Mechanisms of Action of Docosahexaenoic Acid in the Nervous System

Norman Salem, Jr.*, Burton Litman, Hee-Yong Kim, and Klaus Gawrisch

Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, Maryland

ABSTRACT: This review describes (from both the animal and human literature) the biological consequences of losses in nervous system docosahexaenoate (DHA). It then concentrates on biological mechanisms that may serve to explain changes in brain and retinal function. Brief consideration is given to actions of DHA as a nonesterified fatty acid and as a docosanoid or other bioactive molecule. The role of DHA-phospholipids in regulating G-protein signaling is presented in the context of studies with rhodopsin. It is clear that the visual pigment responds to the degree of unsaturation of the membrane lipids. At the cell biological level, DHA is shown to have a protective role in a cell culture model of apoptosis in relation to its effects in increasing cellular phosphatidylserine (PS); also, the loss of DHA leads to a loss in PS. Thus, through its effects on PS, DHA may play an important role in the regulation of cell signaling and in cell proliferation. Finally, progress has been made recently in nuclear magnetic resonance studies to delineate differences in molecular structure and order in biomembranes due to subtle changes in the degree of phospholipid unsaturation.

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DHA COMPOSITION

In the 1960s, the very high level of docosahexaenoic acid (DHA, 22:6n3) in the mammalian brain was already appreciated (1,2) although the first description by Thudichum (3) was nearly a century earlier [see review by Salem *et al*. (4)]. Yabuuchi and O'Brien (5) described the positional distribution of brain phosphoglycerides in 1968, detailing both the high concentration of DHA in position *sn*-2 and its concentration in the aminophospholipids, phosphatidylserine (PS), and phosphatidylethanolamine (PE). By the early 1970s, the very high concentration in brain synaptosomal plasma membranes (6) and synaptic vesicles (7) was described by Breckenridge and co-workers. Table 1 presents the DHA composition in the aminophospholipids of brain and other selected mammalian tissues (1,6–18). It is apparent that the DHA content of the nervous system is very high. The retina not only contains a very high level of DHA in the rod outer segment (ROS) membranes, but also contains a very considerable amount of di-DHA species (19) as well as ones with DHA coupled to other highly unsaturated fatty acids (HUFA). The sperm is another compartment enriched in DHA. Every mammalian cell contains DHA, and phospholipids of internal organs and muscles have a significant content. Human milk contains a relatively low content of DHA with a higher percentage in phospholipids than triglycerides, the main lipid component of milk.

It has long been known that when an adult mammal consumes a diet low in DHA and its n-3 precursors, the nervous system content of DHA is much less altered than are other organs, i.e., DHA is said to be tenaciously retained once neural development has occurred (for reviews, see Refs. 4,20). However, animal studies have shown that when n-3 fat sources are inadequate during early neural development, then the levels of brain and retinal DHA decline (4,20–24). This has also been confirmed in autopsy studies of human infants that were fed a vegetable oil-based formula with low n-3 fat sources vs. breast-feeding in which preformed DHA was present (25–27). This has naturally led to an interrogation of the functional consequences of neural DHA loss.

ANIMAL STUDIES

Representative studies in the animal literature (28–42) concerning the n-3 fatty acid deficiency syndrome are presented in Table 2. Only studies that focus on neural functions, notably brain and retinal functions, have been included here. Typically, these studies involve a two-generation diet regimen in which the mother is raised on an n-3-deficient diet and her offspring are then studied. Such treatment has generally been found to be necessary to induce a marked decline in brain and retinal DHA; a decline of 50–80% is typical of those associated with a change in neural function. A variety of different tasks show impairment, including those in both the visual and olfactory modalities (Table 2). In addition to decrements in performance in simple associative learning types of tasks, losses in spatial memory (39) and olfactory set learning have been reported recently (43). Thus, the loss in brain DHA may be said to affect cognition, at least to the extent that it can be ascertained in the rat.

*To whom correspondence should be addressed at 12420 Parklawn Dr., Room 150, Rockville, MD 20852. E-mail: nsalem@dicbr.niaaa.nih.gov

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DPAn-6, docosapentaenoic acid; DROSS, dipolar recoupling on-axis with scaling and shape preservation; HUFA, highly unsaturated fatty acids; LCP, long-chain polyunsaturates; α-LNA, α-linolenate; LO, lipoxygenase; M, metarhodopsin; MAS, magic angle spinning; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; PC, phosphatidylcholine; PDE, phosphodiesterase; PE, phosphatidylethanolamine; PS, phosphatidylserine; ROS, rod outer segment.

TABLE 1
Docosahexaenoic Acid (DHA) Content of Aminophospholipids in Various Mammalian Tissues

				Phospholipid class (% DHA)	
Ref	Species	Tissue	Fraction	Phosphatidylserines	Phosphatidylethanolamines
1	Human	Brain	Gray matter	36.6	24.3
1	Human	Brain	White matter	5.6	3.4
8	Bovine	Brain	Gray matter	28.7	—
8	Bovine	Brain	White matter	7.6	—
6	Rat	Brain	Synaptic plasma membrane	34.1	32.4
7	Rat	Brain	Synaptic vesicles	37.0	30.6
9	Human	Retina	—	18.5	22.2
10	Bovine	Retina	Rod outer segment	37.7	38.7
11	Ram	Sperm	—	—	40.5
12	Bovine	Sperm	—	—	37.8
13	Rat	Heart	—	—	23.4
14	Rat	Liver	Plasma membrane	14.1	6.9
15	Rat	Muscle	—	—	36.1
16	Human	Platelet	—	2.1	4.1
17	Human	Erythrocyte (infant)	—	—	5.9
18	Human	Milk	—	—	0.08

DHA RECOVERY

It has long been known that once depleted, the brain recovers its DHA rather slowly (44,45). A recent study in rats provided the time courses of DHA recovery and the reciprocal decline in docosapentaenoic acid (DPAn-6, 22:5n-6) in the retina, brain, liver, and serum when the rats were repleted with a diet containing both α-linolenate (LNA) and DHA (46). The half-times for brain and retinal recovery of DHA were 2.9 and 2.1 wk, respectively, even though the liver and plasma half-times were only 0.3 and 0.5 wk, respectively. This suggests a rather slow transport of DHA into the brain/retina even in the case of a DHA-deficient nervous system (47).

