis, organogenesis and progressive establishment of the two vascular circulations within the placenta in preparation for the second phase of pregnancy of fetal growth (Dantzer *et al.*, 2000). The placenta is a vascular system with the maternal blood circulating on one side of a membrane and that of the embryo/fetus on the other, i.e. the novelty in the evolution of placental mammals was a sudden increase in vasculogenesis.

This change in reproductive plan is unlikely to have been the result of a single mutation. It is more likely to have resulted from conditions which favoured vascularisation rather than calcification

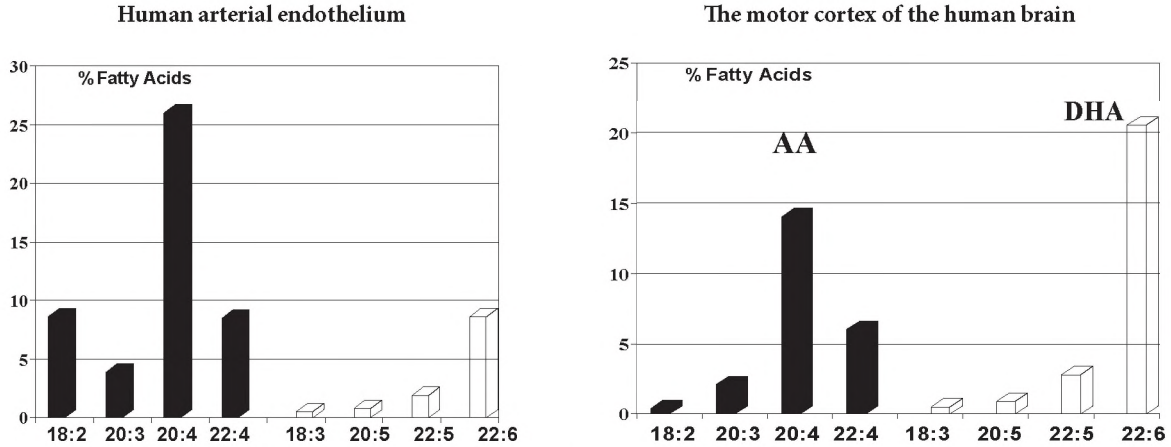

Fig. 4. Inner cell membrane lipid (ethanolamine phosphoglycerides) in the artery and in the brain. ■: ω-6 □: ω-3

(egg laying). Imprinting of the genetic mechanism would have followed. The end result leaves little doubt. The placenta is an extensive and extremely fast growing vascular network. In all mammals, this processing by the placenta supervises the early phases of brain development that eventually reached a peak in *H. sapiens.* The continuous perfusing of the product of conception provided a substantial biological advantage for brain growth compared to the one shot, external egg. The question to be asked is what was it that enabled the development of the placenta to take place?

The interesting question is how did the system of reproduction change from egg laying to placental mammals?

The difference between laying the egg externally and the entrapment of the embryo which adheres to the wall of the uterus could be explained by the several adhesion and angiogenic molecules that would have arisen along side the increasing concentrations of arachidonic acid (AA). The hypotheses we suggest is that this novelty in evolution was driven by omega-6 fatty acids and in particular AA. There is much money made and publicity given today about the value of fish oils, eicosapentaenoic acid (EPA) in particular, to suppress AA activity. Hence significant amounts

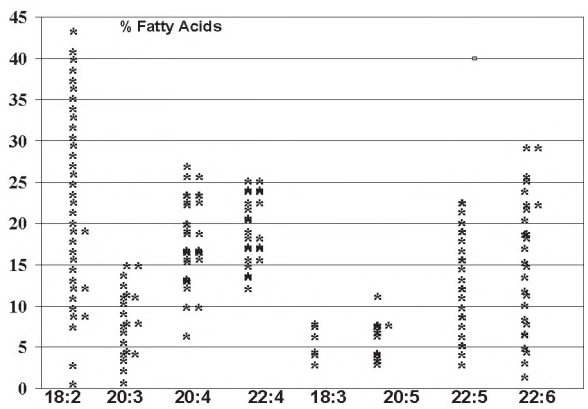

Fig. 5. Liver essential fatty-acid composition (ethanolamine phosphoglycerides).

of AA would have been necessary to change the biochemical scene. The inner cell membrane lipid of the human endothelium is AA rich as opposed to that of the brain which is DHA rich, cf. Fig. 4. The omega-3 fatty acids would have dominated the food chain during previous evolutionary epochs as the first 3 billion years of life was fed from photosynthesis of the marine type and on land by photosynthetic green leaves, resulted in an omega-3 rich food chain.

The evolution of the mammals coincided with the evolution of flowering plants with protected seeds. The seeds contained oil stored as energy for the growth of the new plant in the next season. For the most part the seed oil contained linoleic acid, the parent omega-6 fatty acid. Previously the fish living in an omega-3 rich environment required omega-3 fatty acids. A new evolutionary break point emerged alongside the introduction of a rich source of omega-6.

Whereas the omega-3 fatty acids are essential for the reproduction of fish, omega-6 is required for mammalian reproduction. The omega-6 family includes eicosatrienoic and eicosatetraenoic (AA) acids. Both these membrane fatty acids are precursors for vasodilatory, anti-platelet adhesion and anti-thrombotic eicosanoids. At the same time AA is the precursor for adhesion, thrombogenic and smooth muscle tone molecules. Moreover, the membrane lipids of the vascular endothelium and the placenta itself are very rich in AA. To test if this high concentration of AA in the placenta was either a collection during the course of pregnancy or intrinsic we studied the earliest placentas from elective abortions. In fact the proportions of AA in the membrane phosphoglycerides were higher at the beginning of pregnancy than at term, demonstrating that these high concentrations were intrinsic and not acquired (Bitsanis *et al.*, 2006). Indeed, the concentrations of AA in the earliest placentae were actually the highest we have seen in any mammalian, tissue phosphoglyceride.

Studies on the lipid language of the liver lipids cf. Fig. 5, in some 42 mammals described a striking species variability which was clearly dependent on the type of food eaten and velocity of body growth: the faster the rate of body growth the less DHA was synthesised. Even the rat pup brain incorporates DHA early and preferentially to its synthesis with an order of magnitude greater efficiency (Sinclair and Crawford, 1972a; 1972b).

If you are a large fast-growing mammal, the only solution to accumulating DHA is to eat it. Fish and sea food being the richest source suggests that the evolution of *H. sapiens* would have had a substantial advantage from being coastal over others who were living inland (Crawford, 1992; Leigh Broadhurst *et al.*, 1998; 2002; Crawford *et al.*, 1999). All the large inland mammals and primates simply loose DHA and brain capacity shrinks as they evolved larger and larger bodies with increased growth velocities. Small mammals such as the squirrel, *Sciurus carolinensis*, and cebus monkey, *Cebus capucinus*, have about 2.5% of their body weight as brain. The chimpanzee, *Pan troglodytes*, has 0.4%, the much bigger rhinoceros, *Perissodactyla rhinocerotidae*, less than 0.02%. The only parallel example of a large mammal with a relatively large brain body weight ratio is the dolphin, *Tursiops truncates*, which has a brain weight of 1.8 kg, a ratio of just over 1%, the sperm whale has 8 kg of brain. Albeit in a very large body it is massively greater than any seen on land. The dolphin 1.8 kg compares with the brain weight of a similar sized-lands mammal as for example that of the zebra, *Equus quagga*, is only about 350g. Modern humans with 1.4 kg have a brain capacity of 2% which is a bit smaller than that of the squirrel. So the chimpanzee losing out with 1 kg less of brain compared to *H. sapiens* can be explained by the lack of availability of preformed DHA in its chosen food web. The dolphin on the other hand is obviously constrained by the low availability of arachidonic acid as both AA and DHA would have been required to the successful evolution of the brain (see Fig. 6). The point is that *H. sapiens* does not really have a large brain compared to a squirrel.

What *H. sapiens* did was to find an ecological niche which enabled both body size and brain size to advance in a harmony of growth. The only niche that satisfies the biochemical requirements is the land-water interface, which during the period of evolution would have been the richest food resource on the planet.

The fatty-acid composition of the motor cortex of the brain in 42 species, cf. Fig. 6, shows that it is closely identical regardless of species and dietary strategies (Svennerholm, 1968; Crawford and Sinclair, 1972; Crawford *et al.*, 1993; 1976; Williams *et al.*, 1987). The variable is the extent to which the brain evolved not the chemistry. Variation is a determinant of function (Messeri *et al.*, 1975). In this context AA and its elongation product contribute quantitatively to neural structures similar to the total omega-3 content. Moreover, there is more long chain omega-6 than omega-3 in the brain where the DHA is mostly concentrated in the synapses where it is preferentially taken up (Suzuki

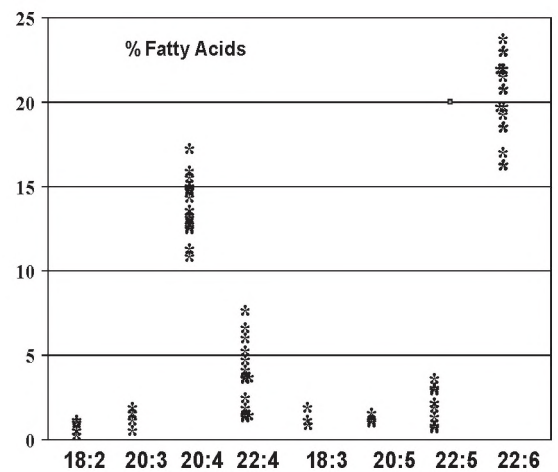

Fig. 6. Brain essential fatty-acid composition (ethanolamine phosphoglycerides). Adapted form Crawford *et al.* (1976) and subsequent publications and data.

et al., 1997). Consequently the addition of a rich source of omega-6 fatty acids in the build up to mammalian evolution by the Cretaceous period the evolution of the omega-6 requiring mammals, vascular and cerebral expansion is unlikely to be a coincidence. It is more likely that the disparate evolutionary paths of relative brain size on land and in the sea were defined by the availability of AA and DHA preformed together, with their associated micronutrients such as iodine, selenium, copper and zinc etc. Today, according to the World Heath Organisation, there are about 1.6 billion people at risk to iodine deficiency disease, the simplest way to produce mentally retarded children. That is nearly a fifth of the human population and the people at risk are inland communities. As the same communities are those at high risk to vitamin A deficiency it would be unsurprising of they we also relatively deficient of omega-3 fatty acids, DHA in particular.

A good test of the concept of lipids as evolutionary determinants is the radiation of the land mammals from their omega-6 food web into the omega-3 rich marine environment which started over 50 million years ago. We reported significant amounts of AA in the muscle, liver and brain of dolphins (Williams *et al.*, 1987). However, the dolphin was one of the most recent migrations into the sea, the gray whale one of the most ancient. The Table 1 below shows results from work being done on the migration of the gray whale from the Bering Sea in the Arctic to their breeding lagoons in Bahía Magdalena (Caraveo-Patiño *et al.,* 2008).

	Blubber biopsies n=6			Liver n=2		Muscle biopsies n=4			
Fatty Acid	Triglyceride			PC	PE	PC		PE	
18:2 ω6	1.8	±	0.4	0.39	0.22	0.39	±0.02	0.17	±0.06
18:3 ω3		nd		0.02	0.02	0.01	±0.02	0.02	±0.02
20:4 ω6	1.5	±	0.3	5.76	16.93	4.72	±0.20	10.08	±0.49
20:5 ω3	18.1	±	7.9	2.71	5.52	7.47	±0.37	15.44	±0.33
22:6 ω3	7	±	2.8	6.44	8.07	1.18	±0.03	3.49	±0.31

Table 1. Gray whale muscle and liver contain significant amounts of AA (data from a paper by Caraveo-Patiño *et al.* (2008)).

As the cold polar food web where the whales spend most of the summer are particularly omega-3 rich, the observations by Caraveo-Patiño pose the question from where are they getting their AA which exceeds the DHA in liver and muscle inner cell membrane lipid the ethanolamine phosphoglycerides (PE). The AA is less but still prominent in the largely outer membrane lipid, the choline phosphoglycerides (PC).

These whales are large animals and the proportion of AA in an adult gray whale works out at about 0.2 ton of AA rich lipid with about 1/10 as AA. Caraveo-Patiño *et al.* (2008) speculate that the reason for this longest of migrations is to obtain AA which is present in significant amounts in the warm water food web. This surprising conserva-

tion of AA in marine mammals again emphasises the role of lipids in determining evolutionary directions. What is interesting about this example is that the lipid requirements are not only constraining the biochemistry but also the non-genomic behaviour in the migration of this large mammal. Note that the blubber contains little detectable AA which is all sequestered for cell membranes.

The evidence on (i) the extreme conservation of DHA in the photoreceptor, neurones and synapse taken together with (ii) the coincidence evolution of omega-6 fatty acids with the mammals raise interesting possibilities that the lipids were actually determinants of evolution. Similarly, the use of AA for mammalian reproduction has been conserved for the best part of 200 million years and in the case of the gray whale, conserved despite living in an omega-3 rich environment for over 50 million years. That is, lipid conservation outstretches the genomic changes over the last 600 million years with DHA and the last 100-200 million years for AA in mammalian evolution. No matter what genomic changes occurred in the 600 million years since the Cambrian explosion, gene mutation did not find an alternative to DHA. So was it the other way round? Was the genomic capability constrained by the lipidomics and other environmental factors? Are these constraints, examples of Darwin's "conditions of existence"?

This evidence bears strongly on the final evolution of the human brain which could not have been on the savannahs of Africa and had to be at the land-water interface to satisfy these powerful twin requirements for AA and DHA. The land water ecological niche provided the best of two world's omega-6 from land and omega-3 from the marine and fresh water systems. As Philip Tobias said at his seminal "Dual Congress" on biology and paeleo-anthropology, in Sun City, South Africa in 1998 "wherever man was evolving, he had to have water to drink".

It is clear that the constraints by chemistry and physics on evolution are not just confined to lipids. Many other examples can be found such as the use of iron in heme proteins and indeed was a thesis of Ernst Baldwin (1947) then professor of biochemistry in London and discussed at a PNAS conference in 1954 (Woodring, 1954). In 1954 the influence of nutrition on the cell membrane and its impact on signaling and gene expression was unknown. The evolutionary paradigm was the Weismann (1893) "all sufficiency of natural selection" so people bent over backwards to interpret systems in this context. Stephen J. Gould raised a serious question about the all sufficiency paradigm pointing out the sudden breaks in evolution followed by long periods of stasis, "punctuated evolution" which seemed inconsistent with the Weisman view adopted by many modern evolutionary biologists (Gould, 1982). It therefore seems that there were forces at work in evolution which as Darwin claimed were more powerful than natural selection and by implication than genomics. This force was Darwin's "conditions of existence" and his pangenomics which now finds evidence in epigenetics and the influence of external conditions on membrane physics, signaling and gene expression. The compelling fact about the lipids is to repeat what has been said, the DNA does not change in a century so nor do the proteins, it is the membrane lipids that changed and were associated with a change in size shape and disease pattern in one century and with still more to come. The introduction of cell complexity and organisation depending on the elaboration of complex lipid membranes in the Cambrian explosion, suggests that lipids have played a pivotal role in evolution since then.

The implication for nutrition, food and health policy in this century and the questions raised by the dependence of the human brain and vascular systems on specialised lipids cannot be underestimated. With brain disorders having overtaken all other burdens of ill health in the EU and

predicted by the Global Forum of Health (www.g;obalforumhealth.org) to be in the top three burdens of ill health worldwide by 2020 the gravity of the present situation is supreme. At stake is the future of children yet to be born.

Summary

Unlike DNA, lipid composition varies with nourishment and environment (chemistry, temperature and pressure). The DNA does not change in composition in response to diet so neither does protein composition change. The cell factor that readily changes in response to both environment and diet is the lipid component. Just as the environmental medium set the unchanging life forms for the first 2.5 billion years of prokaryotic life on the planet, is it possible that the influence of environment and the chemistry of the food web on lipids affected protein function, signaling and gene expression in a manner that actually set the boundaries and paths for evolution? In that way the lipids would have been regulating and defining the subsequent aerobic evolutionary gene potential and metabolic pathways consistent with Darwin's concept of "conditions of existence". If so, then with the changing lipid environment since the domestication of plants and animals in the Fertile Crescent 10,000 years ago, it is likely that human evolution is still being manipulated by lipidomics. The changing pattern of non-communicable disease is evidence of this evolutionary process in action. There is little doubt in Europe and America people have changed in shape, size and disease pattern in one century. That change is not due to a change in genome. The continued evolutionary change of *Homo sapiens* is beginning to be suggested by others (World Science, 2007) as affecting physical and mental states. The concern today is the rise in brain disorders which have now outstripped all other burdens of ill health in the European Union and pose what is by far the most serious threat in relation to health in society. This discussion raises concerns about present and hence future food and aquatic policies which have yet to take into consideration the impact of nutrition on neural development and degeneration.

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Omega-3 fatty acid deficiencies and the global burden of psychiatric disorders

Joseph R. Hibbeln

Abstract

Neuropsychiatric illnesses constitute a large burden of global ill health and nutritional deficiencies of omega-3 essential fatty acids are emerging as reversible and preventable risk factors. Recommendations by the American Psychiatric Association support treatment efficacy for major depression in adulthood. Emerging clinical intervention data indicate that homicide, personality and substance abuse disorders respond to alleviation of these deficiencies. Neuropsychiatric manifestations deficiencies in long chain omega-3 in the fetus may include low verbal IQ in childhood and abnormal social behaviors, potential developmental indicators of a deviant social trajectory towards substance abuse and violence. Public advisories for fertile or pregnant women to limit seafood intake may encourage nutritional deficiencies in lipids critical to optimal neural development and inadvertently increase risk for the harm they intent to prevent. In the US alone supplementation with 1,800 mg/d of long chain omega-3 fat is estimated to prevent 384,303 hospitalizations due to cardiovascular disease and save USD 3.1 billion over 5 years. Similar reductions in misery and economic costs could be expected for the reduction in neuropsychiatric illnesses.