It could be hypothesized then that if the functional consequences of dietary n-3 fatty acid deficiency were due to the loss in DHA, at least some neural functions may be restored as the neuronal and retinal DHA level is restored. Others may not be reversible due to missed opportunities in sequential development or changes in structural features of the brain (48). The first such study of functional recovery by Connor and

TABLE 2
Animal Studies of Effects of Low n-3 Fatty Acid Diets on Neural Functions

Task	Reference
Rodent studies	
Reduced amplitude of a- and b-waves	Wheeler and Benolken, 1975 (28)
Y-maze performance	Lamptey and Walker, 1976 (29)
Active avoidance task	Mills *et al.*, 1988 (30)
Brightness discrimination	Yamamoto *et al.*, 1991 (31)
Shock avoidance	Bourre *et al.*, 1989 (32)
Death after neurotoxin	Bourre *et al.*, 1989 (32)
Exploratory activity	Enslen *et al.*, 1991 (33)
Scopolamine-induced locomotion	Nakashima *et al.*, 1993 (34)
Age of eye opening (mice)	Wainwright *et al.*, 1991 (35)
Morris water maze (mice)	Nakashima *et al.*, 1993 (34)
Electroretinogram, a-wave, peak-to-peak	Weisinger *et al.*, 1996 (36,37)
Delayed acquisition of olfactory discrimination	Sheaff-Greiner, *et al.*, 1999 (38)
Spatial task acquisition and memory	Moriguchi *et al.*, 2000 (39)
Cat study	
Electroretinogram, a- and b-wave implicit time	Pawlosky *et al.*, 1997 (40)
Primate studies	
Reduced visual acuity, longer implicit time	Connor and Neuringer, 1984 (41)
Impaired recovery of dark-adaptation	Neuringer *et al.*, 1986 (42)

Neuringer (49) indicated that electroretinographic changes associated with low retinal DHA persisted after DHA repletion. However, Moriguchi *et al*. (50) recently presented evidence that spatial task acquisition and memory are reversible and, to a first approximation, correlate well with the level of brain DHA. However, Weisinger *et al*. (51) reported that when n-3-deficient guinea pigs were subsequently given a diet containing LNA, changes in mean arterial blood pressure and electroretinographic changes in the a-wave were not reversed even when the DHA levels were indistinguishable from the control levels. Thus, it appears that there may be no general answer to the question of reversibility of losses in function due to DHA losses in early development; the answer will depend on the type of function involved.

HUMAN STUDIES

As mentioned above, formula-feeding of infants has been associated with a loss in brain DHA with respect to the level in those breast-fed (25–27). The pre-existing animal literature would predict that formula-fed infants would have a functional deficit if the neural DHA loss were of a sufficient magnitude. Of course, there are many differences between breast-feeding and formula-feeding; these involve not only differences in nutrients, but also maternal contact and care, and the association of breast-feeding with socioeconomic factors. Nevertheless, it appears that the DHA variable can explain a good portion of the benefits associated with breast-feeding.

Studies of preterm infants have generally shown a benefit when DHA is added to the formula in controlled experiments (Table 3) (52–59). The studies included here are limited to controlled studies of formula-feeding with or without addition of DHA or DHA plus arachidonic acid (AA). Also, only those in which neural outcomes were included are listed; studies of growth and other anthropometric measures are not included. Studies of preterm infants (52–58) indicated, with one exception (59), that there was a benefit to adding long-chain polyunsaturates (LCP) to formulas that contain only the 18-carbon essential fatty acids found in vegetable oils. In addition, a meta-analysis of visual acuity differences in premature infants at 2 and 4 mon of age found a benefit of LCP of 0.47 and 0.28 octaves, respectively (60). These observations, in combination with studies indicating the safety of the ingredients used to supply these nutrients, lead us to conclude that preterm infant formulas must contain DHA/AA.

Studies of full-term infants are listed in Table 4 (61–72). In half of these studies, the LCP supplement supported an increased visual acuity (61,63,67), neurodevelopmental score

TABLE 3
Effect of Formula Supplementation with Docosahexaenoic Acid (DHA) or DHA and Arachidonic Acid (AA) on Brain and Retinal Function in Preterm Infants[a]

Authors	Reference	Year	Outcome tested	Results		Age
Uauy *et al.*	52	1990	ERG threshold, V_{max}	LCP > F	LCP = BF	36 wk PCA
Birch *et al.*	53	1992	VEP, FPL	LCP > F	LCP = BF	36, 57 wk PCA
Carlson *et al.*	54	1993	FPL	LCP > F		2, 4 mon
Carlson *et al.*	55	1996	FPL	LCP > F		2 mon
Werkman and Carlson	56	1996	Fagan NPT	LCP > F		6.5, 9, 12 mon
Carlson and Werkman	57	1996	Fagan NPT	LCP > F		12 mon
Faldella *et al.*	58	1996	ERG latency	LCP > F	LCP = BF	52 wk PCA
Bougle *et al.*	59	1999	Motor nerve conduction	LCP < F	LCP < BF	30 d

[a]ERG, electroretinogram; LCP, long-chain polyunsaturates, i.e., DHA or AA/DHA; F, formula-fed; BF, breast-fed; PCA, post-conceptional age; VEP, visual evoked potential (log MAR, where MAR = minimum angle of resolution); FPL, forced choice preferential looking (Teller cards); NPT, novel preference test or Fagan test of infant intelligence.

TABLE 4
Studies of Formula Supplementation with DHA or DHA and AA on Retinal and Brain Function in Full-Term Infants[a]

Authors	Reference	Year	Outcome tested	Results		Age
Makrides *et al.*	61	1995	VEP	LCP > F	LCP = BF	16, 30 wk
Agostoni *et al.*	62	1995	Brunet-Lezine[b]	LCP > F	LCP = BF	4 mon
Carlson *et al.*	63	1996	FPL	LCP > F	LCP = BF	2 mon
Agostoni *et al.*	64	1997	Brunet-Lezine	LCP = F	LCP = BF	24 mon
Auestad *et al.*	65	1997	FPL, VEP	LCP = F	LCP < BF	2, 4, 6, 9, 12 mon
Hornby Jorgensen *et al.*	66	1998	VEP	LCP = F	LCP = BF	4 mon
Birch *et al.*	67	1998	VEP	LCP > F	LCP = BF	6, 17, 52 wk
Willatts *et al.*	68	1998	Means-end problem solving	LCP > F	—	10 mon
Scott *et al.*	69	1998	MCDI	LCP < F	—	14 mon
Lucas *et al.*	70	1999	Bayley MDI, PDI	LCP = F	LCP = BF	18 mon
Birch *et al.*	71	2000	Bayley MDI	LCP > F	—	18 mon
Makrides *et al.*	72	2000	VEP, Bayley MDI	LCP = F	LCP < BF	34 wk, 2 yr

[a]VEP, visual evoked potential (log MAR); MCDI, Minnesota Child Development Inventory; PDI, psychomotor development index; MDI, mental development index. For other abbreviations see Table 3.
[b]Brunet-Lezine derived from Gesell test for psychomotor development.

(62,71), or problem-solving ability (68). In five of the trials, no effect was observed for the LCP supplement (64–66,70,72). In one trial, infants with the LCP supplement appeared to perform more poorly in a vocabulary test administered to 14-mon-old children (69). However, a recent trial with a larger number of preterm infants reported a positive effect of LCP-supplemented formula on vocabulary scores (73). San Giovanni *et al*. (74) performed a meta-analysis of the trials involving visual acuity and concluded that there was a 0.32 octave difference in visual acuity when supplemented and unsupplemented formula groups were compared, with the DHA-fed groups having the higher acuity. A somewhat larger difference (0.49 octaves) was observed when breast-fed infants were compared with unsupplemented formula-fed infants.

In most of these trials, a rather low level (0.1–0.35% of total fatty acids) of DHA supplement was given, corresponding to a "Western" level of DHA in milk. In a recent review, Jensen (75) calculated the average for DHA content in mature milks in Western and non-Western women to be 0.45 and 0.88% of total fatty acids, respectively. Thus, it is likely that more of the trials would have observed a benefit of DHA if given at a higher level that is more consistent with the range of present-day worldwide human milk values. Viewed from this perspective, it is rather surprising that some trials can succeed in demonstrating a benefit of a fat component that is only 0.1–0.2 % of the total fatty acids. This suggests that these LCP are potent and essential nutrients for optimal development.

It was also of interest to note that Jensen (75) found the ratios of AA/DHA to be very close to 1 in his averages of human milk from both Western and non-Western women. The average levels found in non-Western women of ~0.9% each AA and DHA may be a good starting point for future research. This is believed by some to represent a better standard than that of Western women because the composition is strongly influenced by the diet and Westerners have in the last century or two shifted their consumption of fats toward n-6 fats and away from n-3 fats due to the availability of linoleic-rich vegetable oils. Estimates of the Paleolithic diet indicate a much greater intake of LCP and a ratio of n-3/n-6 fats close to 1 (76). Thus, human infants likely received a much higher intake of DHA and other LCP from their mother's milk during human evolution. In modern times, this ensured supply of DHA during neurodevelopment has been abrogated by formula-feeding and by a very low maternal intake of n-3 fats in many modern women. Given the present state of knowledge from human and animal studies of changes in neural function associated with a low DHA status, coupled with biochemical and nutritional studies indicating the loss of DHA in both peripheral tissues and the nervous system when preformed DHA is not fed and the safety of ingredients (70,77) used to supply DHA, it is clear that a prudent course of action would be to supply sources of preformed DHA in the infant diet.

MECHANISMS OF ACTION OF DHA

From the above, it should be clear that many effects of DHA status have been observed relating to physiologic and behavioral functions of the nervous system. What has been unclear are the mechanisms underlying DHA function. Perhaps the most perplexing aspect of this question relates to the phenomenal degree of specificity that is apparent in this effect. It must be recalled that these studies did not involve essential fatty acid deficiency and that there were adequate and often excessive amounts of n-6 fats present, usually in the form of linoleic acid (LA). There is a well-known reciprocal replacement of DHA with DPAn-6, in this case in the brain (78) and retina (37,46,49). These two fatty acids differ only with respect to the absence of the Δ-19 double bond in the DPAn-6 molecule; both are 22-carbon HUFA with their first five double bonds in the same positions with respect to the carboxyl end of the molecule. There is little in the modern disciplines of biochemistry, biophysics, and neuroscience to offer a conceptual framework to understand this extraordinary specificity.

The first and most reasonable hypothesis was that a cyclooxygenase or lipoxygenase (LO) product of DHA but not DPAn-6 was produced that had an important function in the central nervous system. This hypothesis was explored extensively after the early reports that cyclooxygenase products of DHA were produced in the rainbow trout gill (79). Early investigations indicated that products made by rat brain were sensitive to LO inhibitors (80–83). A correction of the trout gill work indicated that the DHA products were not prostaglandins but rather LO products (84). Aveldano and Sprecher (85) observed that platelet LO produced a monohydroxylated form of DHA, and Bazan *et al.* (86) found a similar product after incubations with rat retinas. However, Kim and co-workers (80,81) demonstrated that the brain DHA products were a racemic mixture and thus unlikely to be enzymatic products. They also demonstrated that many of the products observed in the brain were a result of the failure to remove platelets and other blood cells by perfusion of the brain before *in vitro* experiments. Apparently, what was being measured *in vitro* may have corresponded to the low level of nonenzymatic fatty acid peroxidation that is known to occur. This is not to deny the existence of LO in the capillary beds in the brain, because Moore *et al*. (87) demonstrated 12-*S*-LO activity in a microvessel fraction. Also, Sawazaki *et al*. (82) found a 12-LO product of AA and DHA in the rat pineal gland and Zhang *et al*. (88) subsequently observed that the formation of these products is regulated by the light–dark cycle and melatonin through the modulation of both 12-LO (88) and cytosolic phopholipase A_2 expression (89). They also reported that n-3 fatty acid deficiency had profound effects not only on the pineal lipid profile but also on pineal biochemical activity, resulting in significantly fewer LO products (90).