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Key words: fatty acids omega-3, fatty acids omega-6, depression, violence, aggression, neurodevelopment, burden

Introduction: World burden of illness potentially attributable to omega-3 deficiencies

Neuropsychiatric disorders, primarily major depression, substance abuse disorders and violence, currently account for 14% of the global burden of disease (Prince *et al.*, 2007). In 2020, the top three leading causes of disability-adjusted life-years are projected to be ischemic heart disease, unipolar major depression and motor vehicle accidents which are often substance abuse related (Murray and Lopez, 1997). Violent and excessively aggressive behaviors are significant threats to public health, and the prevention of injury has been identified as a policy priority by the U.S. Surgeon General (Satcher, 1995; US Department of Health and Human Services, 2005). Developmental pathologies of the brain (Liu and Wuerker, 2005), potentially exacerbated or caused by nutritional deficiencies in omega-3 essential fatty acids

(Hibbeln and Salem, 1995) may in part explain the increased prevalence of these prevalent afflictions. While other major morbidities, such as cardiovascular disease, are wide accepted to be linked to dietary factors (Hu and Willett, 2002) the perception of mental ill health as an emergent symptomotology of nutritional deficiencies of omega-3 essential fatty acids has been slow to emerge despite knowledge of their importance to biophysical, biochemical and neurodevelopmental mechanisms (Salem *et al.*, 2001). Omega-3 long-chain fatty acids (omega-3 LCFAs: eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids), found primarily in fish oil, are essential nutrients for humans and therefore must be obtained through the diet. Western society may be in a global state of nutritional deficiency of these nutrients, especially compared to diets which permitted evolution of hominid encephalization and optimal neuronal function (Crawford and Sinclair, 1971; Cunnane *et al.*, 2007). Global economic changes in the use of seed oils rich in the omega-6 fatty acid linoleic acid may cause functional deficiencies in omega-3 fatty acids by displacing eicosapentaenoic acid and docosahexaenoic acid from membranes and preventing efficient elongation and desaturation (Hibbeln *et al.*, 2006). The understanding on the utility of long chain omega-3 fatty acids and the competitive effects of omega-6 fatty acids could lead to the development of treatments and prevention strategies to reduce the burden of depressive and aggressive disorders at low cost with global applicability. Here we will examine the hypothesis that omega-3 fatty acid deficiency in at least two developmental periods increases risk for aggressive and depressive behaviors.

Treatment efficacy for major depression

First, the treatment efficacy for long chain omega-3 fatty acids in major depression in adults has recently been supported by treatment recommendations issued by the American Psychiatric Association (Freeman *et al.*, 2006). The large effect size ($g=0.57$, $p<0.0008$) confirmed in a meta-analysis of 9 randomized placebo controlled trials, which was a larger treatment effect size for most antidepressant medications. Because depression is frequently co-morbid with cardiovascular disease and obesity, the treatment recommendations were formulated to follow the American Heart Association Recommendations for primary and secondary prevention (Kris-Etherton *et al.*, 2003). Three similar meta-analyses have found similar effect sizes for the treatment of major depressive illnesses (Appleton *et al.*, 2006; Lin and Su, 2007; Ross *et al.*, 2007). These data indicate that major depressive illnesses may, in part, be a reversible manifestation of omega-3 fatty acid deficiency in adulthood. Given this evidence of treatment efficacy, a new perspective can be taken on the ecological data which indicates that the risk of major depression is 50-fold greater among countries with the lowest seafood consumption (Hibbeln, 1998). Causal interpretations need not be debated regarding these ecological data; alternatively they can be used to estimate the burden of depression potentially treatable by ensuring adequate essential fatty acid nutrition.

Emerging data in homicide, personality disorders and substance abuse

Additional neuropsychiatric manifestations deficiencies in long chain omega-3 in adults may include a predisposition for personality disorders, substance abuse and impulsive or violent behaviors. Treatment effect sizes for the reduction of violent and impulsive behaviors appear to be a large as for major depression: for example a 50 % reduction in anger among 35 aggressive polysubstance abusers (Budens-Branchey Hibbeln *et al.*,

in press) and a 45 % reduction in time contemplating suicide among patients who had been referred to an emergency room for self injury (Hallahan *et al.*, 2007). In ecological studies homicide can be considered as a surrogate measure of aggression as it is an extreme case of violent behavior. Mortality data are particularly useful as the definition of homicide is consistent across countries and data are prospectively collected for whole populations. Cross-national ecologic data indicate that there is an inverse relationship between seafood consumption, a surrogate of omega-3, LCFA intake, and rates of death by homicide ($r = 0.63$, $p<0.0006$, $n = 36$ countries) (Hibbeln, 2001). Whereas tissue compositions of EPA and DHA can be increased by greater seafood consumption, these levels can be decreased by greater consumption of competing omega-6 fatty acids, in particular linoleic acid, found principally in seed oils (Lands *et al.*, 1992). In addition, greater linoleic acid consumption, estimated from economic disappearance data, has been found to have a direct relationship with homicide rates across five countries ($r=0.93$, $p<1 \times 10^{-40}$) between 1960 and 1999 (Hibbeln *et al.*, 2004). Differences in apparent intake of linoleic acid ranged from approximately 1% of calories in the United Kingdom in 1960 to nearly 10% of calories in the U.S. in 1999 and represent the world's diversity of linoleic acid intake (Hibbeln *et al.*, 2006) and correlates with a 100-fold difference in rates of homicide mortality (Hibbeln *et al.*, 2004).

Observational and intervention studies of human subjects are consistent with the cross-national data described above, suggesting that low omega-3 levels are associated with aggression. Virkkunen and others were the first to report that violent and impulsive offenders had lower plasma concentrations of DHA than non-impulsive offenders and healthy controls (Virkkunen *et al.*, 1987). Previously, Fiennes *et al.* (1973) has reported severe behavioural pathology and hepatic lipid infiltration in a primate model of omega-3 deficiency in the presence of excess linoleic acid. Higher adipose concentrations of linoleic acid were strongly associated with Type A personality among Cretan adults (Mamalakis *et al.*, 1994). Lower plasma DHA correlated with greater neuroticism and lower agreeableness on the NEO personality inventory in a normative population (Conklin *et al.*, 2007). In 6- to 12-year old boys, a greater number of behavior problems, temper tantrums and sleep problems were associated with lower total omega-3 fatty acid concentrations in comparison to controls (Stevens *et al.*, 1996). The CARDIA study found that greater seafood consumption amongst 4,000 subjects was associated with lower scores on the Cook-Medley hostility scale, irrespective of gender or ethnicity (Iribarren *et al.*, 2004). Several double-blind, placebo-controlled intervention trials have been conducted to assess the efficacy of omega-3 fatty acids in reducing hostility, an affective state closely related to anger and aggression. Although not specifically designed to assess psychometric changes, hostility and depression scores were reduced by a high fish diet over the course of five years (Weidner *et al.*, 1992). Hamazaki *et al.* (1996), reported that 1.5 to 1.8 g/d of DHA reduced measures of hostility in a picture frustration test among Japanese students, indicating that omega-3 fatty acids may reduce aggression during stress in normal subjects. It is interesting to note that the baseline plasma DHA composition of this group was 3.0%, compared to typical American levels of approximately 1.5%. Small decreases in hostility measures among Thai University employees after two months of supplementation with 1.5 g/d of DHA compared to placebo (Thienprasert *et al.*, 2000). Investigators in Boston (Zanarini and Frankenburg, 2003) reported large decreases in verbal and physical aggression among women with borderline personality disorder with EPA monotherapy. Although not primarily examining aggression, investigators at Oxford, UK (Richardson and Montgomery, 2005) found decreases in

disruptive behavioral disorders among children with developmental coordination disorders and a high prevalence of attention deficit hyperactivity disorder upon supplementation of 558 mg/d EPA and 174 mg/d DHA. In a study of Japanese children (9-12 y/o), (Itomura *et al.*, 2005) one group supplemented with fortified foods providing 840 mg/week EPA and 3,600 mg/week DHA, while the control group ate unfortified foods. Measures of hostility and symptoms of attention deficit hyperactivity increased in girls in the control group who had no changes in EPA and DHA from baseline, but whose RBC linoleic acid levels increased. These data may indicate that foods low in EPA/DHA and high in linoleic acid increase the risk of hostility and attention deficit hyperactivity disorder. One of the most provocative reports has been that a cocktail of multivitamins, mineral and essential fatty acids (including about 180 mg of EPA plus DHA) reduced felony level violence among prisoners by 37% (Gesch *et al.*, 2002). These studies suggest that residual behavioral problems may be reduced or reversed in childhood, adolescence and adulthood or at least concurrently treated by the increased use of omega-3 and decreased use of omega-6 essential fatty acids.

Early development

Neuropsychiatric manifestations deficiencies in long chain omega-3 in the fetus may include low verbal IQ in childhood and abnormal social behaviors, potential developmental indicators of a deviant social trajectory towards substance abuse and violence. Deficiencies in long-chain essential fatty acids during critical periods of prenatal and childhood neurodevelopment may result in a residual predisposition towards aggressive and depressive behaviors (Hibbeln *et al.*, 2006). Possible mechanisms include impaired neuronal migration, connectivity, timed apoptosis, and dendritic arborization, such that there is an irreversible disruption in the neuronal pathways that regulate behavior (Sinclair *et al.*, 2007). Although it is well established that omega-3 fatty acids are important for optimal brain function during infancy (Willatts *et al.*, 1998), data regarding the persistence of these neurodevelopmental effects into childhood and/or adulthood are just beginning to emerge in the literature.

The developing fetal nervous system is especially at risk of neurodevelopmental abnormalities when mothers do not eat sufficient long chain omega-3 fatty acids. The richest sources of long chain omega-3 fatty acids are from seafood for which there is strong (Crawford and Sinclair, 1971; Broadhurst *et al.*, 1998) and increasing (Marean *et al.*, 2007) evidence for the contribution of seafood to the evolution of human encephalzation, socialization and intellect. Marean *et al.* (2007) have recently discovered fossil evidence for what is described as "the earliest appearance of a dietary, technological and cultural package that included coastal occupation, bladelet technology, pigment use and dietary expansion to marine shellfish, and is dated to a time close to the biological emergence of modern humans". In 2004 however, the US Government, advised pregnant women to limit fish intake to less than 340 gm/w to avoid potential harm exposure to trace levels of methylmercury. However, this advisory did not consider the potential harm to the fetus caused by deficient intakes of long chain omega-3 fatty acids. Thus, we considered both the risks and benefits of limiting seafood consumption among a population of 14,541 mother and infant pairs in a large longitudinal study in England (Hibbeln *et al.*, 2007).

When mothers were in compliance with the advisory and consumed less than 340 g/wk of seafood, their children were more likely to have low verbal IQ at age 8, and greater risk of abnormal social behaviors throughout childhood. These findings remained significant after considering 29 potential confounding variables of social class

dietary food group groups and dietary intakes of methyl-mercury. Inclusion of this variable increased the risks of low verbal IQ attributable to low seafood consumption (odds ratio for no maternal seafood consumption 1·98, 95% CI 1·39–2·81, and for 1–340 g/week 1·34, 1·05–1·72, compared with >340 g/week; trend p=0·0001) despite a small risk of low verbal IQ from methyl-mercury exposure (odds ratio for one SD increase 1·14, 95% CI 1·02–1·27, p=0·0229). When expressed as verbal IQ points, children of mothers with no seafood consumption were at risk of having -2.15 (lower) verbal IQ points (95% CI -4.33, -0.04) compared to children whose mothers exceeded 340 g/w and children of mothers who consumed between 1 and 340 g/ w of seafood were at risk of having - 0.61 (lower) verbal IQ points (95% CI -2.08, 0.86) compared to children whose mothers consumed more than 340 g/w, with a three group trend of $p<0.05$. The estimated linear effect for methyl-mercury was –0.14 (lower) verbal IQ points [95% CI -0.82, 0.54] per μg of intake with an overall loss of 0.32 verbal IQ points (despite a gain of 2.15 verbal IQ points) comparing children with no maternal intake to those with more that 340 g/w.

Perhaps if women ate seafood with no methyl-mercury another 0.32 verbal IQ points may have been gained. However, the nutritional benefits of sea food intake outweighed the small adverse effect of methyl-mercury, confirming our conclusion that to limit consumption to 340 g/week is probably detrimental and nutritionally inadequate. Here we consider a minimum to 340 g/w of seafood during pregnancy as necessary to meet criteria for nutritional adequacy as defined by protecting the majority of the population from risk of harm, defined as increased risk of lower verbal IQ.

Calculation of dietary intakes needed to obviate deficiencies

Essential poly-unsaturated fatty acids compete with each other as the same enzyme systems are used by both omega-6 and omega-3 fatty acids for elongation desaturation and transformation in to biologically active eicosanoids and docosanoids. Excessive linoleic acid in the international food supply appears to have created functional deficiency of long chain omega-3 fatty acids (Lands *et al.*, 1992). In order to calculate healthy intakes of omega-3 fatty acids that meet RDA criteria, the worldwide diversity of dietary intakes of omega-6 and omega-3 fatty acids influences tissue compositions of omega-3 LCFA were considered by (Hibbeln *et al.*, 2006). Deficiency in omega-3 LCFA's was defined as attributable risk from 13 morbidity and mortality outcomes, including all causes of death before the age of 75, coronary heart disease, stroke, cardiovascular disease, homicide, bipolar disorder, and major and post-partum depressions. Dietary availability of omega-3 LCFAs from commodities for 38 countries and tissue composition data were correlated by best fit to each illness in deficiency risk models. We found that the potential attributable burden of disease ranged from 20.8% (all-cause mortality in men) to 99.9% (bipolar disorder). Omega-3 LCFA intake for Japan (0.37% of energy, or 750 mg/d) met criteria for uniformly protecting 98% of the populations worldwide. Omega-3 LCFA intakes needed to meet a tissue target representative of Japan 60% omega-3 in LCFA, ranged from 278 mg/d (Philippines, with intakes of 0.8% of energy as linoleate, 0.08% of energy as alpha-linolenate, and 0.06% of energy as arachidonic acid) to 3667 mg/d (United States, with 8.91% of energy as linoleate, 1.06% of energy as alpha-linolenate, and 0.08% of energy as arachidonic acid). With caveats inherent for ecologic, nutrient disappearance analyses, a healthy dietary allowance for omega-3 LCFAs for current US diets was estimated at 3.5 g/d for a 2000-kcal

diet. This allowance for omega-3 LCFAs can likely be reduced to one-tenth of that amount by consuming fewer omega-6 fats. As the availability of omega-6 fatty acids, which compete with omega-3 LCFAs for incorporation in cell membranes, in the international food supply has increased, so have homicide rates in 26 countries. Thus, these ecological data are consistent with current observational and interventional data indicating that a substantial proportion of depressive and violent behaviors may be manifestations of nutritional deficiencies in omega-3 essential fatty acids and considered as targets for public health interventions.

Conclusion

A large global burden of ill health due to cardiovascular disease and neuropsychiatric illnesses appears to be attributable to diets deficient in omega-3 LCFA and potentially exacerbated by excesses in dietary intakes of the omega-6 fatty acid linoleic acid. We note that our estimations of adequate dietary intakes may be conservatively low as we used tissue targets of phospholipid composition in Japan, where in a similar population the JELIS trail reported that supplementation with an additional 2 g/d of ethyl ester EPA resulted in significant reductions in cardiovascular events (Yokoyama *et al.*, 2007). It is remarkable that further prevention effects were seen in this population where plasma EPA levels may be 5-fold higher than in the US (Yokoyama and Origasa, 2003). While efficacy has been well described for reduction in cardiovascular mortality and, from existing trials for major depression, aggressive and substance abuse disorders also seem to be substantially decreased by ensuring nutritional adequacy. The Lewin group Inc. recently used US Congressional budget office techniques to conduct an economic analysis balancing the cost of supplementing seniors in the US alone with omega-3 LCFAs and savings benefit to cardiovascular health (DaVanzo *et al.*, 2006). The estimate of the five year net savings in hospital expenditures and physician charges resulting from a reduction in the occurrence of coronary heart disease among the over age 65 population through daily intake of approximately 1800 mg of omega-3 fatty acids was $3.1 billion. They calculate that approximately 384,303 hospitalizations due to CHD could be avoided across the five years. Perhaps even more money can be saved, and misery prevented, by the simultaneous reduction in neuropsychiatric illnesses which cost the European Union States 386 Billion Euros for the 25 member states at 2004 prices (Andlin-Sobocki *et al.*, 2005).

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Mechanisms of adaptation of the retina to oxidant stress

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Abstract

One of the dilemmas in research on omega-6 and omega-3 poly-unsaturated fatty acid (PUFA) metabolism is how these fatty acids, which are so susceptible to non-enzymatic oxidation, can be neuroprotective in an oxidant stress environment. To address this issue in the retina, we designed a series of experiments to test the hypothesis that the products of PUFA oxidation up-regulate the expression of endogenous neuroprotective pathways. 4-Hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) were produced during light stress and decorated retinal proteins. When provided in cell culture in sub-lethal amounts, 4-HNE protected the cells from subsequent challenge with hydrogen peroxide. The transcription factor Nrf2 was found to be up-regulated in the cells incubated in 4-HNE, and was responsible for increased expression of three proteins, thioredoxin (Trx), thioredoxin reductase (TrxR), and heme oxygenase-1 (HO-1).

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Key words: retinal light adaptation, 4-hydroxynonenal (4-HNE); 4-hydroxyhexenal (4-HHE), nuclear-factor-E2-related factor 2 (Nrf2), lipid peroxidation, thioredoxin, thioredoxin reductase, heme oxygenase-1 (HO-1)

Introduction

The photosensitive rod outer segment (ROS) membranes of the retina contain the highest level of DHA of any mammalian membrane (Fliesler and Anderson, 1983). Over 30 years ago, we established a role for DHA in retinal function by showing that dietary restriction of omega-3 PUFA in rats led to a reduced a- and b-wave of their electroretinogram (Benolken *et al.*, 1973; Wheeler *et al.*, 1975). Other laboratories made the same observation in rats (Watanabe *et al.*, 1987; Bourre *et al.*, 1989) and guinea pigs (Weisinger *et al.*, 1996).

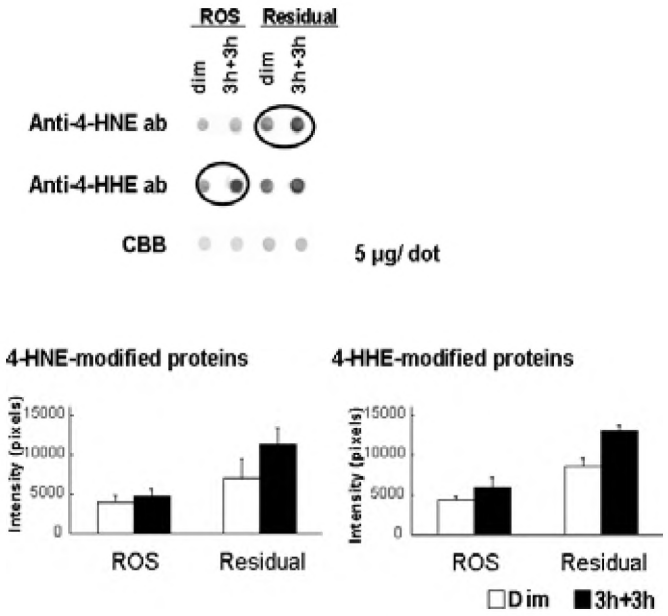

Fig. 1. Western dot blot for 4-HNE- and 4-HHE-protein modifications in ROS and residual retinal fractions. The eyes were enucleated before (dim, 5 lux) and after 3h of light exposure at 5,000 lux followed by 3h of dim light. The circled areas illustrate dim/bright differences. (Upper panel)-Representative western dot blots of ROS and residual retinal fractions. CBB, Coomassie Brilliant Blue. (Lower panel)- Densitometric analysis of dots. [Reprinted from Tanito *et al.* (2005a) with permission from Invest. Ophthalmol. Vis. Sci.]