It is still quite possible that a "magic bullet" type of molecule may be found for DHA, i.e., a function for the nonesterified fatty acid or a metabolite that is extremely potent. Physiologic experiments, for example, have demonstrated that DHA or an anandamide analog of DHA has a potent effect on the K^+ channel (91–93). Leaf and co-workers showed that the nonesterified form of DHA has a potent effect on Na^+ (94–96) and Ca^{2+} channels (96,97). Also, synaptic transmission (98) and long-term potentiation (99,100) in the hippocampus as well as

N-methyl-D-aspartate responses in the cerebral cortex (101) are altered by DHA. However, what is not clear is whether these actions of DHA and its analogs are operative *in vivo*. Moreover, the substrate specificity required to explain the n-3 deficiency syndrome has generally not been found in these studies.

The failure of this initial hypothesis led to proposals that are based on the concept that the active form of DHA is in the form of a phospholipid (102–104). Little progress was made on this intractable problem until workers focused on this hypothesis. Several approaches have been used including cell biological, biochemical, and biophysical attacks. Examples of each of these will be summarized in turn below with reference to apoptosis, protein-lipid interactions, and the physical state and membrane properties of DHA-phospholipids.

The first topic that will be taken up is G-protein signaling with a focus on rhodopsin. This line of inquiry is central to an understanding of the function of DHA-lipids in the visual system; it also serves as a model of other G-protein-coupled signaling receptor systems that helps us to understand how DHA may function in the brain.

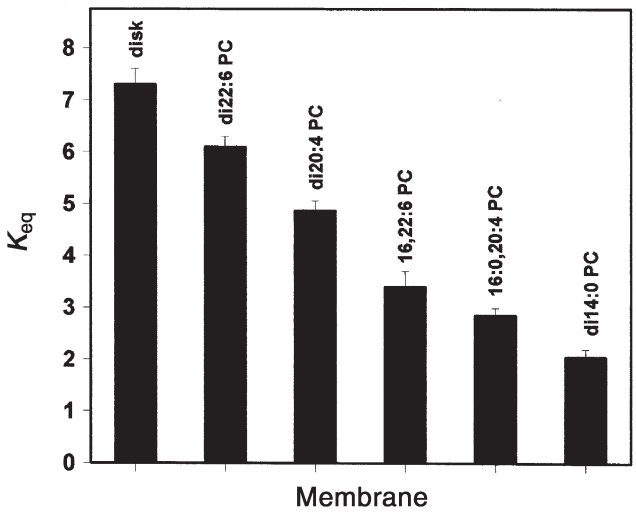

FIG. 1. The compositional dependence of the metarhodopsin MI↔MII equilibrium constant, K_{eq}, determined for rhodopsin in a series of compositionally defined bilayers varying in degree of unsaturation at 37°C. *Source*: Reference 107. M, metarhodopsin; PC, phosphatidylcholine.

PROTEIN-LIPID INTERACTIONS: G-PROTEIN SIGNALING

Intercellular signaling is initiated through the activation of ligand-specific receptors imbedded in the lipid bilayers of cellular membranes. An understanding of the factors that govern the efficiency of signaling processes requires elucidating how the lipid composition of the membrane modulates the interactions of these receptors with the other membrane-bound protein components in the signaling pathway. To elucidate the role of n-3 fatty acids in the nervous system and visual process, the phospholipid acyl chain dependence of several steps in the visual transduction pathway was studied (105). This was accomplished by purifying several components of the visual transduction system and reconstituting them in phospholipid vesicles of defined lipid composition (106).

The visual transduction pathway is initiated by the absorption of a photon by rhodopsin, a prototypical member of the family of G-protein-coupled receptors that includes many neurotransmitter receptors such as those for serotonin and dopamine. Metarhodopsin (M)II is the conformation of photoactivated rhodopsin that binds and activates the visual G-protein, G_t, which in turn activates a cGMP-specific phosphodiesterase (PDE) (105). Hydrolysis of cGMP by the PDE results in the closing of cGMP-gated channels in the ROS plasma membrane, changing the transmembrane potential and initiating the neuronal response to light. In n-3-deficient animals, a reduced amplitude and delayed response are observed in the leading portion of the a-wave of electroretinograms (37,40,41). This portion of the a-wave is associated with the transduction pathway. To determine whether these observations can be linked to changes in membrane composition, we examined the bilayer dependence of MII formation, the kinetics and extent of MII-G_t complex formation, and resulting PDE activity as a function of phospholipid acyl chain composition and cholesterol content of the bilayer. These topics will be treated in turn below.

K_{eq} measures the extent of MII formation, which represents the formation of the activated ligand-bound receptor state, for the MI-MII equilibrium. This parameter depends critically on the level of acyl chain unsaturation (Fig. 1) (107). For both mixed-chain phosphatidylcholines (PC) and symmetrically substituted PC, the highest levels of MII formation were seen in DHA-containing bilayers. The addition of 30 mol/100 mol phospholipid to these systems lowered the level of MII formation. However, the lowest percentage reductions were obtained in the DHA-containing systems, suggesting that DHA-containing phospholipids are best able to buffer the inhibitory effects of cholesterol.

The first amplification step in the visual cascade is the activation of G_t. The initial step in this activation is the binding of G_t to MII and is characterized by the association constant, K_a. This process is also affected by acyl chain composition. The value of K_a in 18:0,22:6-PC is more than two times greater than that in 18:0,18:1-PC, indicating that twice as much MII-G_t complex is formed in the DHA phospholipid than in the monounsaturated bilayer (Niu, S., Mitchell, D.S., and Litman, B.J. unpublished results). The number of G_t molecules activated in the two bilayers should be proportional to the amount of complex formed, suggesting that at equivalent levels of MII and G_t, a higher signal amplitude will be observed in the DHA-containing bilayer.

Another important aspect of signaling is the response time. This aspect of signaling was addressed by measuring the kinetics of both MII and MII-G_t formation in several bilayers. An important characterizing parameter in these measurements is the ratio of the rate of formation of MII-G_t to that of MII. This parameter represents the lag time in appearance of the

complex after MII has formed and is a measure of the efficiency of the interaction of the receptor and G_t protein. This ratio is 1.4 in the native ROS disk membrane, indicating a rapid complex formation after the appearance of MII (106). Complex formation in 18:0,22:6-PC and 18:0,18:1-PC is characterized by ratios of 3.5 and 4.9, respectively. Although the DHA PC phospholipid is not as efficient as the disk system, it does provide for more efficient MII-G_t formation than the less unsaturated bilayer. Some of the enhanced efficiency of MII-G_t formation in the disk membrane may be attributable to the more complex mixture of phospholipid classes that contribute a net negative surface charge to the membrane.