Studies in monkeys (Neuringer *et al.,* 1984) and human infants (Carlson *et al.,* 1993; Uauy *et al.,* 1990; Birch *et al.,* 1992; 1998) demonstrated the importance of dietary sources of omega-3 fatty acids in the development of the visual system. Thus, development and optimal electrical function of the retina depend on an adequate supply of DHA in the developing fetus and in the diet thereafter.

DHA has also been found to have a neuroprotective function in retinal neurons. Supplementation of cultured retinal neurons with DHA protects the cells from oxidant stress-induced apoptosis (Rotstein *et al.,* 1997; 2003). A molecular explanation for this effect was recently demonstrated in a seminal paper by Mukherjee *et al.* (2004) that described the stress-induced production of a bioactive molecule from DHA, which was named neuroprotectin D1 (see review by Bazan (2007) and Dr. Bazan's chapter in this volume).

The retina exists in a paradoxical state. On the one hand, it is challenged daily with light and a high oxygen flux, which work in concert to produce reactive oxygen species that can destroy PUFA and lead to cell death. On the other hand, high levels of DHA are necessary for development and optimal function of the retina. To survive, the retina has established elaborate defense mechanisms that can protect it from oxidant stress-induced cell death.

Role of oxidant stress in retinal degeneration

Previous studies have suggested that oxidant stress plays a major role in retinal degenerations, including age-related macular degeneration (Winkler *et al.,* 1999; Beatty *et al.,* 2000) and diabetic retinopathy (van Reyk *et al.,* 2003; Kanwar *et al.,* 2007). We

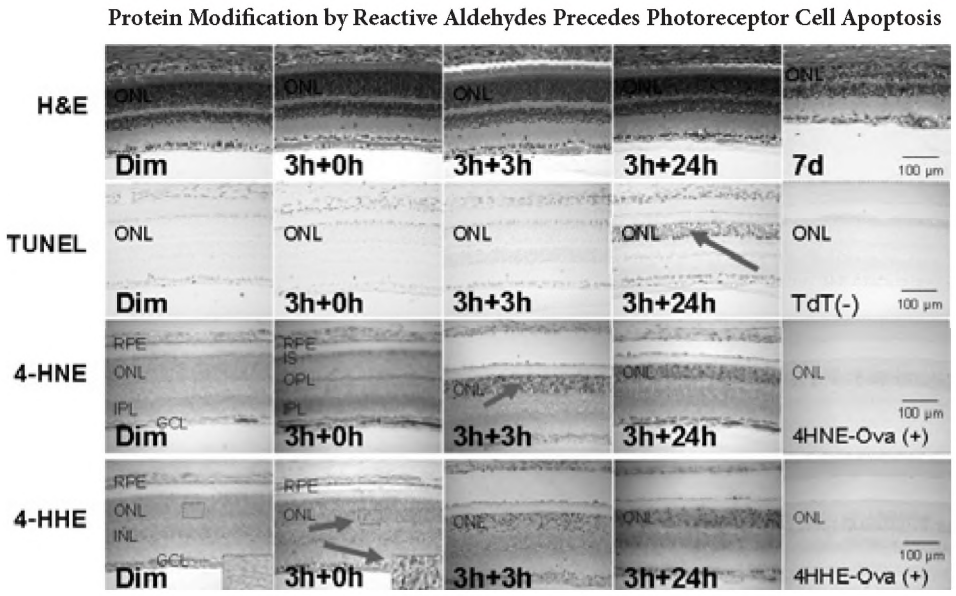

Fig. 2. H&E, TUNEL, and immunohistochemical staining for 4-HNE- and 4-HHE-protein modifications. Eyes were enucleated before (dim, 5 lux) and at 3h+0h (time in light stress followed by time in dim light), 3h+3h, 3h+24h, and 3h+7d of light exposure at 5,000 lux. Representative images at 1 mm superior from the optic nerve head are shown (5 rats analyzed in each group). (First panel)- H&E staining. Severe loss of ONL is observable at 3h+7d. (Second panel)- TUNEL staining. Nuclear staining was observed in ONL at 3h+24h (red arrow). TdT(-), no TdT enzyme (control) on the same section of 3h+24h. (Third panel)- Immunohistochemistry for 4-HNE-modified proteins. Nuclear staining was observed in the ONL at 3h+3h and later (blue arrow). The 4-HNE-OVA(+) blocking experiment was done on a 3h+24h section. (Lower panel)- Immunohistochemistry for 4-HHE-modified proteins. Nuclear/perinuclear staining was observed in the ONL (inset) at 3h+0h (blue arrows). Nuclear staining in the ONL became more dramatic at 3h+3h and 3h+24h. The 4-HHE-OVA(+) blocking experiment was done on a 3h+24h section. [Reprinted from Tanito *et al.* (2005a) with permission from Invest. Ophthalmol. Vis. Sci.]

study oxidant stress in the retina using an acute light damage model (Ranchon *et al.,* 2001). Albino rats born and raised in dim cyclic light will loose a significant number of their rod photoreceptors if placed in bright (usually 3,000-5,000 lux) for 3-6 hours. Under these conditions, the central superior region of the retina is most susceptible to damage. The role of lipid peroxidation in retinal degeneration was determined by studying the production and distribution of 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE), which are reactive aldehydes derived from the non-enzymatic oxidation of omega-6 and omega-3 PUFA, respectively. This review summarizes some of our recent studies (Tanito *et al.,* 2005a; Tanito *et al.,* 2006; Tanito *et al.,* 2007).

Albino rats were stressed in 5,000 lux light for 3 hours followed by 3 hours in dim light, after which retinas were removed, homogenized, and assessed by densitometric analysis of semi-quantitative western dot blots using 4-HNE and 4-HHE specific antibodies. Protein modifications by 4-HNE and 4-HHE increased in retinal tissues after exposure of rats to high intensity light (Fig. 1), showing that acute light stress leads to lipid peroxidation in the retina.

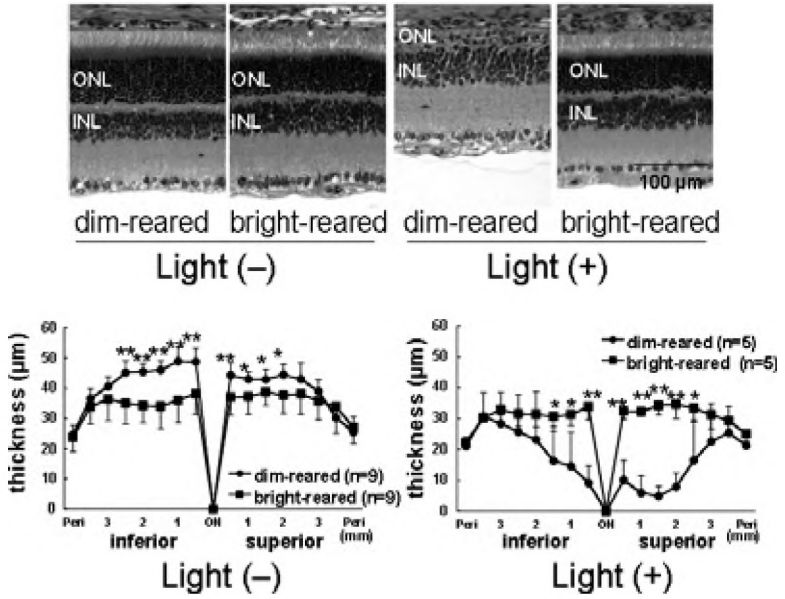

Fig. 3. Quantification of the outer nuclear layer thickness and area. (Upper panel)- Representative retinal sections of at 1-1.5 mm superior to the optic nerve head stained with H&E. ONL, outer nuclear layer; INL, inner nuclear layer. Note the severe loss of rod nuclei (ONL) in the dim-reared exposed retina. (Lower panels)- ONL thickness in dim light-reared and bright light-reared groups without (left panel) and after (right panel) damaging light exposure. The mean (±SD) thickness is shown. The * and ** indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between the dim light-reared (5 lux) and the bright light-reared (400 lux) groups using an un-paired t-test. The number of animals analyzed is indicated in each graph. Significant differences are indicated by * and ** for $p<0.05$ and $p<0.01$, respectively, using a 1-way ANOVA followed by Scheffe's posthoc test. The number of animals analyzed is indicated in each graph. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

To determine the retinal localization of 4-HNE and 4-HHE following bright light stress, albino rats were exposed to 5,000 lux light for 3h and, at various times thereafter, the levels and localizations of aldehyde-modified proteins in retinas was determined by immunohistochemistry using 4-HNE- and 4-HHE-specific antibodies (Fig. 2). Increases in 4-HNE- and 4-HHE-modified proteins were more prominent at 3h than at 24h following light exposure, and preceded rod photoreceptor cell apoptosis, determined by TUNEL staining. We conclude that intense light exposure increases 4-HNE- and 4-HHE-protein modifications in the retina, suggesting that free radical initiated, non-enzymatic reaction oxidation of omega-6 and omega-3 PUFA is involved in this process. These modifications are early events that precede photoreceptor cell apoptosis, consistent with our hypothesis that oxidant stress leads to photoreceptor cell death in our light stress model.

The dilemma

In light of the above-described studies, how then can PUFA be neuroprotective in the retina under oxidant stress conditions (Rotstein *et al.,* 1997; Rotstein *et al.,* 2003; Bazan, 2007)? We have previously shown that albino rats born and raised in dim (5 – 10 lux) cyclic light were more susceptible to light-induced apoptosis that albino rats born

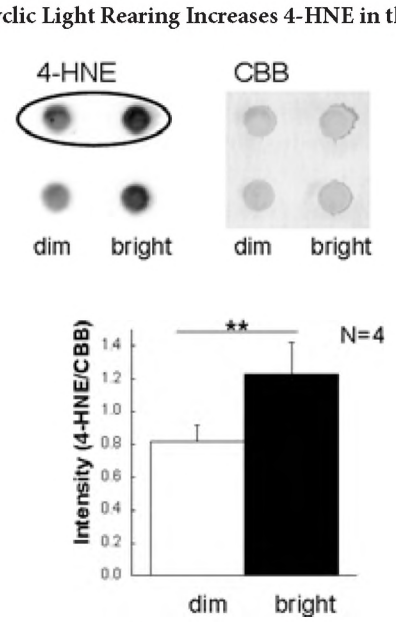

Fig. 4. Western dot blots and immunohistochemical localization of 4-HNE in the retina. (Upper panel)- Representative dot blots of protein modifications by 4-HNE for the dim light-reared (5 lux) and the bright light-reared (400 lux) groups. Note the difference between the circled dot-blots. CBB staining served as a loading control. (Lower panel)- Densitometric analysis of blots. The mean (±SD) densities standardized using CBB staining are shown for the dim light-reared and the bright light-reared groups (n=4 rats in each group). The ** indicates significant differences ($p<0.01$) between the two groups, using an un-paired t-test. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

and raised in bright (300-800 lux) cyclic light (Penn *et al.,* 1987). Biochemical studies showed an up-regulation of antioxidants and the activity of glutathione-metabolizing enzymes (Penn and Anderson, 1987). In a later study, we continued and expanded these earlier investigations to address the molecular mechanisms involved in this phenomenon. Specifically, we tested the hypothesis that stress responses mediated by the Nrf2-antioxidant responsive element (ARE) pathway are involved in the initiation of retinal neuroprotection provided by bright cyclic light-rearing (Tanito *et al.,* 2007). Albino rats born and raised in dim (5 lux) or bright (400 lux) cyclic light were exposed to damaging light (3000 lux, 6 h). After exposure, the outer nuclear layer (ONL) thickness and area, and electroretinogram a- and b-wave amplitudes were significantly reduced in the dim light-reared rats compared to the bright light-reared rats, confirming a light adaptation neuroprotection phenomenon (Fig. 3).

Retinas from albino rats born and raised in dim or bright cyclic light were removed and analyzed for the presence of 4-HNE. Although protected from light stress-induced cell death, the retinas from rats raised in 400 lux had higher levels of 4-HNE (Fig. 4), indicating that the chronic light stress led to an increased non-enzymatic oxidation of omega-6 PUFA in the retina.

These results suggested that the production of

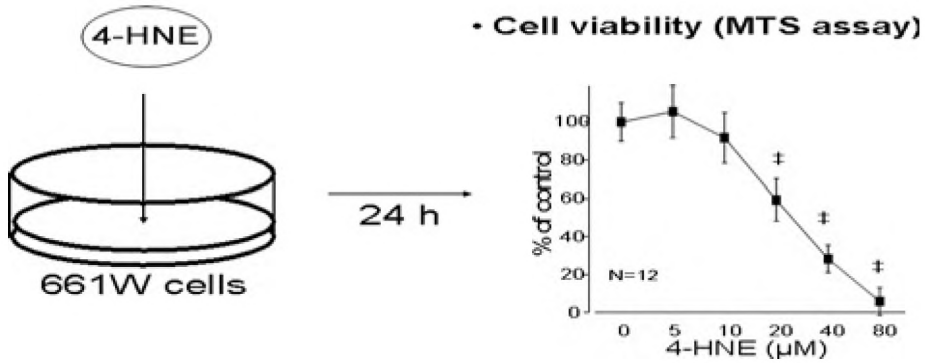

Fig. 5. Cell viability assay using 661W cells treated with various concentrations of 4-HNE for 24 h. The mean (±SD) for cell viability is shown (n=12 in each group). The ‡ indicates significant differences ($p<0.01$) in comparisons to cells that were not treated with 4-NHE using a 1-way ANOVA followed by Scheffe's posthoc test. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

4-HNE in the bright cyclic light-raised rats may be involved in some paradoxical way in providing neuroprotection to the retinal photoreceptors when challenged by damaging levels of light. To test this hypothesis, we used cell culture of an immortalized line of cone photoreceptors (called 661W cells). There was a dose-dependant loss of cells treated with 4-HNE for 24 hr, indicating that this product of lipid peroxidation, shown above to accumulate during light stress, was cytotoxic to these cultured retinal neuronal cells (Fig. 5). However, at low doses, between 0 and 10 μM, 4-HNE was not cytotoxic.

We then tested the hypothesis that pretreatment with a sub-lethal dose of 4-HNE could protect against stress-induced cell damage. Cells were pretreated with 5 μM 4-HNE for 24 hours, after which they were exposed to different concentrations of H_2O_2 for an additional 24 hours (Fig. 6). Although cell death occurred at all peroxide concentrations, there was a significant protective effect of pretreatment with 4-HNE.

Tanito *et al.* (2005b) had previously shown that sulforaphane, an extract from broccoli, up-regulated retinal levels of Trx in rat retinas. Using our cell culture paradigm, we looked for the up-regulation of Trx, TrxR, and HO-1 following treatment with 5 μM 4-HNE for 24 hours (Fig. 7). All three were up-regulated, as was Nrf2, a nuclear transcription factor previously shown to be involved in Trx up-

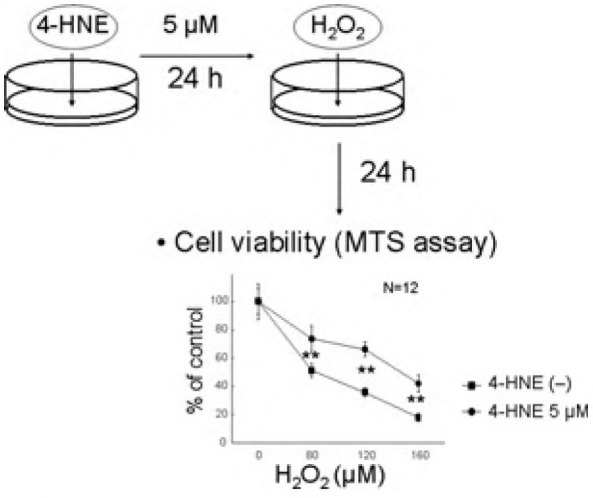

Fig. 6. Viability of 661W cells pretreated with 5 μM 4-HNE for 24 h followed by various concentrations of H_2O_2 for 24 h. The mean (±SD) for cell viability is shown (n=12 in each group). The ** indicates a significant difference ($p<0.01$) between the 4-HNE treated and untreated cells using an un-paired t-test. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

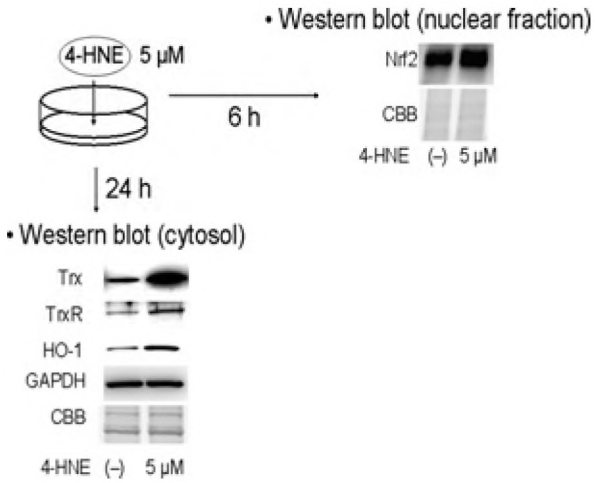

Fig. 7. Effect of 4-HNE on protein expression. (Upper right)- Western blot for Nrf2 in the nuclear fraction from 661W cells treated with or without 5 µM 4-HNE for 6 hours. CBB, Coomassie Brilliant Blue. (Lower left)- Western blots for Trx, TrxR, HO-1, and GAPDH using the cytosolic fraction from cells treated with or without 5 µM 4-HNE for 24 h. Note the increased immunoreactivity in the treated lane. (Lower right)- Densitometric analysis of blots from the western blots. The mean (±SD) densities standardized using GAPDH are shown (n=3 samples for each group). The * and ** indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between the 4-HNE treated and untreated cells using an un-paired t-test. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

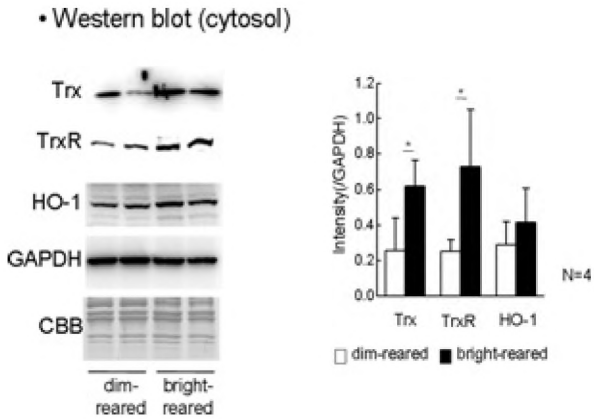

Fig. 8. Western blots for Trx, TrxR, HO-1, and GAPDH in the retinal cytosolic fraction from both the dim (5 lux) and bright (400) cyclic light-reared groups. CBB, Coomassie Brilliant Blue. (Right panel)- Densitometric analysis of Western blots. The mean (±SD) densities were standardized against GAPDH (n=4 rats for each group). The * indicates significant differences ($p<0.05$) between the two groups using an un-paired t-test. [Reprinted from Tanito *et al.* (2007) with permission from Elsevier]

regulation. Prevention of Nrf2 expression using siRNA technology also reduced the up-regulation of Trx and TrxR, but apparently not HO-1 (results not shown).

To determine if bright light rearing up-regulated Trx, TrxR, and HO-1 in animals, retinas from rats born and raised in 5 lux or 400 lux cyclic light were taken for semi-quantitative Western blot analysis (Fig. 8). Trx and TrxR were significantly increased in bright raised rat retinas. HO-1 was also higher, but the difference was not significant in the small sample size (n=4). The level of Nrf2 was also higher in nuclear extracts of retinas from rats born and raised in 400 lux cyclic light, compared to dim-reared (5 lux) controls (not shown).

Conclusions

Our studies show that products of the non-enzymatic oxidation of omega-6 and omega-3 PUFA can lead to the death of neural cells. However, we also demonstrate that sub-lethal doses of these products (in the reviewed studies we tested 4-HNE), which would occur in the initial phases of oxidant stress, can up-regulate endogenous neuroprotective pathways. We therefore suggest that in their day-to-day course of metabolic activity, cells challenged with an oxidant stress respond by up-regulating a host of endogenous neuroprotective mechanisms, which include Trx, TrxR, HO-1, GSH-peroxidase, GSH-reductase, GSH-S-transferase, vitamin E, and vitamin C, to name a few.

These, along with the enzymatic products of PUFA metabolism, such as neuroprotectin D1 (Bazan, 2007), serve to maintain the cell in a reduced state and protect against oxidant stress-induced apoptosis.

Acknowledgments

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The ω -3 poly-unsaturated fatty acids and the function of the brain and retina in infants

Lotte Lauritzen, Camilla T. Damsgaard, Anders D. Andersen & Kim. F. Michaelsen

Abstract

The central nervous system of human infants has a uniquely high content of docosahexaenoic acid (DHA, 22:6ω-3), which is accreted during the brain growth spurt that occurs during the first year of life. Based on results from randomized controlled trials on visual acuity it is presently agreed that preterm infants have a conditional need for preformed DHA, but the data for term infants are inconclusive. The term infant studies are in general performed more recently and with higher levels of α-linolenic acid (LNA, 18:3ω-3) in the control formulas. A meta-regression analysis of the data has shown that differences in the dose of ω-3 poly-unsaturated fatty acid (PUFA) are an important factor in explaining the inconsistencies in the functional outcomes. Thus, the data on both term and preterm infants are in agreement with a classical dose-response relationship, but it is unknown at this stage whether dietary LNA could meet the ω-3 PUFA requirements. Moreover, the potential long-term implications of the early improvements in visual function are not known. ω-3 PUFA intake in the first year of life is also believed to affect infant cognitive development, although this question remains unresolved. Breast-feeding has been shown to confer a long-term advantage in cognitive performance of approximately 3 IQ-points relative to that in formula-fed subjects, but this difference could be due to confounding as well as specific components of human milk. The DHA-content of human milk depends on the maternal fish intake, which in many countries does not support optimal levels of DHA in the milk. Most maternal fish oil supplementation trials report no advantage to infant mental development during the first year of life, but many of these studies find positive associations between breast-milk DHA and neuro-developmental outcomes, mirroring the results of observational studies. Some of these studies indicate possible negative effects of ω-3 LCPUFA, e.g. on language development, but the interpretation is complicated by lack of knowledge of the long-term predictive role of the employed early tests on cognitive development. Apart from direct mental effects, changes in the fatty acid composition of the central nervous system may also influence other types of behavior and body functions, such as the regulation of blood pressure. Early intake of ω-3 PUFA has been shown to affect blood pressure later in life in both LCPUFA-supplemented formula-fed infants and ω-3 PUFA deficient rats. This line of research is up-coming and we expect the next decade will provide us with new aspects of the effect of ω-3 PUFA that need to be taken into consideration when making dietary recommendations for PUFA-intake during growth.

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Key words: PUFA, fish oil, cognitive function, infancy, mental, programming, brain, breast-feeding, blood pressure

Introduction: ω-3 PUFA and the developing brain

Humans have a very high growth rate during the first year of life, particularly in the central nervous system (CNS). The CNS has a uniquely high content of docosahexaenoic acid (DHA, 22:6ω-3) that accretes during its growth spurt. Infancy is characterized by a high growth velocity of the head and brain, which increases in weight from 200 g at birth to 1 kg at 1 year of age, when the growth spurt starts to fade out (Martinez, 1992), and DHA is accreted in this period. This accretion is not only due to the increase in brain size but also to an increase in the relative content of DHA (Martinez, 1992). It has been estimated that approximately half of the DHA accumulation within the body of a breast-fed infant appears in the brain (Cunnane *et al.,* 2000), which has been calculated to accumulate as much as 4 g DHA during the first year of life (Cockburn, 1997). In order to judge the magnitude of this accumulation one has to consider both the intake and the efficiency of incorporation.

The efficiency of DHA-incorporation from dietary alfa-linolenic acid (LNA, 18:3ω-3) appears to be extremely low in rats, partly due to recycling of carbon into non-essential components in the brain (Cunnane *et al.,* 1999). A number of primate studies (Sheaff Greiner *et al.,* 1996; Sheaff Greiner *et al.,* 1997; Su *et al.,* 1999) have shown that dietary DHA is incorporated in the CNS about 10 times more efficiently than LNA, but that only a small fraction of the supplied ω-3 fatty acids are incorporated, regardless of the source. A number of studies investigating the brain fatty acid composition of infants that died from sudden infant death syndrome (SIDS) show that brain DHA-accretion confers a dietary ω-3 PUFA demand (Farquharson *et al.,* 1992; Farquharson *et al.,* 1995; Jamieson *et al.,* 1999; Makrides *et al.,* 1994). Also, an increase in the relative DHA-content in the brain was observed in breast-fed infants, whereas no increase was seen in formula-fed infants (Makrides *et al.,* 1994). This indicates that the infant formulas did not sustain brain DHA-accretion, at least not to the same extent as breast-milk. The important question is whether the differences in DHA-accretion in the CNS affect brain function.

ω-3 PUFA in breast-milk and infant formula

Infant formula is designed to match breast-milk in its overall fatty-acid composition, i.e. its content of saturated, mono-unsaturated and poly-unsaturated fats (PUFA). However, as the composition of breast-milk is variable, no defined standard exists for the composition of formula, especially not with respect to the content of specific PUFAs. The PUFA-content of formulas has changed over the years as evidence of the importance of ω-3 PUFA has increased. A decade ago most infant formulas contained linoleic acid (18:2ω-6) and LNA as the only sources of PUFA. In the early studies from the 1980-1990'ies, which examined the functional effects of infant formula, the LNA-content was as low as 0.2 % of the energy or less (Faldella *et*

al., 1996; Uauy *et al.,* 1990). Today many formulas contain some long-chain (LC)PUFA, most often both DHA and arachidonic acid (20:4ω-6). In 2003, all term infant formulas on the Danish market contained linoleic acid and LNA in a ratio of approximately 8:1 (Straarup *et al.,* 2006). Human milk always contains some DHA and other LCPUFAs, but the amount of ω-3 LCPUFA varies greatly with diet (Brenna *et al.,* 2007; Lauritzen *et al.,* 2001). Breast-milk from women with special diets, such as vegans who ingest no LCPUFA and Eskimos who consume large amounts of ω-3 LCPUFA, differs only 3-fold in their content of linoleic acid, arachidonic acid and LNA, but differs more than 15-fold in their content of DHA. Reports on the DHA-content of breast-milk from individual women range from 0.1 to 3.5 % of the fatty acids (FA%) (Brenna *et al.*, 2007; Koletzko and Rodriguez-Palmero, 1999).

ω-3 PUFA and visual acuity

Research on functional CNS-effects of ω-3 PUFA-intake in infancy has mainly focused on visual acuity. This is due to: 1) The exceptional high levels of DHA in the retina, 2) Visual acuity being a well-described symptom of ω-3 PUFA-deficiency (Neuringer *et al.,* 1984), and 3) Visual acuity develops rapidly during early infancy and is relatively easy to measure (Lauritzen *et al.,* 2004a). Visual acuity is normally assessed by the general physician as the ability to read letters or symbols from a certain distance. This method is however not applicable to infants, who instead are presented with stripes in order to judge their tendency to gaze at these (the Teller card method). Alternatively infant visual acuity can be determined as the visually evoked electric potentials in their visual cortex by the use of electrophysiological methods (VEP or SWEEP-VEP) (Neuringer *et al.,* 1994).

As reviewed in (Lauritzen *et al.,* 2001), several randomized controlled trials have shown an improvement in visual acuity in formula-fed preterm infants after addition of DHA to formula. A meta-analysis showed a slower visual acuity maturation in preterm infants (born <37th week of gestation) given formulas with a LNA-content of less than 2 FA% compared to those given human milk or formulas with DHA, alone or in combination with ω-6 LCPUFA (SanGiovanni *et al.,* 2000b). The difference in visual acuity between infants who were fed formulas with or without DHA was observed at 2 and 4 months of age. Only a few studies compared visual acuity between breast-fed and formula-fed preterm infants (Birch *et al.,* 1992; Birch *et al.,* 1993), but these studies and a number of similar studies in term infants have shown better visual acuity at 4 months with breast-feeding (SanGiovanni *et al.,* 2000a). Since many randomized controlled trials in preterm infants show positive effects on infant visual acuity when DHA is added to infant formula, it is generally agreed that preterm infants have a conditional need for preformed DHA (e.g. (Fleith and Clandinin, 2005)). In contrast, the data from term infants is not believed to be conclusive.

Various reviews of the trials in term infants report that only half of the studies show a faster visual acuity maturation after DHA-addition, while the other half show no effects (Eilander *et al.,* 2007; Lauritzen *et al.,* 2001; SanGiovanni *et al.,* 2000a). However, it is important to keep in mind that as evidence has accumulated, the ω-3 PUFA-content of formulas has increased (Lauritzen *et al.* 2001). The first three preterm infant trials used formulas (with 0.25-0.5 FA% LNA) that would now be considered to be ω-3 PUFA-deficient. The studies in term infant are in general performed more recently and with considerably higher levels of LNA in the control formulas (around 2-3 FA%). The DHA-content that was used in the experimental formulas has also varied. One of the early trials with preterm infant used large amounts of fish oil (as much as 0.5-1 FA% ω-3 LCPUFA) (Carlson *et al.,* 1991), whereas lower amounts (down to 0.1-

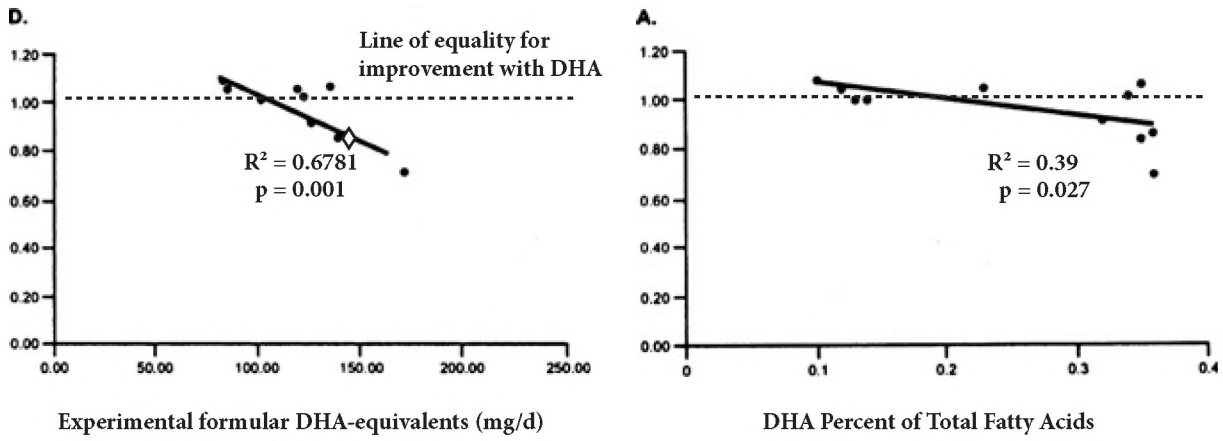

Fig.1. *Left:* Dose-response: term infant DHA intake *vs.* visual acuity improvement. The plot shows the results from the meta-regression analysis of Uayu *et al.* (2003). The experimental formula content is given in DHA-equivalents (mg/d) assuming a 10% conversion of LNA to DHA. The result from the study of Birch *et al.* (2005) (◊) has been superimposed on the fit from the meta-regression analysis. *Right:* the results of a crude analysis that do not consider formula content of LNA.

0.2 FA% DHA) were used in the trials with term infants (Auestad *et al.*, 2001; Carlson *et al.*, 1996). The more recent studies seem to have realized the need for larger DHA-doses and provided 0.3-0.4 FA% DHA in their experimental formulas. The difference between the results of the term and preterm infant trials are much less obvious when we look at only the most recent trials, that have used similar standard formulas and similar doses of DHA (Auestad *et al.*, 2001; Birch *et al.*, 2005; Innis *et al.*, 2002; O'Connor *et al.*, 2001; Wezel-Meijler *et al.*, 2002).

In a review of the effect of ω-3 PUFA in the function of the brain and retina in infants, we hypothesized that the differences in the dose of ω-3 PUFA (both LNA and DHA) could be an important factor in explaining the inconsistencies in the functional outcomes in the term infant trials (Lauritzen *et al.*, 2001). This has later been confirmed in a meta-regression analysis (Uauy *et al.*, 2003). Uauy and his coworkers found a highly significant association between the observed effects on visual acuity and the combined intake of LNA and DHA in the term infant trials. The group tested different theoretical proportions (0-10 %) of LNA-conversion to DHA and the best fit was achieved with a DHA-equivalence factor of 10% for LNA. This is in agreement with data on DHA-incorporation in the CNS of primates. The meta-regression analysis included seven trials that assessed visual acuity at 4 months of age (Fig. 1) and explained as much as 68 % of the variation between the results of the randomized controlled trials that tested formulas with and without DHA in term infants (p=0.001) (Uauy *et al.*, 2003). Also, the results of a more recent trial (Birch *et al.*, 2005) fits nicely with the regression (Fig. 1). Thus, in our opinion the present data from both term and preterm infants are in agreement with a classical dose-response relationship, suggesting that the dietary need may be around 100 mg/d or 0.4 FA% DHA in the milk, regardless of gestational age at birth. At this stage it is unknown whether LNA-intake alone could meet the ω-3 PUFA-requirements of infants. To our knowledge only one study has investigated the effects of a formula with a LNA-content above 4 FA% (Innis *et al.*, 1997).

Since breast-milk DHA varies with maternal

diet, a way to look at the developmental effects of infant DHA-intake is to perform randomized trials with lactating mothers. We and others have conducted such trials in which the DHA-content of breast milk was manipulated by supplementing the lactating mothers with fish oil in order to look for functional effects on visual acuity and other outcomes in the infants (Table 1). The breast-milk DHA content in our trial correlated significantly with the maternal intake of ω-3 LCPUFA and was raised from a mean of 0.4 FA% in the olive oil-supplemented (control) group to around 1 FA% in the fish-oil supplemented group (Lauritzen *et al.*, 2004b). In our study, the fish-oil supplement did not have any immediate effect on the SWEEP-VEP visual acuity of the infants at 2, 4 or 9 months of age, i.e. no significant differences were observed between the two randomized groups. However, we did see a significant association between the DHA content of infant erythrocytes (a well-accepted biomarker of DHA intake) and their visual acuity at 4 months. The erythrocyte DHA-content together with the degree of breast-feeding, gestational age and number of siblings were found to account for 24 % of the variance in infant visual acuity (around 4 % by DHA alone). The low degree of explanation is in part due to noise in the SWEEP-VEP testing of visual acuity in the infants. When the random variation of the SWEEP-VEP method is removed, the effect of DHA is estimated to increase to a much larger fraction of the variance (Lauritzen *et al.*, 2004a).

The two other randomized controlled trials looking at the effects of ω-3 LCPUFA-supplementation during lactation did not find any group-differences in infant visual function (Gibson *et al.*, 1997; Jensen *et al.*, 2005) and neither did a maternal supplementation trial in which ω-3 LCPUFA-supplements were given during pregnancy or during both pregnancy and lactation (Malcolm *et al.*, 2003a). Accordingly, the most obvious conclusion would be that maternal DHA-supplementation does not markedly affect infant visual development (Eilander *et al.*, 2007). However, the pregnancy trial is questionable as no biochemical effect of the intervention was observed in breast milk or infant blood at delivery (Malcolm *et al.*, 2003a). In contrast, a more recent trial in which mothers were supplemented with DHA during pregnancy did find an effect on infant visual acuity at 4 months, although not at 6 months of age (Judge *et al.*, 2007a). This study was performed in the US and used a very low DHA-dose. Since both intake of DHA and thus the breast-milk content of DHA are lower in the US than in Scandinavia, this study may indicate that the trials should be re-evaluated in order to look at dose-response-relations, like the formula trials. However, the new pregnancy DHA-supplementation trial was only a small study and the result could be a chance-finding. Data from one of the published trials with maternal DHA supplementation in pregnancy (Malcolm *et al.*, 2003a; Malcolm *et al.*, 2003b) and a new Canadian trial (Innis, 2007a) show associations between visual functions (ERG, VEP-latency and visual acuity) in infants and biochemical biomarkers of DHA intake and a few observational studies in breast-fed infants have also observed that DHA in breast-milk or infant erythrocytes was positively associated with visual acuity (Innis *et al.*, 2001; Jørgensen *et al.*, 2001; Makrides *et al.*, 1993). This supports the association found in our maternal supplementation trial and underlines the need for a dose-response approach when evaluating results. Furthermore, the evidence indicates that the optimal milk DHA level is higher than that found in breast-milk from women with a low intake of ω-3 LCPUFA.

Apart from the optimal dose, another unresolved matter is whether or not the effect of ω-3 LCPUFA on visual acuity is long-lasting. Only a few randomized trial have looked at the effects on visual acuity beyond the first year of life (Auestad *et al.*, 2003; Birch *et al.*, 2007; Singhal *et al.*, 2007).