The overall measure of the signaling pathway is the dose-response curve, generated by determining the level of PDE activity brought about by increasing levels of rhodopsin activation. In these experiments, both G_t and PDE were reassociated with rhodopsin-containing vesicles. At light exposure levels at which 1 in 1000 rhodopsin molecules was activated, ROS disks yielded 87% of their maximal PDE activity. Under similar light exposure conditions, 59 and 26% of maximal disk activity was obtained in 16:0,22:6-PC and 16:0,18:1-PC, respectively (106). Although not reaching the same activity as native disk membranes, the DHA-containing bilayer yields twice the activity of the monounsaturated bilayer. Thus, in the integrated function of the pathway, the DHA-containing bilayer yields higher activity levels than the monounsaturated bilayer.

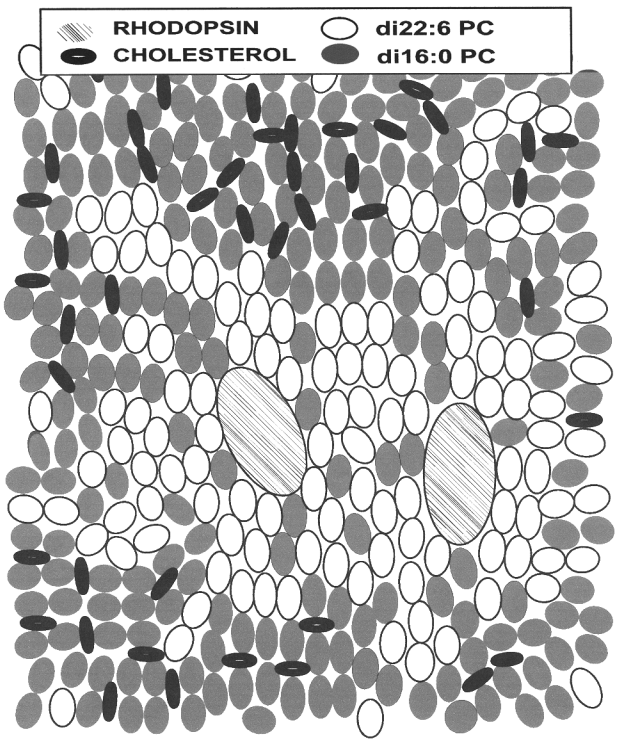

FIG. 2. Domain structure of rhodopsin-lipid model membranes. Fluorescence energy transfer studies of model membranes composed of a 3:7:3 mixture of di22:6-phosphatidylcholine (PC)/di16:0-PC/cholesterol and varying levels of rhodopsin show the presence of lateral domains. These domains are composed of a rhodopsin-containing region highly enriched in di22:6-PC and a second region highly enriched in di16:0-PC and cholesterol. The formation of these domains requires the presence of both rhodopsin and cholesterol, demonstrating the complex nature of the molecular interaction responsible for domain formation. These include a rhodopsin preference for docosahexaenoic acid (DHA) acyl chains and a preference of cholesterol for saturated acyl chains (108).

Recent studies suggest that lateral domain formation may play a critical role in the requirements for DHA-containing phospholipids (Fig. 2) (108). Fluorescence energy transfer experiments have provided evidence of the formation of lateral domains in reconstituted membranes consisting of di22:6-PC, di16:0-PC, cholesterol, and rhodopsin. In these domains, the lipid composition around rhodopsin is highly enriched in di22:6-PC, whereas the di16:0-PC is highly enriched in cholesterol. Domain formation requires the presence of both rhodopsin and cholesterol, indicating the complex nature of the interactions that drive lateral segregation of the constituents of this system. If the other components of the signaling pathway have the same preferable partitioning into a DHA-rich lipid domain as rhodopsin, then this would raise their local concentration and provide a greater efficiency for the signaling pathway.

The results presented here demonstrate that the visual signaling pathway is greatly dependent on the acyl chain composition of the bilayer. The steps in this signaling process involve unimolecular conformation changes required for rhodopsin activation and several protein–protein interactions for the activation of G_t and PDE. Both of these types of processes were enhanced in bilayers containing DHA. The studies reported here were carried out in PC bilayers of varying acyl chain composition. The disk membrane contains ~42–45% of both PC and PE and ~10–12% PS. The differences in activity observed between the pure PC system and native disk membrane might be attributable to the lack of a surface potential supplied by the presence of PS or perhaps specific properties contributed by the PE. It should be noted that both PE and PS contain the highest levels of symmetrically substituted di-DHA species in the retina. Despite these differences, the results reported here suggest an explanation for the observations in the electroretinograms of n-3-deficient animals. The delay in the development of the leading edge of the a-wave is likely related to the increased lag time observed in the formation of the MII-G_t complex, whereas the reduced amplitude may be explained on the basis of the reduced association constant observed for MII-G_t complex formation.

ANTIAPOPTOTIC EFFECT OF DOCOSAHEXAENOIC ACID

Next, we turn to a consideration of the effects of DHA at a cell biological level. Unlike AA, DHA is not easily released from neuronal membranes, but instead is retained by membrane phospholipids (109–111). In contrast, astroglia cells, which are known to support neuronal survival, release this fatty acid readily (110–113). This suggests that DHA fatty acid may act as a trophic factor, and enrichment of this fatty acid in neuronal

membranes may be an important aspect in neuronal survival. In neuronal membranes, DHA is highly enriched in aminophospholipids, especially PS (1,2,4,5–10,114). We and others have previously demonstrated that the enrichment of DHA in cell membranes increases PS synthesis and, conversely, that depletion of this fatty acid by an n-3-deficient diet or by chronic ethanol exposure decreases the accumulation of PS (115–118). Considering the fact that PS is the major negatively charged phospholipid class in many mammalian cell membranes and many of the signaling proteins such as protein kinases are influenced by PS (119–121), this alteration of PS content may have significant implications for cellular function.

In contrast to the well-documented apoptotic effect of DHA (122–127), only a few studies have indicated an antiapoptotic function for this fatty acid (128–131). In each case, an antiapoptotic effect was observed only after preincubation with DHA before the induction of apoptosis. Because DHA is prone to oxidation during the incubation period and much of the apoptotic effect is mediated through oxidative stress (126,127), it is difficult to successfully enrich cultured cells with DHA without accompanying lipid peroxidation. Therefore, to observe the antiapoptotic effect of DHA, it is crucial that an antioxidant such as vitamin E be added along with DHA to the culture medium (130,131).

In studies by Kim *et al*. (131) in which PC-12 or Neuro-2A cells were exposed to AA (1–25 μM) during serum deprivation, apoptosis determined by genomic DNA fragmentation decreased in a dose-dependent manner, but treatment with DHA or oleic acid had no effect. The insensitivity of the protective effect of AA to indomethacin or nordihydroguaiaretic acid indicated that the observed protective effect of AA was not mediated by either cyclooxygenase or LO derivatives, but rather through the direct action of AA (132). In contrast to the effect of AA, DHA became protective only after a prolonged period of incubation. In Neuro-2A cells, the protective effect required at least 24 h of enrichment, and a longer incubation led to an enhancement of the protective action of DHA. During this period, DHA was steadily incorporated into PS, and total cellular PS was increased. The protective effect is related to the extent of cellular PS accumulation. When cells were enriched with DHA in a serine-free medium, the PS content did not increase significantly and the antiapoptotic effect was diminished significantly.

Caspase-3 activity, which has been shown to mediate mammalian apoptosis, increased as the starvation proceeded, with the exception of cells enriched with DHA. Both AA- and DHA-treated cells initially showed less caspase-3 activity in comparison to nonenriched control or oleic acid-enriched cells. However, prolonged serum starvation abolished the protective effect of AA, and only DHA-treated cells maintained caspase-3 activity at a level similar to that of control cells kept in the 5% serum medium. The DNA fragmentation data and DNA ladder formation obtained after at least 48 h of serum starvation showed consistent results (Fig. 3). It was also observed that the 17-kDa active fragment of caspase-3 increased under serum-free conditions, and supplementation of Neuro-2A cells with DHA before serum starvation effectively prevented the increase of the 17-kDa fragment (131).

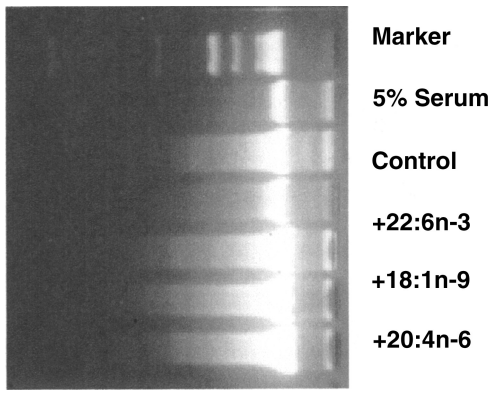

FIG. 3. Protection from DNA fragmentation after the enrichment of cells with docosahexaenoic acid for 48 h.

Enrichment of cells with DHA also altered expression of various proteins at the gene level because the levels of mRNA for caspase-3 decreased, whereas mRNA of Raf-1 increased (131,132). DHA has been shown to affect transcriptional activities through nuclear hormone receptors such as the peroxisome proliferator-activated receptor (133) or the retinoid X receptor (134). The antiapoptotic effect of DHA enrichment suggests that ensuring the survival of neuronal cells may be one of the reasons for the high level of DHA in brain. Alterations of the membrane PS content by DHA will influence not only the receptor activities but also the translocation of various signaling proteins as well as their activation (118–120,131). Although the release of DHA in neuronal cells may be minimal, it may be possible to reach a local concentration of intracellular DHA sufficient to activate nuclear receptors involved in transcriptional activity. It is likely that the antiapoptotic effect of DHA is the result of multiple regulations at various signaling stages, ranging from the plasma membrane to nuclear events, most of which have yet to be discovered.

BIOPHYSICAL PROPERTIES OF POLYUNSATURATED LIPID MEMBRANES

As referred to above, there has been a lack of a conceptual basis in biophysics in which a meaningful difference in function could be predicted with respect to a DHA- vs. a DPAn-6-containing lipid. In fact, there have been few biophysical studies that discern appreciable differences in the properties of various polyunsaturated species. It has often been said that introduction of a double bond into a saturated lipid results in a large change in physical properties. Introduction of a second double bond also results in an additional effect; however, it is of a much smaller magnitude than that of the first double bond. Thereafter, introduction of additional double bonds (for a total of three or more) has little effect. However, the n-3 fatty acid deficiency syndrome described above indicates that there are significant biological effects measurable in the whole organism, e.g., by

behavioral or physiologic means, when DPAn-6 is substituted for DHA. Because these are pentaenoic and hexaenoic lipids, it would appear that a physical basis must exist for the differential biological function of various highly unsaturated fatty acids. Recent studies have begun to demonstrate that all highly unsaturated lipids indeed do not have the same physical properties. Some of the techniques and research approaches as well as data obtained from these investigations are presented below.

MAGIC ANGLE SPINNING (MAS)

Solid-state nuclear magnetic resonance (NMR) spectroscopy on models of polyunsaturated membranes enables the measurement of dozens of parameters, probing every segment of the lipid bilayer with atomic resolution. This became possible after improving the performance of magic angle spinning (MAS) probes in combination with very high magnetic field strength. MAS NMR reduces the linewidth of lipid resonances to ~10 Hz (135). That is equivalent to or better than resolution of resonances for very small unilamellar liposomes that tumble rapidly enough to eliminate anisotropic interactions. However, in contrast to studies of liposomes, for MAS NMR, the membranes are not required to have small radii of curvature, and water content does not matter as long as the lipids remain in the liquid-crystalline state. Just a few milligrams of sample is sufficient to provide very high signal-to-noise ratios as evidenced by the spectra in Figure 4 (136).