Study	a) Experimental supplement b) Control oil c) Period of supplementation	DHA in breast-milk (FA%)	Performed assessments (n in all random groups)	Observed group-differences (relative to control)	Description of observed associations with DHA in breast-milk or RBC
Supplementation in pregnancy					
Malcolm *et al.*, 2003a; Malcolm *et al.*, 2003b	a) 250 mg/d ω-3 LCPUFA/d (fish oil) b) High oleic acid sunflower oil c) Gestation wk 15 to delivery	a) 0.2 nmol/l b) 0.3 nmol/l at 0 wks	1) Infant plasma & RBC at 0 wks (53) 2) ERG within 1 wk (41 or 44) 3) Flash VEP at 0, 2.5 & 6 mo (55, 52, 51) 4) t-VEP 2.5 & 6 mo (?)	1) NS: RBC-DHA increased by 5% 2) No differences 3) No differences 4) No differences in acuity or parameters	2) Significant associated with RBC ω-3 LCPUFA status at birth 3) No associations 4) Association between peak latency and RBC-DHA at birth
Tofail *et al.*, 2006	a) 3 g/d ω-3 LCPUFA (fish oil) b) Soy oil c) Gestation wk 25 to delivery	Not assessed	1) BSID at 10 mo (249) 2) Infant behavior at 10 mo (249)	1) No differences 2) No differences	No biochemical measures of the intervention included
Judge *et al.*, 2007a Judge *et al.*, 2007b	a) 340 mg/d ω-3 LCPUFA (functional foods) b) Corn oil c) Gestation wk 24 to delivery	Not assessed	1) Teller at 4 & 6 mo (30, 26) 2) Fagan at 9 mo 3) PS at 9 mo	1) Better acuity at 4, but not at 6 mo. 2) No differences 3) Higher score	No biochemical measures of the intervention included
Supplementation in pregnancy and lactation					
Helland *et al.*, 2001 Helland *et al.*, 2003	a) 2.2 g/d (cod liver oil) b) Corn oil c) Gestation wk 18 to 12 wks post partum	a) 1.2 b) 0.47 at 12 wks	1) Infant plasma PL at 0, 4 & 12 wks (74, 82, 75) 2) EEG at 2 days & 12 wks (148, 122) 3) Fagan at 6 & 9 mo (262, 245) 4) K-ABC at 4 y (84)	1) ω-3 PUFA increased by 60% at 4 wks 2) No differences 3) No differences 4) Higher score	2) Mature and immature 2d-EEG was associated with differences in ω-3 LCPUFA status at birth 3) No associations 4) Association with ω-3 LCPUFA status at 0 & 4 wks and maternal DHA-intake.

Table 1. Overview of randomized trials on the visual and cognitive effects in children after maternal fish oil-supplementation during pregnancy and/or lactation.

Study	a) Experimental supplement b) Control oil c) Period of supplementation	DHA in breast-milk (FA%)	Performed assessments (n in all random groups)	Observed group-differences (relative to control)	Description of observed associations with DHA in breast-milk or RBC
Supplementation in lactation					
Makrides *et al.*, 1996 Gibson *et al.*, 1997	a) 0-1.3 g/d DHA (algal oil) b) None used c) Day 5 to 12 wks post partum	a) 0.21-1.13 at 12 wks	1) Infant plasma & RBC at 12 wks (52) 2) VEP at 12 & 16 wks (26, 36) 3) BSID at 1 & 2 y (51,49)	1) RBC-DHA increased by >70% 2) No differences	1) Asymptotic dose-response with milk-DHA 2) No association with milk or RBC-DHA 3) MDI positively associated with milk & RBC-DHA at 1y, but not at 2 y.
Jensen *et al.*, 2005	a) 200 mg/d DHA (algal oil) b) Soy/Corn oil (50:50) c) Day 5 to 4 mo post-partum	a) 0.35 b) 0.20 at 4 mo	1) Infant plasma PL at 4 mo (159) 2) Teller at 4 & 8 mo & SWEEP-VEP at 4 mo (147,147, 160) 3) t-VEP at 4 & 8 mo (168, 153) 4) CAT, CLAMS, DQ at 12 & 30 mo (165, 147) 5) BSID at 30 mo (133)	1) PL-DHA increased by 35% 2) No differences 3) Lower amplitude 4) No differences 5) No difference in MDI, but higher PDI	2-5) No significant correlation with any visual or neuro-developmental outcome and infant PL-DHA at 4 mo
Lauritzen *et al* 2004 & 2005	a) 1.3 g/d ω-3 LCP-UFA (functional foods) b) Olive oil c) 1 wk to 4 mo post-partum	a) 1.34 b) 0.41 at 4 mo	1) Infant RBC at 4 mo (78) 2) SWEEP-VEP at 2 & 4 mo (88, 97) 3) PS at 9 mo (86) 4) CDI at 1 and 2 y (89, 71) 5) Follow-up with blood pressure, immune function and body composition at 2.5 y	1) RBC-DHA increased by 40% 2) No differences 3) Higher score in girls 4) Lower 1y-comprehension, mostly in boys and less sentence complexity in boys at 2y	1) Asymptotic dose-response with milk-DHA 2) Association between visual acuity and RBC-DHA at 4 mo 3) No association with RBC-DHA at 4 mo 4) Active vocabulary at 1 y adversely associated with RBC-DHA at 4 mo, but no asscociattions at 2y

Table 1. (continued)

BSID: Bayley Scales of Infant Development, **MDI**: Mental Developmental Index, **PDI**: Psychomotor Development Index, **CAT**: Clinical Adaptive Test, **CLAMS**: Clinical Linguistic and Auditory Milestone Scale, **DQ**: Developmental Quotient on Gesell Scale Gross Motor scale, **ERG**: Electroretinogram, **K-ABC**: Kaufman Assessment Battery for Children, **NS**: Not significant, **PL**: Phospholipids, **PS**: Problem solving assessed by the Infant Planning Test, **RBC**: Erythrocyte, **SWEEP-VEP**: Swept visual evoked potential assessment of visual acuity, **t-VEP**: Transient visual evoked potential assessment of visual acuity, latency or amplitude.

In one of their trials Birch and her co-workers found that children who during the intervention from 0-4 months of life had received standard unsupplemented formula still had poorer visual acuity at 12 month (Hoffman *et al.*, 2000) and 4 years of age (Birch *et al.*, 2007), whereas Auestad *et al.* and her co-workers found no effect on visual acuity neither in infancy nor at 3½ years of age (Auestad *et al.*, 2003). In the largest long-term randomized trial so far, no effect of formula LCPUFA-supplementation was observed on the proportion of children with low visual acuity at 4-6 years of age (Singhal *et al.*, 2007). Many of the trials have measured visual acuity at different ages during the intervention period, typically at 2, 4, 6, 9 and 12 months. The results from these studies appear to separate into two types. Some studies (e.g. (Carlson *et al.*, 1993)) and two meta-analyses (SanGiovanni *et al.*, 2000a; SanGiovanni *et al.*, 2000b) indicate that the unsupplemented children catch up, since significant differences are generally observed at 2 and 4 months, but not at older ages. Other studies show a more permanent effect, with persistent differences in visual development between the supplemented and unsupplemented groups, or even increased differences over time (e.g. (Birch *et al.*, 2007)). The meta-analysis of term infant trials indicate that the ability to detect differences in visual acuity may depend on the method of visual acuity assessment (SanGiovanni *et al.*, 2000a). It has been suggested that the differences are explained by differences in sensitivity of the visual acuity test or by differences in the amount of DHA included in the experimental formula (Cheatham *et al.*, 2006). However, in our opinion the observed inconsistencies in the persistency of the effect are not explained by gestational age at birth, DHA-dose or visual acuity test-type, as there is no systematic difference in these variables between the studies that found an effect of DHA and those that did not. However, the inconsistent results could be due to other aspects of testing. In our experience, the children are harder to test with the SWEEP-VEP method when they are older than 8 months of age, resulting in noise and improper measurements, probably because the children are impatient and easier distracted during the test at this age. Therefore, we propose that the lack of a persistent effects of DHA despite continued DHA supplementation could be due to testing-noise. Even if the effect of DHA on visual acuity should prove to be truly transient, two studies report that other aspects of visual function later in childhood may depend on infant nutrition (Singhal *et al.*, 2007; Williams *et al.*, 2001). Both of these studies found that breast-feeding (Singhal *et al.*, 2007) was associated with a greater likelihood of achieving a mature foveal stereoacuity at 3-6 years of age (Singhal *et al.*, 2007; Williams *et al.*, 2001). Furthermore, one of these studies found that children whose mothers ate oily fish during pregnancy were also more likely to have a high degree of stereoacuity at 3½ years of age (Williams *et al.*, 2001), whereas the other study observed no effect of formula LCPUFA-supplementation on foveal stereoacuity (Singhal *et al.*, 2007). Even if the visual effect turns out not to be persistent, an accelerated maturation of visual acuity could maybe affect maturation of higher cognitive functions or it could reflect general differences in the information processing that may also affect other CNS functions.

ω-3 PUFA and cognitive function

Apart from visual function, the intake of ω-3 LCPUFA in the first year of life is also believed to affect the cognitive development of the infants. In a meta-analysis, breast-feeding was shown to confer a long-term advantage in cognitive performance of approximately 3 IQ-points relative to formula-feeding (Anderson *et al.*, 1999). The effect in preterm infants was much stronger than in term infants, supporting that the effect is caused

by ω-3 LCPUFA. In a recent WHO meta-analysis the effect was 4.9 IQ points (Horta *et al.*, 2007). One study showed that breast-feeding also had an effect of the same magnitude in young adults (Mortensen *et al.*, 2002). An effect of breast-feeding of 3-5 IQ-points is not large compared to the effect of genetic and social conditions, but a shift in IQ in of this magnitude in a population may have a large impact on the number of disadvantaged children. Breast-feeding is a choice of the mother and is also determined by socioeconomic factors. These must be controlled for before we can conclude if the milk itself has causal effects on cognitive function. Proper adjustment for confounding requires collection of information on many potential confounding factors, including parental IQ and measures of parenting skills and the home environment. In a recent analysis of a large cohort from the US, which included sibling pair analysis and control for maternal IQ, the conclusion was that there was no effect of breast-feeding on IQ (Der *et al.*, 2006). However, mean duration of breast-feeding was only 3 months and more than half of the children were not breast-fed. Furthermore, it was based on a US population, where breast-milk DHA levels are likely to be sub-optimal (Brenna *et al.*, 2007). Thus, although there is a plausible mechanism for an effect of breast-feeding on cognitive development through breast-milk DHA content, there is still a discussion about the potential influence of residual confounding by factors such as the quality of parental care, which goes along with a healthy diet.

Epidemiological studies have described positive associations between maternal intake of marine foods and verbal IQ measured at 8 years of age (Hibbeln *et al.*, 2007). However, permanent adverse associations with brain function have also been described, especially in countries with a high intake of marine mammals with high levels of methyl-mercury (Debes *et al.*, 2006; Gochfeld and Burger, 2005). Interestingly, in populations exposed only through fish intake, similar levels of maternal methyl-mercury exposure are not associated with adverse outcomes (Davidson *et al.*, 2006). Benefits from fish consumption are confounded by socioeconomic variables and by the replacement of other foods that could potentially influence the outcome. Thus, additional studies with better dose-construction and preferably bio-monitoring of both ω-3 LCPUFA-status and neurotoxin exposure are needed in order to evaluate the composite benefit-risk dose-curve of fish on infant CNS development (Gochfeld and Burger, 2005). Furthermore, differences caused by breast-feeding *versus* formula-feeding could be confounded by socio-economic factors or other specific components of human milk, rather than being a true effect of ω-3 LCPUFA (McCann and Ames, 2005). Therefore, randomized controlled intervention trials are needed to prove any causal effects of ω-3 LCPUFA.

Some randomized trials support an association between IQ and ω-3 PUFA intake, but the results are diverse. Rather than making definite conclusions, we will therefore exemplify and discuss some of the relevant issues in the field. The two most recent reviews conclude that 1) evidence from several types of studies, particularly animal studies, suggest that changes in brain DHA-concentrations are positively associated with changes in cognitive or behavioral performance (McCann and Ames, 2005) and 2) that randomized trials in preterm formula-fed infants indicate a beneficial effect of LCPUFA on cognitive development, but that further studies are needed to assess the effect in term formula-fed infants (Eilander *et al.*, 2007). Many of the studies investigating the effects of DHA on cognitive function have used tests such as the Griffiths Scales or the Bayley Scales of Infant Development (BSID), which were originally developed to identify children with serious developmental problems (Cheatham *et al.*, 2006). These well-established but very general tests may not be

well-suited for detecting small differences within the normal range of behavior, the development of which in early infancy is characterized by memory and speed of information processing (Cheatham *et al.*, 2006). A couple of studies have investigated the effects of infant DHA-supplementation on specific developmental measures of information processing and detection of novelty (Auestad *et al.*, 2001; O'Connor *et al.*, 2001; Willatts *et al.*, 1998b). By use of a specific problem solving test, the Infant Planning Test, designed for children at exactly the age at which they were tested (Willatts, 1999), Willatts and his coworkers found that infants, who were fed infant formula supplemented with LCPUFA during the first 4 months of life had better overall test-scores at 10 months than those who were given standard formula (Willatts *et al.*, 1998a). In this test the child had to overcome some physical barriers (e.g. remove a cloth) in order to find a toy and the effect was mainly seen in the final step of the test (Willatts *et al.*, 1998a) as the less mature children never made it to this step. It has been suggested that the cognitive effect of dietary DHA may be easier detectable at older ages, when cognitive tests are more sensitive and reliable (e.g. by (Eilander *et al.* 2007)). One of the term trials on LCPUFA-supplemented infant formulas found increased performance in the Wechsler Preschool and Primary Scale of Intelligence-test at a long-term follow-up at 4 years of age (Birch *et al.*, 2007).

Six of the seven randomized trials with ω-3 LCPUFA during pregnancy and/or lactation tested the cognitive effects in the children (Table 1). Most of the maternal fish oil-supplementation trials report no clear advantage to infant mental development during the first two years of life, but some of the studies found positive associations between DHA in breast-milk and neuro-developmental outcomes, mirroring the results of observational studies (Innis, 2007b). Our maternal fish oil-supplementation trial showed better problem solving at 9 months in the intervention group, but only in girls (Lauritzen *et al.*, 2005). In the lactation trial by Gibson's group, they found an associations between RBC and milk DHA-levels and mental function, measured by global tests of infant development, although this was only observed at 1 year and not at 2 years of age (Gibson *et al.*, 1996). Jensen *et al.* found a positive effect of a low dose of DHA during lactation on the psychomotorical score on the Bayley scale at 1½ years of age (Jensen *et al.*, 2005) and a recent maternal supplementation-trial found an improvement of Infant Planning Test scores in infants 9 months after the pregnant mothers had ingested DHA-enriched functional foods (Judge *et al.*, 2007b). Data from a Norwegian trial with cod liver oil-supplementation during pregnancy and lactation showed that the children from the cod liver oil-group did significantly better in the over-all score when they examined their problem solving abilities at 4 years of age (Helland *et al.*, 2003). They used the K-ABC test, which consists of a number of tasks on sequential problem solving, simultaneous problem solving and non-verbal abilities. In contrast, results from a Dutch observational study do not provide evidence for a positive association between LCPUFA-status at birth or at 7 years (Bakker *et al.*, 2003) and cognitive performance at 7 years of age, but showed a significant negative correlation between perinatal DHA status and internalizing problem behavior (Krabbendam *et al.*, 2007).

One of the highly discussed issues is the timing of the "open-window"-period, *i.e.* what stage in infant or fetal life is most vulnerable to effects of deficiencies in the DHA-supply (Eilander *et al.*, 2007). However, most of the positive results on cognitive development so far are observed in post-partum maternal supplementation trials (Eilander *et al.*, 2007). This is in contrast to the general belief that the earlier during development, the more sensitive is the brain. This belief is based on the assump-

tion that DHA is affecting the formation of nerves and synapses early in fetal life, and that once the CNS is established, the effect is limited. It could, however, be hypothesized that early post-natal life is a more vulnerable period, because although the relative growth of the fetus is large in pregnancy, the absolute amount of DHA that is accreted in the brain is much larger during the first months after birth. Randomized trials with follow-on formulas or DHA-enriched baby foods in term breast-fed infants after weaning have found positive effects on visual acuity (Birch *et al.*, 2002; Hoffman *et al.*, 2004). This suggests that the critical period, in which the dietary DHA-supply can affect the maturation of cortical function, extends beyond fetal life and the early lactation period. Furthermore, a combined analysis of some of the LCPUFA-trials has demonstrated that the duration of the LCPUFA-supplementation period was positively associated with improvements in visual acuity (Morale *et al.*, 2005). Thus, it is likely that CNS function is affected by the LCPUFA-intake throughout the first year of life (Morale *et al.*, 2005).

Some of the studies on the effect of early LCPUFA-intake and infant cognitive function indicate possible negative effects of ω-3 LCPUFA, e.g. on language development. However, the interpretation of these results is complicated by the lack of detailed knowledge on infant cognitive development and the long-term predictive role of the employed early tests on later cognitive development (Cheatham *et al.*, 2006). Our maternal supplementation trial showed a negative effect of fish oil on language development at the follow-up examinations at 1 and 2 years of age (Lauritzen *et al.*, 2005). Language development was examined with MacArthurs parent-assessed Communicatory Development Inventory (CDI)-questionnaire, in which the parents report the number of words that the child is able to understand and say. The active and passive vocabulary at 1 year was found to be lower in the children from the fish oil-supplemented group and at 2 years of age there was still a negative effect in boys, who are known to mature at a slower rate than girls. A similar inverse relationship between ω-3 LCPUFA and language acquisition assessed by the CDI-questionnaire was shown in an observational study and in a randomized trial in preterm formula-fed infants (Auestad *et al.*, 2003; O'Connor *et al.*, 2001; Scott *et al.*, 1998). This has been taken as adverse effects, but we and others have found slower language development in children of parents with higher education (Lauritzen *et al.*, 2005), who are likely to have better verbal and educational skills later in life. Fast language development may intuitively be interpreted as a sign of high cognitive function, but it is also possible that a slower language development reflects a different state of mind – e.g. that the children so to speak "think before they speak".

The point is that there are many things we do not know about infant cognitive and verbal development. John Colombo's group found that the attention-score was differently related to the DHA-content in umbilical cord blood (a measure of the DHA-status at birth) at two different ages (Colombo *et al.*, 2004). In the their test the child was given a complicated electronic toy with wheels, buttons, sounds etc. and the investigator assesses the time the child spends looking at the toy. Those with high DHA-status paid less attention to the toy than those with low DHA at 12 months, whereas it was opposite at 18 months, resulting in a significant age×DHA-effect. From other tests the group could show that long duration of attention at 12 months was positively associated with less mature behavior and reflects low processing efficiency, whereas it at 18 months was associated with more mature behavior and reflected the ability to stay focused. These results indicate that effects on cognitive function manifest differently at different ages. Colombo speculated that the effect of e.g. DHA could be evident only on the function that is most demanding to the infant at that spe-

cific age– i.e. staying awake, holding attention and IQ later in childhood (Wainwright and Colombo, 2006).