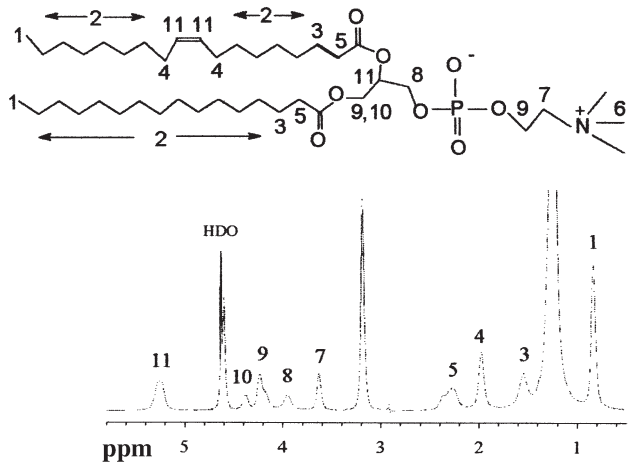

FIG. 4. ^{1}H magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectrum of 16:0,18:1 PC in 50 wt% D_2O recorded at ambient temperature and a spinning speed of 10 kHz. Signal assignment is provided by numbers in the spectrum and the formula. *Source*: Reference 137.

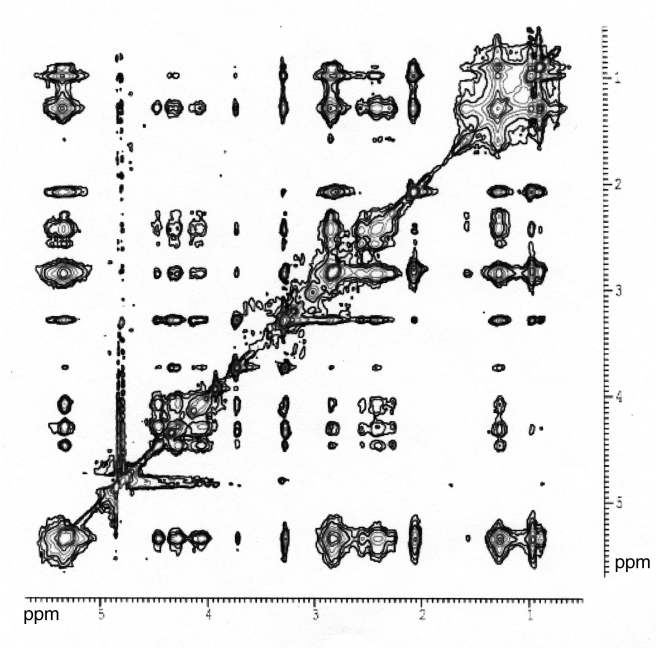

FIG. 5. Two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectrum of an 18:0-22:6 phosphatidylcholine (PC)/18:0_{d35}-22:6 phosphatidylethanolamine (PE)/18:0_{d35}-22:6 phosphatidylserine (PS)/cholesterol$_{d7}$ (4:4:1:3, by vol) mixture.

NUCLEAR OVERHAUSER ENHANCEMENT SPECTROSCOPY (NOESY)

The excellent resolution of lipid resonances allows application of techniques that probe magnetization transfer between protons such as nuclear Overhauser enhancement spectroscopy (NOESY), well known for its important contribution to the structural determination of soluble proteins (137).

Magnetization transfer is the result of interactions between the magnetic dipoles of the protons in lipids. Rates of transfer become observable when the protons approach each other to within distances ≤5 Å (138). In the two-dimensional NOESY contour plot shown in Figure 5 (139), the peaks along the diagonal correspond to the one-dimensional resonances shown in Figure 4. The rate of magnetization transfer is reflected by the intensity of the off-diagonal crosspeaks. The surprising observation has been that magnetization is transferred between all lipid resonances, but at different rates. Even the most distant protons such as methyl groups of the choline headgroup and methyl groups at the end of lipid hydrocarbon chains exchange magnetization. For a long time, such surprising transfers were ascribed to a process called spin diffusion. Spin diffusion relays magnetization *via* coordinated flip-flops of magnetization along the proton network of a lipid molecule. In a series of experiments on protonated lipids in deuterated matrices, we demonstrated recently that this interpretation is incorrect (140). In the biologically relevant liquid-crystalline state, membranes are truly disordered to the point that lipid headgroups and ends of hydrocarbon chains meet once in a while, albeit with lower probability than membrane segments that are located closer to each other. The experiments also demonstrated that the unexpected long-range contacts are not taking place within one lipid molecule, but rep-

resent magnetization transfer to neighboring lipid molecules that surround the emitter (141).

Experiments on *sn*-1 chain perdeuterated 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (18:0_{d35},22:6-PC) not only confirmed that polyunsaturated chains in membranes are similarly disordered, but also offered evidence that conformational disorder of the remaining degrees of freedom of a polyunsaturated chain is higher (135,142). Furthermore, the intensity of cross- and diagonal peaks as a function of mixing times allows judgment about motional correlation times in the polyunsaturated membranes on the time scale from pico- to milliseconds (138). The spin-lattice and spin-spin relaxation rates of polyunsaturated chains are lower, indicating more rapid motions of these protons. This result is surprising because polyunsaturated chains have long been perceived as rigid and bulky owing to the loss of degrees of freedom.

DIPOLAR RECOUPLING ON-AXIS WITH SCALING AND SHAPE PRESERVATION (DROSS)

MAS NMR techniques with application of radio-frequency pulses, which are synchronized with the phase of the spinning rotor, enable recoupling of anisotropic interactions, e.g., the magnetic dipolar interaction between ^{1}H and ^{13}C nuclei. The strength of this interaction depends on the time-averaged orientation of the ^{1}H-^{13}C bond vector with respect to the lipid bilayer normal.

In the two-dimensional dipolar recoupling on-axis with scaling and shape preservation (DROSS) experiments (Fig. 6), this technique allows assignment of order parameters to resolved ^{13}C resonances of lipid segments (143). Conducting this experiment on ^{13}C nuclei benefits from the much greater resolution of ^{13}C chemical shifts. Throughout the molecule, >20 order parameters can be assigned to specific regions within the polyunsaturated lipid, without isotopic labeling.