ω-3 PUFA and other central nervous system effects

The CNS regulates many functions in the body *via* the autonomic nervous system. Thus, apart from direct mental effects, changes in the DHA-content in the brain could also influence other types of behaviors and body functions, such as the development of blood pressure control. It has been speculated that perinatal ω-3 PUFA intake may have a programming effect, reducing the risk of cardiovascular diseases in later life (Das, 2003). Compared with formula-feeding breast-feeding has been shown in two large systematic reviews and meta-analyses to be associated with a 1.2-1.4 mm Hg lower systolic blood pressure later in life (Horta *et al.*, 2007; Martin *et al.*, 2005). Moreover, in a unique study, Singhal *et al.* randomized preterm infants to either banked breast-milk or preterm infant formula and found that blood pressure at 13-16 years of age was negatively associated with the intake of breast-milk (Singhal *et al.*, 2001). This latter study indicates an effect of specific components in the milk rather than confounding factors often related to voluntary breast-feeding. Not surprisingly only a few randomized trials have examined long-term programming effects of such specific breast-milk components in humans. However, an early intake of ω-3 PUFA has been shown to affect blood pressure later in life in both LCPUFA-supplemented formula-fed infants (Forsyth *et al.*, 2003) and ω-3 PUFA-deficient rats (Armitage *et al.*, 2003). Forsyth *et al.* found that infants receiving dietary supplementation with LCPUFA added to infant formula had lower blood pressure at 6 years of age than those who had been given the standard control formula (Forsyth *et al.*, 2003). This is in accordance with data from the rat study, showing permanently increased blood pressure in offspring that had been exposed to ω-3 PUFA-deficiency during early life (Armitage *et al.*, 2003; Weisinger *et al.*, 2001). The control-formula group in Forsyth's study may also have been ω-3 PUFA-deficient, as the formula did not contain the currently recommended 2 FA% LNA and had a very high ω-6/ω-3 PUFA-ratio (>16:1) (The Commission of the European Communities, 2006). We do not know whether the blood-pressure lowering effect in that study was caused by ω-3 LCPUFA, since the formula in Forsyth's study contained both DHA and arachidonic acid (20:4ω-6). Our maternal fish oil-supplementation trial did not show any convincing effects on blood pressure at 2½ years (Larnkjær *et al.*, 2006) but we did find a tendency towards an effect on heart rate (Larnkjær *et al.*, 2006). Furthermore, we recently found an acute blood-pressure lowering effect of 3 months fish-oil supplementation in healthy infants at 12 months of age (Damsgaard *et al.*, 2006). As with the effects of ω-3 LCPUFA on cognitive functions, the evidence on blood pressure shows many interesting indications, but we need more randomized controlled trials in order to make firm conclusions about the long-term effects of ω-3 LCPUFA on blood pressure regulation.

Despite the lack of firm evidence, plausible mechanisms exist. Programming depends on some sort of imprinting or memory mechanism and it is most likely that long-term vascular effects would be mediated via the development and regulation of the autonomous nervous system. Changes in the hypothalamic-pituitary-adrenal axis have been suggested as a possible mechanism of early programming (Wintour *et al.*, 2003). Pharmacological studies in preterm infants have shown that antenatal treatment with glucocorticoids such as β-methasone may persistently influence the hypothalamic-pituitary-adrenal regulation (Davis *et al.*, 2006). An effect of ω-3 PUFA on the au-

tonomic control of heart rhythm, blood pressure and other physiological functions may therefore provide a common link between possible effects on later health and on the CNS development. The observed long-term effect of ω-3 LCPUFA-supplementation on blood pressure (Forsyth *et al.*, 2003) and heart rate (Larnkjær *et al.*, 2006) could be interpreted as an indicator of accelerated CNS maturation. Exactly how DHA affects CNS functions remains to be elucidated, but a number of potential mechanisms are supported by data from *in vitro* studies and animal experiments (Innis, 2007b).

Conclusions

In summary, early intake of ω-3 LCPUFA from human milk and infant formula has been shown to affect DHA accretion in the tissues, but it is presently unclear to what extent neural function is affected by tissue composition and what the optimal levels of DHA in various tissues are. Human milk DHA-content depends on maternal diet, but often provides a better supply of ω-3 PUFA than formulas. Formula-fed infants have been shown to have poorer visual acuity than breast-fed infants and the visual acuity in formula-fed infants has been shown to be associated with the overall ω-3 PUFA intake. Dietary LNA appears to correspond to 1/10 of preformed DHA and the optimal intake in preterm and term infants may be around 100 mg/d DHA. The potential long-term implications for visual function are unknown, but other CNS functions may be affected. Breast-feeding is associated with a cognitive advantage relative to formula. However, both observational studies relating maternal fish intake with child IQ, randomized studies investigating cognitive effects of DHA-enriched formula, and studies with ω-3 LCPUFA in pregnancy/lactation are inconclusive with regard to the mental benefits for the infants. Changes in CNS ω-3 LCPUFA may also influence behavior and physiological function, such as blood pressure. Some studies indicate possible negative effects, e.g. on language, but we lack randomized trials and knowledge about cognitive development and the predictive value of early tests of cognitive and verbal function and markers of health. Hopefully, the next decade will provide new evidence that will contribute to elucidate the dietary needs for PUFA during optimal growth and development.

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Dietary poly-unsaturated fatty acids and bioactive lipids

Harald S. Hansen

Abstract

The poly-unsaturated fatty acids of the omega-6- and omega-3-fatty acid families are essential fatty acids that serve a number of important functions in the multi-cellular mammalian organism. Thus, lack of linoleic acid in structural *O*-acylated ceramides of the epidermis is the reason for usual essential fatty acid-deficiency symptoms observed in young experimental animals, i.e. growth retardation, scaly skin, and increased trans-epidermal water loss. However, arachidonic acid also serves essential functions particular in cellular signaling *via* its precursor role for numerous oxygenated derivatives like prostaglandins, leukotrienes, hepoxilins and other eicosanoids. Furthermore, arachidonic acid is also a structural part of the molecules called endocannabinoids (anandamide and 2-arachidonoylglycerol) that have signaling functions in relation to modulation of neurotransmitter release, which may involve physiological and patho-physiological phenomena as regulation of appetite, energy metabolism, pain perception, memory and learning. Omega-3 fatty acids in form of docosahexaenoic acid serve structural functions in phospholipid membranes of neuronal cells, and to a minor degree docosahexaenoic acid is also be precursor for signaling molecules like neuroprotectin D1. As all these fatty acids are ultimately derived from the diet, one can ask what the effect is of different dietary fats on the degree of formation of these bioactive signaling molecules. Generally, *in vivo* eicosanoid production from arachidonic acid can be increased and decreased by prolonged feeding with pharmacological levels of arachidonic acid and long-chain omega-3 fatty acids, respectively. However, changes in the level of these two fatty acids within the usual human diet do hardly affect this eicosanoid production. The beneficial effect of 0.85 mg/day of long-chain omega-3 fatty acids on death from coronary heart disease seems not to be mediated by changes in eicosanoid production. Preliminary data suggests that endocannabinoid formation is equally difficult to affect by the dietary intake of these two fatty acids.

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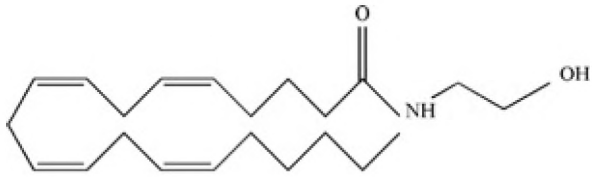
Key words: diet, eicosanoid formation, endocannabinoid formation, anandamide, 2-arachidonic acid, essential fatty acids, prostaglandin formation

Introduction

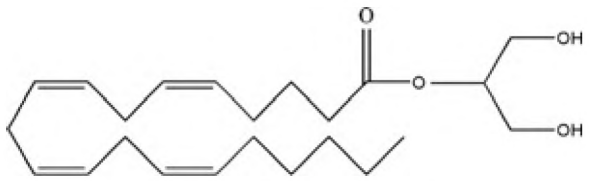
Poly-unsaturated fatty acids belonging to the omega-6- and omega-3-fatty acid families are considered as essential fatty acids, i.e. if deficient in the diet of young growing animals, a number of pathological deficiency symptoms will appear. Thus, the deficiency symptoms of the omega-6 fatty acids involve initially scaly skin, decreased growth, increased trans-epidermal water loss, and increased urinary excretion of vasopressin, and all these symptoms seem to be attributable to an essential structural function of linoleic acid in the epidermal water permeability barrier (Hansen *et al.,* 1986; Hansen, 1986; Hansen, 1989; Phinney *et al.,* 1993). A dietary intake of around 1 energy% should be enough to prevent these symptoms. Early symptoms of deficiency of omega-3 fatty acids involve delayed visual development probably caused by a defective structural function of docosahexaenoic acid in the retina (Lauritzen *et al.,* 2001; Lauritzen and Hansen, 2003; Wheeler *et al.,* 1975; Muskiet *et al.,* 2004). Docosahexaenoic acid is also essential for proper brain function. Different cell lines maintained in culture can exist without incorporation of any poly-unsaturated fatty acids (Stubbs *et al.,* 1992; Urade *et al.,* 1985) indicating that these fatty acids are not absolutely necessary for the basal function of cellular membrane structures of non-communicating cells in an optimal environment.

Bioactive lipids

Poly-unsaturated fatty acids acylated into membrane lipids like diacylglycerol have signaling roles (Wang, 2006) that may be influenced by the type of poly-unsaturated fatty acid present, i.e. arachidnonic acid versus docosahexaenoic acid (Madani *et al.,* 2004). The type of poly-unsaturated fatty acids in phospholipids of cellular membranes may also affect the function of selected integral enzymes in the membranes (Ruf *et al.,* 2006; Turner *et al.,* 2006). However, relatively little is known on this subject.



Anandamide
(AEA)



2-Arachidonoylglycerol
(2-AG)

Fig. 1. Structure of two endocannabinoids that can activate cannabinoid receptors.

Poly-unsaturated fatty acids as free acids, especially arachidonic acid, are also precursors for a vast number of enzymatically formed oxygenated derivatives, e.g. prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids, epoxy-derivatives, neuroprotectins, resolvins, and hepoxilins (Funk, 2001; Montuschi *et al.,* 2004; Pace-Asciak, 2005; Spector and Norris, 2007; Bazan, 2007). Generally, those oxygenated compounds that are derived from arachidonic acid are all together called eicosanoids while those derived from docosahexaenoic acid are called docosanoids. Besides these enzymatically formed eicosanoids and docosanoids, non-enzymatically oxidation of phospholipid-bound arachidonic acid and other long-chain poly-unsaturated fatty acids can lead to formation of isoprostanoids and neuroprostanes that after hydrolysis from the phospholipids may have biological functions during oxidative stress (Montuschi *et al.,* 2004).

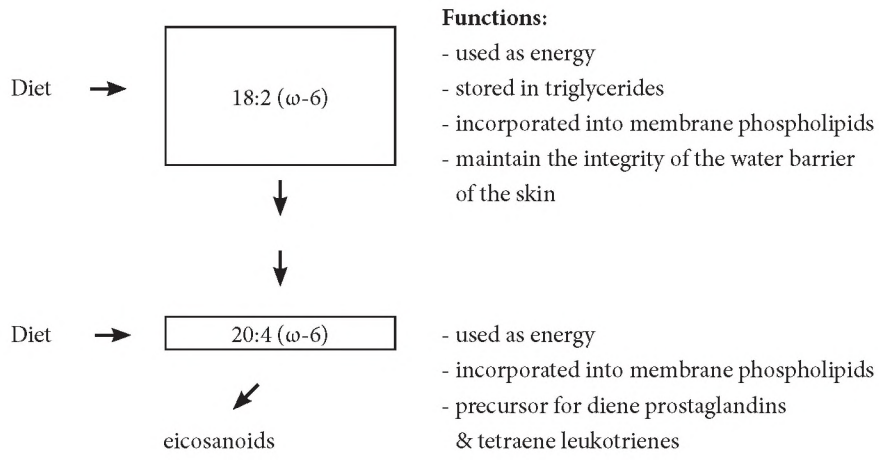


Fig. 2. Turnover and functions of omega-6 fatty acids. The figure illustrates the large dietary intake of linoleic acid (18, 2(ω-6)) and the corresponding large pool of linoleic acid in the body as typical for humans in western societies. A very minor amount of this linoleic acid can be elongated and desaturated to arachidonic acid (20, 4(ω-6)) that are found as a much smaller pool in the body. Omnivorous humans have a small dietary intake of arachidonic acid from animal products. The figure also illustrates the very small daily production of eicosanoids that is far smaller than the endogenous formation plus the dietary intake. Besides the eicosanoids, arachidonic acid is also precursor for endocannabinoids that probably also is formed in minute daily amounts.

Within the last decade, arachidonoylethanolamide (AEA, also called anandamide) and 2-arachidonoylglycerol (2-AG) (Fig. 1) have been demonstrated to be agonists for the cannabinoid receptors (Devane *et al.,* 1992; Mechoulam *et al.,* 1995; Sugiura *et al.,* 1995) that are involved in such diverse functions as regulation of appetite, neurotransmitter release, bone formation and pain (Hansen *et al.,* 2006; Pacher *et al.,* 2006; Sugiura *et al.,* 2006). Especially 2-AG appears to function as a retrograde messenger in regulating neurotransmitter release in the synapse (Hashimotodani *et al.,* 2005; Katona *et al.,* 2006). AEA is always formed *in vivo* together with other acylethanolamides, e.g. oleoylethanolamide (OEA) and palmitoylethanolamide, especially during tissue injury, and they have a variety of biological actions including modulation of food intake and neuroprotection (Hansen *et al.,* 2002; Hansen *et al.,* 2000; Sun *et al.,* 2007; Lo Verme *et al.*, 2005; Degn *et al.,* 2007; Petersen *et al.,* 2006).

It is astonishing that poly-unsaturated fatty acids, especially arachidonic acid, can serve as precursor for so many different signaling compounds.

Effects of diet on bioactive lipids

One can then ask whether the formation of all these different poly-unsaturated fatty acid-derived bioactive lipids are influenced by variation in the dietary intake of poly-unsaturated fatty acids, especially the dietary intake of the direct precursors, arachidonic acid and docosahexaenoic acid?

Generally, humans in the western world may have a rather large intake of linoleic acid, 18, 2ω6. i.e. 8-25 g/day, and thus also large stores of this fatty acid in both phospholipids, cholesteryl esters and triacylglycerol as shown in Fig 2.

Omnivorous humans also have an intake of

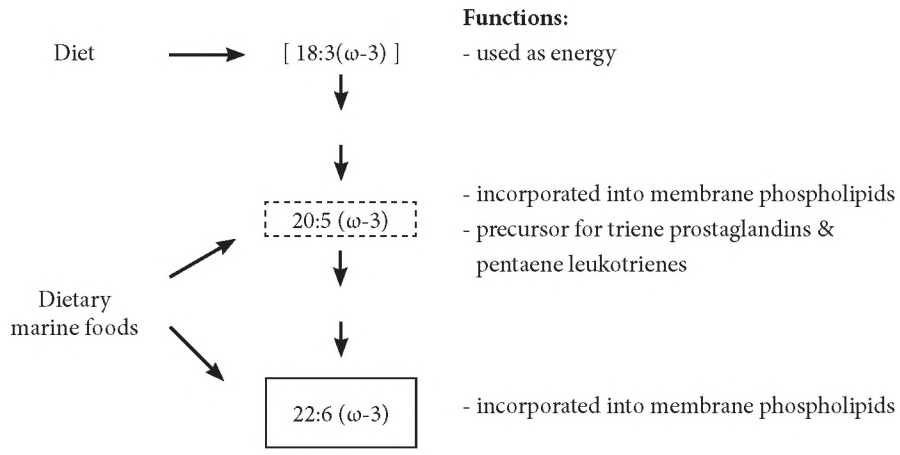


Fig. 3. Turnover and functions of omega-3 fatty acids. The figure illustrates the small dietary intake of alpha-linoleic acid (18, 3(ω-3)) and the nearly absent pool of alpha-linolenic acid in the body as typical of humans in western societies. Most of the ingested alpha-linolenic acid is oxidized and used for energy production while only an extremely small fraction is elongated and desaturated to omega-6 docosahexaenoic acid (22, 6(ω-6)) that is found specifically in relatively high amounts in neurons of the central nervous system. Humans ingesting seafood and fish oil can have a relatively large intake of omega-3 eicosapentaenoic acid (20, 5(ω-3)) and docosahexaenoic acid. Docosahexaenoic acid can also be oxidized and used for energy production. Only extremely small amounts of omega-3 fatty acids are converted to eicosanoids and docosanoids.

arachidonic acid from animal products amounting to 100-300 mg/day (Zhou and Nilsson, 2001). Arachidonic acid is not found in higher plants. Calculation of the endogenous production of arachidonic acid from linoleic acid indicates that this may be a bit higher, i.e. 180-800 mg/d depending among other things on the dietary intake of docosahexaenoic acid (Emken *et al.*, 1999). The daily endogenous prostaglandin formation seems to be quite low as estimated from urinary excretion of prostaglandin metabolites, i.e. in the order of 2-3 mg/day (Hansen, 1983; Zhou and Nilsson, 2001), and adding this up with all the other enzymatically produced eicosanoids, the total production of eicosanoids may probably not exceed 10 mg/day, thus being far lower than the daily arachidonic acid intake and endogenous arachidonic acid production. Humans in the western world ingest far less omega-3 fatty acids (around 1-3 g/day), and the dietary intake of eicosapentaenoic acid and docosahexaenoic acid varies a lot between individuals and populations being mainly related to the dietary intake of seafood (Fig 3).

In the USA, the average intake is around 130 mg/day while it in Denmark is around 500 mg/day (Gebauer *et al.*, 2006; Marckmann *et al.*, 1995; Tjonneland *et al.*, 1993) . In Japan it is around 800 mg/day (Hino *et al.*, 2004). It is clear that eicosapentaenoic and docosahexaenoic acids can inhibit the *in vitro* eicosanoid production from arachidonic acid (Hansen *et al.*, 1983; Rees *et al.*, 2006), but the *in vivo* eicosanoid formation seems to be much less influenced by dietary intake of eicosapentaenoic and docosahexaenoic acids (Murphy *et al.*, 2007; Ferretti *et al.*, 1998) probably because *in vitro* stimuli for prostaglandin formation often are stronger than those occurring *in vivo*. Generally, an intake of several grams per day of eicosapen-

taenoic and docosahexaenoic acid for many weeks is necessary for seeing a moderate decrease in the *in vivo* production of eicosanoids from arachidonic acid. Thus, the beneficial effect of 0.85 mg/day of long-chain omega-3 fatty acids on death frequency from coronary heart disease seems not to be mediated by changes in eicosanoid production (Marchioli *et al.,* 2002). Eicosapentaenoic acid is generally a rather poor substrate for prostaglandin-producing enzymes resulting in only very small levels of eicosapentaenoic acid-derived eicosanoids. Attempts to increase *in vivo* prostaglandin production by dietary supplements of pure arachidonic acid (e.g. 6 g/day for 2-3 weeks) have shown that a slight increase can be seen (Seyberth *et al.*, 1975) but the general impression is that eicosanoid production hardly is influenced by the variations in arachidonic acid content of usual human diets (Ferretti *et al.,* 1997; Pantaleo *et al.,* 2004). Studies of dietary influence on endogenous levels of endocannabinoids and acylethanolamides have been few. One study have shown that feeding of suckling piglets with a milk formula deficient in arachidonic acid decreased the brain levels of AEA and 2-AG and the levels can be increased by adding arachidonic acid to the formula (Berger *et al.,* 2001). Another study found that feeding young mice 10 wt% fish oil for 6 weeks, a diet that is not relevant for humans, resulted in decreased levels of 2-AG in the brain (Watanabe *et al.*, 2003). We have found that a one-week feeding of adult rats with diets containing 36 energy% of different fats (palm oil, safflower oil and olive oil) resulted in changes within both brain and intestine of levels of AEA, 2-AG and oleoylethanolamide. Thus, both type of dietary unsaturated fats increased levels of 2-AG and OEA in the brain and olive oil increased levels of AEA. These changes were seen without changes in the fatty acid composition of total brain phospholipids. All dietary fats decreased levels of acylethanolamides in the intestine (unpublished data).

Conclusion

The overall conclusion is that eicosanoid formation is not easily affected by variation in the dietary intake of arachidonic acid within the range of usual human diets. Prolonged intake of very high amounts of fish oil providing several grams of long-chain omega-3 fatty acids per day to humans will eventually decrease prostaglandin formation and thereby have weak anti-inflammatory and analgesic effects. Endogenous tissue levels of endocannabinoids and acylethanolamides seem in certain cases to be influenced in a complicated way by the type of dietary fat, a mechanism that perhaps is mediated through changes in expression of enzymes involved in the turnover of endocannabinoids and acylethanolamides.

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Physical effects of poly-unsaturated fatty acids on membranes

Ole G. Mouritsen

Abstract

A brief review is given of our current understanding of the physical and physico-chemical effects of poly-unsaturated fatty acids on lipid bilayers as models of biological membranes. Results from a variety of different experimental and theoretical studies will be synthesized into a coherent picture in which the major effects are described in terms of changes in mechanical properties, permeability, lateral pressure profile, and lateral lipid-domain organization. It is pointed out that the addition of an extra double bond in the fatty-acid acyl chains can lead to major changes in the bilayers properties that may reflect on protein- and receptor function.

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Key words: lipid bilayer, fluidity, lateral pressure profile, lipid domains, acyl-chain order, lipid-protein interaction, membrane mechanics, elasticity, permeability, lateral packing, rhodopsin, gramicidin A, docosahexaenoic acid, docosapentaenoic acid

Introduction

The lipid composition of biological membranes has remained an elusive problem in membrane science for decades and it is still a mystery why Nature has embarked on a strategy involving the use of a large diversity of lipids for each type of cellular membrane (Mouritsen 2005; Mouritsen and Andersen, 1998; Heimburg, 2007). Until recently, little was known regarding the actual lipid composition of membranes in terms of lipid species, but at the moment we witness an upsurge in the available data for the exact lipid composition of specific membranes and organelles, mainly obtained by the use of modern lipidomics techniques. Although basically unexplained, lipid composition and diversity have over the years been rationalized by the need for membranes to exert some kind of homeostatic control, e.g. by maintaining some more or less well-defined physico-chemical properties, such as fluidity, hydrophobic thickness, phase state, diffusivity, microviscosity, etc. Chemical lipid-specificity for protein function has been asserted in some cases, where specific binding sites at membrane proteins have been identified, but in the vast majority of cases, the putative requirement of certain lipids for the functioning of a specific membrane protein or receptor has turned out only to be apparent, and full or at least part of the function could be supported by an appropriate combination of other lipids.

The growing interest in determining the details of the trans-membrane structure (Cantor, 1999; 2003) and the small-scale lateral structure of membranes (Jacobson *et al.*, 2007) may be seen as an attempt to unravel in quantitative terms which physical and possibly unspecific mechanisms may be responsible for lipid-protein interactions in membranes. This viewpoint is that of the physical chemist, and the emergence of novel and powerful, quantitative experimental techniques, combined with large-scale computer simulations, has lead to new insight that eventually may give some clue to lipid diversity in membranes.

The effect of unsaturated and in particular poly-unsaturated fatty acids (PUFAs) on membrane properties and protein function holds a prominent position in this complex of problems. Except for very few cases, the surfactant lining of the lung alveoli being a well-known example, virtually all cell membranes of mammalian cells contain a substantial portion of unsaturated lipids. Neural membranes constitute an extreme case where more than half of the lipids are poly-unsaturated, notably docosahexaenoic acid (DHA), eicosapentaenoic acid, and arachidonic acid.

Traditionally, unsaturation has been explained as a need for membranes to stay fluid in the functional state, but it is obvious that fluidity can be controlled in many different ways and that by introducing unsaturation in lipids, other properties in addition to fluidity change at the same time, such as membrane thickness, trans-bilayer stress profile, permeability barrier, and membrane elasticity, just to name a few. Moreover, the concept of membrane fluidity is a best misleading and should be properly defined in each specific context.

Experimental and theoretical methods to study physical properties of model membranes

Quantitative studies of the various physical and physico-chemical effects of PUFAs on membranes proceed conveniently by means of well-defined lipid-bilayer models in the form of vesicles, liposomes, or supported bilayers. These model systems can be studied by experimental physical techniques, such as nuclear magnetic resonance spectroscopy, X-ray and neutron scattering, fluorescence microscopy, micromechanical experiments, and electrophysiological methods, or by theoretical and simulational techniques, such as molecular modeling and atomic-scale molecular dynamics simulation techniques. Interpretation of the experimental data are complicated by the fact, that even simple membranes composed by two or more lipid species have complex phase behavior, with the possibility of both large-scale phase separation as well as small-scale domain- or raft formation. An important regulator of this lateral organization is cholesterol which is abundant in all plasma membranes. The simulation of model systems is mostly restricted to small systems with few different molecular components, and detailed molecular dynamics simulations in particular are hampered by severe restriction in time.

Fluidity, acyl-chain order, phase state, diffusion, mechanics, permeability, and lateral pressure profile

Based on such quantitative experimental as well as theoretical model studies a coherent picture is gradually emerging of the effect of PUFAs on membrane structure and dynamics and how the membrane properties control protein function. Increasing the degree of unsaturation of the fatty acids in the lipids making up a bilayer membrane has a number of generic effects (Stillwell and Wassall, 2003). The most dramatic effect in physical properties occurs when two or more double bonds are introduced. In the case where nothing but the degree of unsaturation is changed, i.e. the acyl-chain length is kept constant and the polar

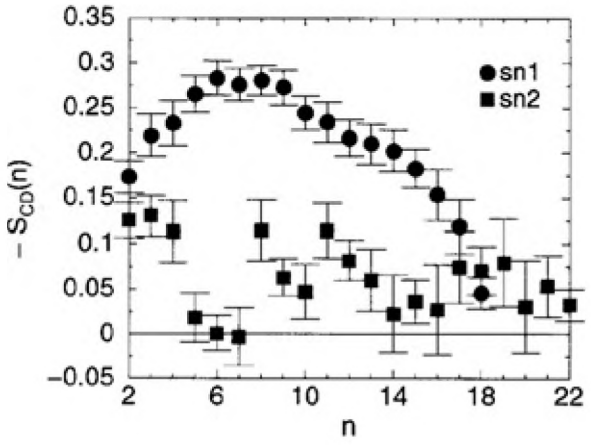

Fig. 1. Acyl-chain segmental order parameter, S_{CD} (n), along the two acyl chains of lipids composed of 18:0 and 22:6 DHA chains. The data derives from an atomic-scale molecular dynamics simulation of a lipid bilayer. Adapted from Saiz and Klein (2001).

head groups remain the same, these effects are as follows. The phase state of the bilayer changes towards a stabilization of fluid phases, specifically liquid-disordered phases, that is phases or phase-separated states of low melting points. The liquid-disordered phases have high lateral molecular mobility, i.e. large molecular diffusion constants and a substantial degree of acyl-chain disorder (Saiz and Klein, 2001; Elho *et al.*, 2003; Martinez-Seara *et al.*, 2007) as illustrated in Fig. 1. In addition, the liquid-disordered phase displays accelerated dynamics of the head groups and the glycerol backbone, as well as augmented acyl-chain rotational dynamics (Ollila *et al.*, 2007). The thickness of the bilayer is deceased in order to allow for the increased acyl chain disorder.

The liquid-disordered phases can be turned into liquid-ordered phases upon introduction of cholesterol, which however retains the diffusional characteristics of a liquid. Upon increasing the degree of unsaturation, the transbilayer passive permeability is diminished substantially (Huster *et al.*, 1997; Olbrich *et al.*, 2000) which is accompanied by a reduction in bilayer bending rigidity and an increase in lateral compressibility (Rawicz *et al.*, 2000; Olbrich *et al.*, 2000; Koenig *et al.*, 1997). These effects are illustrated in Fig. 2.

The lateral pressure profile of the bilayer, cf.

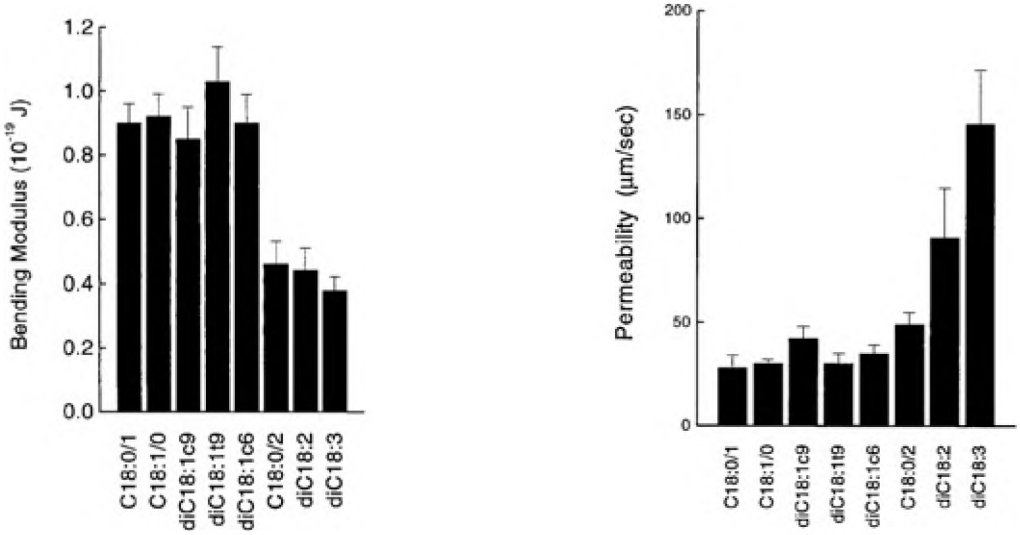

Fig. 2. Left: Elastic bending moduli for di-C_{18} phosphatidylcholine bilayers arranged in order of increasing unsaturation. Adapted from Rawicz *et al.* (2000). Right: Water permeability for di-C_{18} phosphatidylcholine bilayers arranged in order of increasing unsaturation. Courtesy by Evan Evans. Adapted from Olbrich *et al.* (2000).

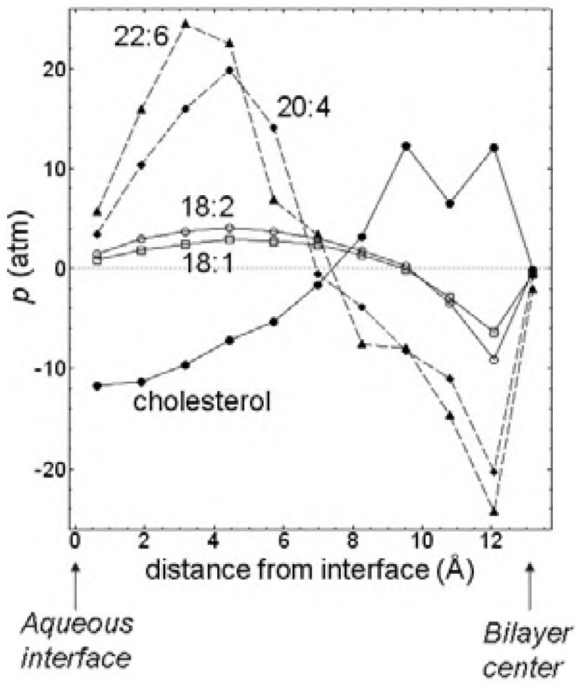

Fig. 3. Theoretical prediction of changes in the lateral pressure profile of lipid bilayers that become incorporated with 1 mol% unsaturated lipid into a 16:0 lipid bilayer. The data are compared to the opposite changes induced by cholesterol incorporation. Courtesy of R. S. Cantor.

Fig. 3, is changed by increasing unsaturation in a manner that shifts the lateral stresses towards the head-group region of the bilayer (Carillo-Trip and Feller, 2005; Michailescu and Gawrisch, 2006; Ollila *et al.,* 2007). This effect implies changes in the curvature stress of the bilayer and an increase in the flip-flop rate between the two monolayer sheets (Armstrong *et al.,* 2003). Possibly even more important, introduction of an extra double bond in the acyl chain, specifically from five to six, has a significant effect on the lateral pressure profile whereas it appears only to have a marginal effect on other bulk bilayer properties (Eldho *et al.,* 2003). Furthermore, the actual position of the double bonds has a significant effect on form of the lateral pressure profile (Ollila *et al.,* 2007).

Lateral molecular organization

Several studies have shown that PUFAs, in particular DHA, participate in lipid-domain formation in membranes and that cholesterol plays a key role in this type of lateral organization. In membranes containing both mono-unsaturated and poly-unsaturated lipids, cholesterol induces a sequestering of the PUFAs into domains depleted of cholesterol and mono-unsaturated lipids (Brzustowicz *et al.*, 2001; Niu and Littmann, 2002; Shaik *et al.,* 2004).

Lipid-protein interactions

Turning then to lipid-protein interactions, two types of quantitative model studies of the effect of PUFAs on the functioning of membrane proteins deserve mention. One type is based on using gramicidin A as a model protein to gauge the relationship between channel function and membrane elastic properties (Bruno *et al.,* 2007). By comparing the effect of oleic acid (OA) and DHA, it is found using single-channel electrophysiological techniques, that although OA partitions more

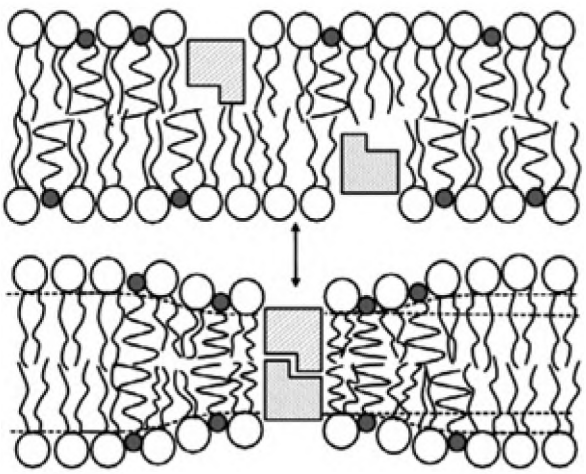

Fig. 4. Schematic illustration of local softening of a lipid bilayer by incorporation of DHA that supports local curvature and formation of a channel dimer of gramicidin A. Courtesy of Olaf Sparre Andersen. Adapted from Bruno *et al.* (2007).

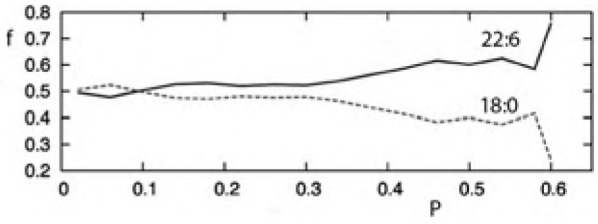

Fig. 5. Difference in packing properties of DHA (22:6) and saturated lipids 18:0 near rhodopsin incorporated in a lipid bilayer as determined from molecular dynamics simulations. The figure shows the fraction, f, of the two lipid species that have a particular packing score, P, at the lipid-protein interface. It appears that DHA packs in a more localized way with rhodopsin. Adapted from Grossfield *et al.* (2006).

strongly into the bilayers than DHA, DHA has a much larger effect than OA on channel function. Since DHA strongly softens the bilayer, it was concluded that bilayer elasticity is a key to understanding the influence of PUFAs like DHA on membrane protein function. DHA does not bind to the channel but is statistically accumulated locally around the channel whereas OA is not. This is illustrated schematically in Fig. 4.

The other type of study is concerned with rhodopsin reconstituted into model membranes (Feller and Gawrisch, 2005) where it was found that the effect of DHA on the structural transitions in rhodopsin could be mimicked by other non-lamellar-forming lipids such as phosphatidylethanolamine (PE) lipids that induce a similar propensity for forming curved structures (Brown, 1997). Furthermore it was found that rhodopsin segregates and packs tightly with the PUFAs, cf. Fig. 5, and this segregation is augmented by the presence of cholesterol (Grossfield *et al.*, 2006; Polozova and Littman, 2000).

Some conclusions

Global effects: Increasing the degree of unsaturation in the lipid-acyl chains softens bilayer membranes and increases the permeability.

Local effects: Unsaturation shifts lateral pressures towards the head-group region and induces small-scale lipid-domain formation organization of lipid membranes. Adding an extra double bond has a big effect and depends on the location of the double bond.

Protein function: The functioning of membrane receptors and channels may depend on the lateral pressure profile and the local domain formation.

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I am grateful to Michael Crawford and Myer Bloom for teaching me about brain evolution and the optimization-of-physics hypothesis for membrane function. I wish to thank the members of MEMPHYS-Center for Biomembrane Physics for many discussions over the years regarding the effect of different lipids on membrane structure and function. MEMPHYS is supported by the Danish National Research Foundation.

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Vision and neurological function supported by the potent, stereospecific mediator neuroprotectin D1 biosynthesized from docosahexaenoic acid

Nicolas G. Bazan

Abstract

The neuroprotective properties of omega-3 fatty acids have been demonstrated during the years by several laboratories. The identification of neuroprotectin D1 (NPD1), a biosynthetic product of docosahexaenoic acid (DHA), in brain and retina as well as the characterization of its bioactivity, is generating a renewed interest on the omega-3 fatty acid's functional role and physiopathological significance.

In experimental stroke, endogenous NPD1 synthesis was found to be upregulated, and the infusion of the lipid mediator into the brain under these conditions revealed neuroprotective bioactivity of NPD1. Furthermore, when DHA was administered i.v. after middle-cerebral artery occlusion, protection was concomitant with NPD1 synthesis on the ipsilateral brain side. In the retinal pigment epithelial (RPE) cells, NPD1 synthesis induction results in cytoprotection against oxidative stress. The bispyridinium bisretinoid, A2E (a byproduct of phototransduction that becomes toxic when it accumulates in RPE cells during aging or in age-related macular degeneration), when added to RPE cells, was found to display attenuated cytotoxicity in the presence of NPD1. Integrity of RPE cells is necessary for photoreceptor cell survival and vision.

Neurotrophins, particularly pigment epithelium-derived factor (PEDF), induce NPD1 synthesis and its polarized apical secretion, implying paracrine and autocrine bioactivity of this lipid mediator. Moreover, DHA elicits a concentration-dependent and selective potentiation of PEDF-stimulated NPD1 synthesis and release through the apical RPE cell surface. The signaling activated by PEDF and DHA uncovered synergistic cytoprotection, with concomitant NPD1 synthesis, when cells were challenged with oxidative stress. Also, DHA and PEDF synergistically activate anti-apoptotic protein expression and decreased pro-apoptotic Bcl-2 protein expression and caspase 3 activation during oxidative stress. Thus, our results identify neurotrophins as regulators of NPD1 biosynthesis and of its polarized apical efflux from RPE cells. Moreover, phagocytosis of photoreceptor outer segments by retinal pigment epithelial cells downregulates oxidative stress-mediated apoptosis with concomitant synthesis of NPD1.

The homeostatic regulation between photoreceptors and RPE preserves RPE cell integrity during successful aging. In fact, RPE cell density is maintained during nine decades if eye pathology does not arise. However, failure of homeostasis results in enhanced DHA peroxidation, drusen formation, lipid peroxide protein adduct accumulation, apoptosis, and pathoangiogenesis. Overall, it is apparent that a breakdown in the balance of protective and potentially cytotoxic factors is involved in various forms of retinal degeneration. NPD1

synthesis is induced under conditions where excessive oxidative stress threatens to disrupt homeostasis, and rescue signals, such as neurotrophins, are released to protect cell integrity.

Hippocampal CA1 regions from Alzheimer's disease (AD) patients (relatively rapidly sampled) show a major reduction in NPD1. Based upon this finding, we developed a human brain cell aging model to study further the significance of NPD1. The aging of human neural progenitor cells (HN cells, neurons and glia) in primary culture during 8 weeks is accompanied by an 8-fold enhanced synthesis and release of Aß40 and Aß42 peptides that resembles Aß deposition during brain aging and in AD. IL-1ß stimulates gamma-secretase-mediated cleavage of ßAPP into Aß peptides. Conversely, DHA suppressed both Aß40 and Aß42 peptide release with concomitant NPD1 synthesis. Moreover, NPD1 inhibits Aß42-induced apoptosis in HN cells. Therefore, DHA neuroprotection in aging human brain cells involves NPD1 synthesis.

Pro- and antiapoptotic proteins are modulators proximal to mitochondria and cell damage. Proapoptotic Bik and Bax were enhanced by Aß42, but not by DHA or NPD1, whereas Bcl-2, Bcl-xl, and Bfl-1(A1) were increased in the presence of DHA. NPD1, on the other hand, promoted a much larger increase in antiapoptotic Bcl-2 proteins. Bfl-1(A1) increased almost 6-fold. These modulatory actions of NPD1 may play critical roles in the survival of aged and terminally differentiated cells and break the mechanistic link between inflammatory signaling and apoptosis. In fact, NPD1 also induces the antiapoptotic Bcl-2 family proteins Bcl-2 and Bcl-xl in oxidatively challenged human retinal pigment epithelial cells and promotes cytoprotection. Thus the interplay of DHA-derived neuroprotective signaling aims to counteract proinflammatory, cell-damaging events triggered by multiple, converging cytokine and amyloid peptide factors in AD.

Neural mechanisms leading toward NPD1 generation from DHA thereby appear to redirect cellular fate toward successful preservation of RPE-photoreceptor cell integrity, and brain cell aging. The Bcl-2 pro- and antiapoptotic gene families, sAPP alpha (and/or other neurotrophins) and NPD1, lie along a cell fate–regulatory pathway whose component members are highly interactive, and have potential to function cooperatively in brain and retina cell survival. Agonists of NPD1 biosynthesis, NPD1 analogs or dietary regimens may be useful for exploring new preventive/therapeutic strategies for neurodegenerative diseases.

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Key words: vision, neuroprotection, neuroprotectin D1, docosahexanoic acid

Introduction

The neuroprotective properties of omega-3 fatty acids have been demonstrated during the years by several laboratories. The identification of neuroprotectin D1 (NPD1), a biosynthetic product of docosahexaenoic acid (DHA), in brain and retina as well as the characterization of its bioactivity (Bazan, 2005; 2006), is generating a renewed interest on the omega-3 fatty acid's functional role and physiopathological significance. In experimental stroke, endogenous NPD1 synthesis was found to be upregulated (Marcheselli *et al.,* 2003), and the infusion of the lipid mediator into the brain under these conditions revealed neuroprotective bioactivity of NPD1 (Marcheselli *et al.*, 2003). Furthermore, when DHA was administered i.v. after middle-cerebral artery occlusion, protection was concomitant with NPD1 synthesis on the ipsilateral brain side (Belayev *et al.,* 2005).

Retinal pigment epithelial (RPE) cells, human progenitor cells, and brain models of injury (e.g., ischemia-reperfusion) respond to oxidative stress or injury by activating the synthesis of an endogenous neuroprotective mediator, NPD1 (Fig. 1) (Mukherjee *et al.,* 2004). The name 'neuroprotectin D1' was suggested based upon its **neuroprotect**ive bioactivity in oxidatively stressed RPE cells and brain, and its potent ability to **in**activate pro-apoptotic and pro-inflammatory signaling. 'D1' refers to its being the first identified neuroprotective mediator derived from DHA. DHA belongs to the omega-3 essential fatty acid family (all of which are derived from linolenic acid, 18:3, ω-3) and, therefore, cannot be made *de novo* in the body. The photoreceptor cells, unlike most other cells of the nervous system, are highly enriched in DHA, tenaciously retaining DHA even during very prolonged periods of omega-3 fatty acid deprivation (Bazan, 2006; SanGiovanni and Chew, 2005; Marszalek and Lodish, 2005).

Previous studies have shown that the retina forms mono-, di-, and trihydroxy derivatives of DHA, and lipoxygenase inhibitors block this synthesis, suggesting an enzymatic process of a lipoxygenase nature (Bazan *et al.,* 1984). Although, at the time, the stereochemistry and bioactivity of these DHA-oxygenated derivatives were not defined, it was proposed that these lipoxygenase products might be neuroprotective (and at the same time, the name 'docosanoids' was suggested) (Bazan *et al.,* 1984; 1985). Upon the advent of liquid chromatography, photodiode array, electrospray ionization, and tandem mass spectrometry-based lipidomic analysis, a collaboration between the group of Charles Serhan (Harvard Medical School) and our group identified oxygenation pathways for the synthesis of the docosanoid NPD1 during brain ischemia-reperfusion (Marcheselli *et al.,* 2003). Moreover, it was also found that RPE cells have the ability to synthesize NPD1 (Mukherjee *et al.,* 2004). NPD1 is formed from free (unesterified) DHA and released from membrane phospholipids by a phospholipase A2 (PLA2). Photoreceptors and RPE cells, although they contain phospholipids richly endowed with DHA (as docosahexaenoyl- or DHA-elongated fatty acyl-chains), display an undetectable quantity of unesterified (free) DHA (as is the case with unesterified arachidonic acid) under basal, unstimulated conditions (Bazan, 2003; Aveldano and Bazan, 1974; 1975; Horrocks and Farooqui, 1994; Sun *et al.*, 2004). This means that the pool size of unesterified DHA is tightly controlled at the levels of its production by a PLA2, by its removal (e.g., by reacylation), and by peroxidation. Free DHA to be incorporated into membrane phospholipids first becomes the substrate of docosahexaenoyl-coenzyme A synthesis for its channeling through acyltransferases that incorporate this fatty acid into phospholipids (Reddy and Bazan, 1984a; 1984b; 1985a; 1985b). The RPE cell thus modulates the uptake, conservation, and delivery of DHA to photoreceptors (Bazan *et al.,* 1985). In addition, the RPE cell utilizes a specific DHA-phospholipid pool as a precursor

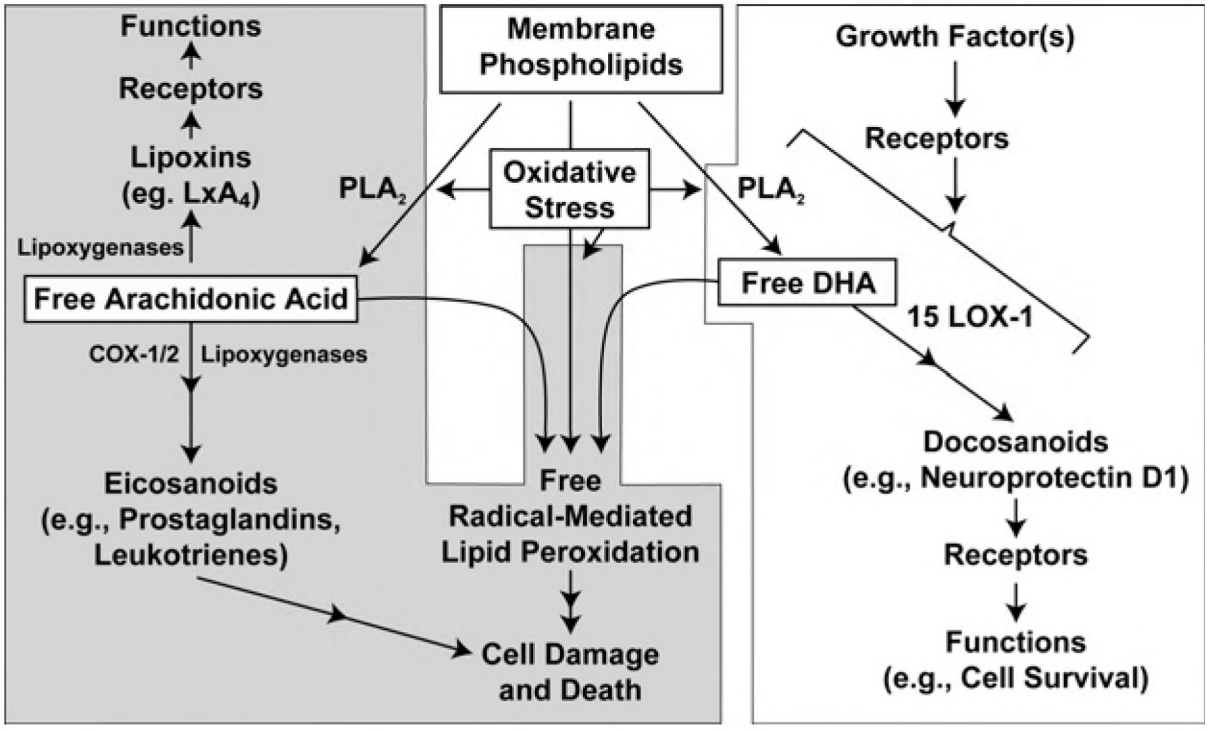

Fig. 1. Fate and bioactivity of derivatives of poly-unsaturated acyl chains (arachidonic acid, C20:4, ω-6; docosahexaenoic acid, C22:6, ω-3) of membrane phospholipids. PLA2 = phospholipase A2. The central arrows indicate that free radical-mediated lipid peroxidation may attack esterifed arachidonoyl or docosahexaenoyl chains, as well as free arachidonic or docosahexaenoic acids. The evolving lipid peroxidation products are highly reactive and promote cell injury. On the left of the diagram (shaded box), phospholipase A2 is depicted releasing free arachidonic acid and leading to the eicosanoid cascade. In the top left portion of the blue box, lipoxin synthesis is indicated. This pathway down regulates inflammation. In the lower portion of the blue box, other eicosanoids may contribute to enhancing inflammation. On the right side of the diagram (unshaded box), this figure illustrates that growth factor-mediated activation of the synthesis of docosanoids leads to biologically active mediators such as neuroprotectin D1, which in turn operate through receptors. Oxidative stress by itself activates both docosanoid synthesis (to counteract cytotoxic actions) and free radical-mediated lipid peroxidation.

for the pathway leading to NPD1 synthesis. Then this stereospecific mediator is synthesized after DHA is released through DHA oxygenation by a PLA2, followed by a 15-lipoxygenase-like activity (Mukherjee *et al.,* 2004). The nature of these enzymes needs to be precisely defined. In Alzheimer's disease (AD) brain of short postmortem time, it was found that cPLA2α and 15 lipoxygenase-1 expression changed in concert with NPD1-decreased content and DHA enhanced pool size in the CA1 area of the hippocampus (Lukiw *et al.,* 2005). In ARPE-19 cells (spontaneously transformed human RPE cells), interleukin (IL)-1β, oxidative stress, or the Ca^{2+} ionophore A23187 activates the synthesis of NPD1 (Mukherjee *et al.,* 2004). In turn, NPD1 might act in an autocrine fashion and/or diffuse through the IPM, to act in a paracrine mode on photoreceptor cells and/or Müller cells (Bazan, 2006).

Neuroprotectin D1: a homeostatic regulator of photoreceptor/retinal pigment epithelial cell integrity

In the RPE cells, NPD1 synthesis induction results in cytoprotection against oxidative stress (Mukherjee *et al.*, 2004). The bispyridinium bisretinoid, A2E (a byproduct of phototransduction that becomes toxic when it accumulates in RPE cells during ageing or in age-related macular degeneration), when added to RPE cells, was found to display attenuated cytotoxicity in the presence of NPD1. Integrity of RPE cells is necessary for photoreceptor cell survival and vision (Bazan, 2006). Neurotrophins, particularly pigment epithelium-derived factor (PEDF), induce NPD1 synthesis and its polarized apical secretion, implying paracrine and autocrine bioactivity of this lipid mediator. Moreover, DHA elicits a concentration-dependent and selective potentiation of PEDF-stimulated NPD1 synthesis and release through the apical RPE cell surface. The signaling activated by PEDF and DHA uncovered synergistic cytoprotection, with concomitant NPD1 synthesis, when cells were challenged with oxidative stress. Also, DHA and PEDF synergistically activate anti-apoptotic protein expression and decreased pro-apoptotic Bcl-2 protein expression and caspase 3 activation during oxidative stress. Thus, our results identify neurotrophins as regulators of NPD1 biosynthesis and of its polarized apical efflux from RPE cells (Mukherjee *et al.*, 2007a). Moreover, phagocytosis of photoreceptor outer segments by retinal pigment epithelial cells downregulates oxidative stress-mediated apoptosis with concomitant synthesis of NPD1 (Mukherjee *et al.*, 2007b).

The homeostatic regulation between photoreceptors and RPE preserves RPE cell integrity during successful ageing. In fact, RPE cell density is maintained during nine decades if eye pathology does not arise (Gao and Hollyfield, 1992). However, failure of homeostasis results in enhanced DHA peroxidation, drusen formation, lipid peroxide protein adduct accumulation, apoptosis, and pathoangiogenesis. Overall, it is apparent that a breakdown in the balance of protective and potentially cytotoxic factors is involved in various forms of retinal degeneration (Bazan, 2006; Rattner and Nathans, 2006; Bok, 2005; Strauss, 2005). NPD1 synthesis is induced under conditions where excessive oxidative stress threatens to disrupt homeostasis, and rescue signals, such as neurotrophins, are released to protect cell integrity (Fig. 1). Triggers of the NPD1 response include A2E and A2E epoxides (oxiranes) that accumulate in the ageing RPE and in Stargardt's disease and other retinal degenerations (Sparrow and Cai, 2001). Therefore, NPD1 is a potent modulator of photoreceptor/RPE cell functional integrity (Bazan, 2007).

Neuroprotectin D1 promotes cell survival during neurodegeneration

Hippocampal CA1 regions from AD patients (relatively rapidly sampled) show a major reduction in NPD1 (Belayev *et al.*, 2005). Based upon this finding, we developed a human brain cell ageing model to study further the significance of NPD1. The ageing of human neural progenitor cells (HN cells, neurons and glia) in primary culture during 8 weeks is accompanied by an 8-fold enhanced synthesis and release of Aß40 and Aß42 peptides that resembles Aß deposition during brain ageing and in AD. IL-1ß stimulates gamma-secretase-mediated cleavage of ßAPP into Aß peptides. Conversely, DHA suppressed both Aß40 and Aß42 peptide release with concomitant NPD1 synthesis. Moreover, NPD1 inhibits Aß42-induced apoptosis in HN cells. Therefore, DHA neuroprotection in ageing human brain cells involves NPD1 synthesis.

Pro- and antiapoptotic proteins are modulators proximal to mitochondria and cell damage. Proapoptotic Bik and Bax were enhanced by Aß42, but not by DHA or NPD1, whereas Bcl-2, Bcl-xl, and

Bfl-1(A1) were increased in the presence of DHA. NPD1, on the other hand, promoted a much larger increase in antiapoptotic Bcl-2 proteins. Bfl-1(A1) increased almost 6-fold. These modulatory actions of NPD1 may play critical roles in the survival of aged and terminally differentiated cells and break the mechanistic link between inflammatory signaling and apoptosis. In fact, NPD1 also induces the antiapoptotic Bcl-2 family proteins Bcl-2 and Bcl-xl in oxidatively challenged human retinal pigment epithelial cells and promotes cytoprotection.

Conclusions

The molecular and functional organization of the cell is being further explored by genomics, proteomics, and metabolomics. Within metabolomics, lipidomics is an evolving and powerful approach for the detailed identification of lipid classes and molecular species, including structural lipids as well as bioactive lipids (mediators of cell signaling). The lipidome of a cell, or part of a cell (e.g., dendrites, specific cellular compartment), defines the complete characterization of the lipids. We are using a lipidomic-based analysis for the decoding of omega-3 fatty acids in the central nervous system. This approach has led to the discovery of neuroprotectin D1 in the RPE cell (Mukherjee *et al.,* 2004) and the uncovering of its bioactivity.

Overall the interplay of DHA-derived neuroprotective signaling (NPD1-mediated) aims to counteract proinflammatory, cell-damaging events triggered by multiple, converging cytokine and amyloid peptide factors in AD (Lukiw, 2005). Neural mechanisms leading toward NPD1 generation from DHA thereby appear to redirect cellular fate toward successful preservation of RPE-photoreceptor cell integrity (Bazan, 2006; 2007), and brain cell ageing (Lukiw, 2005). The Bcl-2 pro- and antiapoptotic gene families, sAPP alpha (and/or other neurotrophins) and NPD1, lie along a cell fate–regulatory pathway whose component members are highly interactive, and have potential to function cooperatively in brain and retina cell survival. Agonists of NPD1 biosynthesis, NPD1 analogs or dietary regimens may be useful for exploring new preventive/therapeutic strategies for neurotrauma, stroke, and neurodegenerative diseases.

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