The experiments showed unambiguously that order parameters of double bond ^{1}H-^{13}C vectors are close to 0. Because of differences in bond geometry between saturated and unsaturated hydrocarbon chains, this result was not unexpected. However, the very-low-order parameters for the five ^{1}H-^{13}C vectors of the methylene groups that are sandwiched between double bonds were surprising. These order parameters must be low as a result of crankshaft-like coordinated motions within the DHA chain. Such motions are likely because of lower potential barriers for rotations about the vinyl-methylene bonds compared with C-C bonds in saturated chains (144). This enables DHA chains to adapt to looped conformations that have shorter length and larger area per molecule (145).

^{2}H NMR

Although the DROSS technique allows determination of assigned order parameters, for technical reasons, the precision in order parameter determination remains about one order of magnitude lower than the precision of order parameters measured by the classical approach, i.e., analysis of effective quadrupolar splittings in the ^{2}H NMR spectra of deuterated lipids (146). Quadrupole splittings are measured with a resolution of ~50 Hz, which corresponds to a precision for order parameters of $\Delta S = \pm 0.0004$. Such small changes are of relevance, e.g., to detect the level of stress that is caused in the lipid matrix as the result of a conformational change of a membrane protein (147). We conducted preliminary ^{2}H NMR experiments on perdeuterated DHA, incorporated at low concentration into bilayers of 18:0,18:1-PC (Fig. 7). Partial assignment of the order parameters to segments of the DHA chain was achieved by taking advantage of the small differences in chemical shift between the deuterium resonances in MAS experiments with partial recoupling of the quadrupolar interaction (Gawrisch, K., Safley, A.M., and Polozov, I.V., unpublished data). The results fully confirmed the observations from the less precise DROSS experiment. Order parameters of all methylene segments between double bonds in the hy-

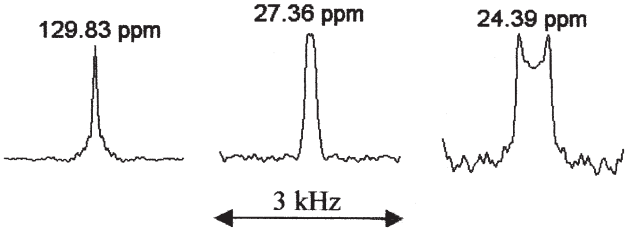

FIG. 6. Extracted columns of a two-dimensional dipolar recoupling on-axis with scaling and shape preservation (DROSS) spectrum corresponding to resonance signals of double bonds (129.83 ppm), methylene groups between the double bonds (27.36 kHz), and the C-17 methylene group of stearic acid (24.39 ppm). The peak doublets are the result of recoupling of dipolar interactions between the ^{1}H and ^{13}C nuclei. The value of order parameters is directly proportional to the magnitude of splittings.

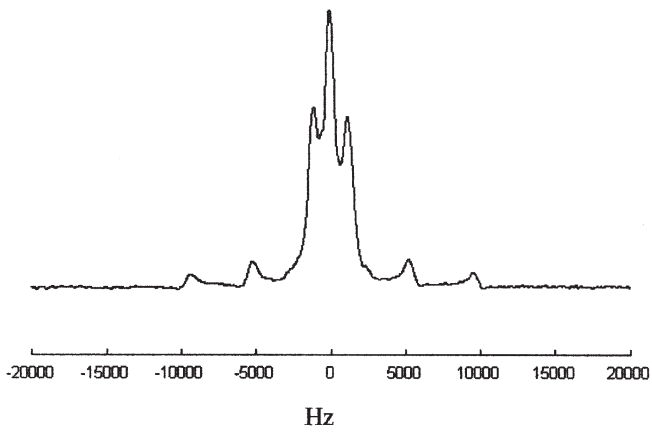

FIG. 7. ^{2}H nuclear magnetic resonance (NMR) spectrum of perdeuterated docosahexaenoic acid in an 18:0,18:1-PC matrix.

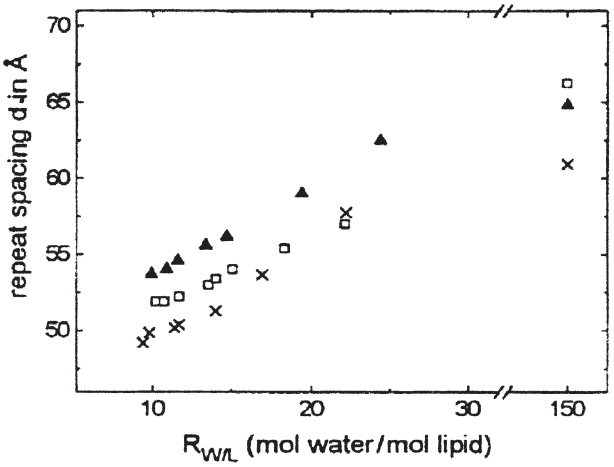

FIG. 8. X-ray repeat spacing of lipid bilayers as a function of water content expressed as the number of water molecules per lipid. *Source:* Reference 142.

drocarbon chain, as well as the order of the majority of the double bonds, are very low. Only the two methylene segments near the carboxyl group of DHA have order parameters that are comparable to values of more saturated chains.

X-RAY DIFFRACTION

Results of X-ray diffraction experiments on 18:0,22:6-PC, recorded as a function of water content, were compared with the results of experiments on 18:0,18:1-PC and di14:0-PC (Fig. 8) (142). The repeat spacing of bilayers with polyunsaturated chains, i.e., the thickness of the lipid layer plus the thickness of the water layer separating two bilayers, is rather similar to the thickness of the di14:0-PC bilayers with only 14 carbon atoms per chain.

Water uptake of polyunsaturated lipids is slightly increased and does not account for this difference (142). We estimated that the polyunsaturated DHA occupies a unit cell with average dimensions of $6 \times 6 \times 14$ Å in the bilayer. Compared with saturated and monounsaturated chains, this is an increase in chain area of ~7 $Å^2$. DHA chains in an extended angle-iron or helical conformation exceed this length by up to 10 Å (148); therefore, they cannot be the only conformation that DHA chains adopt in bilayers. Consequently, we propose that looped conformations, as proposed in a paper by Dolmazon's laboratory (145), are very common in polyunsaturated chains.

We also conducted X-ray experiments as a function of osmotic stress to reduce the sample's water uptake. Controlled dehydration of membranes creates lateral tension, which compresses bilayers laterally. Using a combined NMR and X-ray approach, we followed the changes in area per molecule of saturated and polyunsaturated chains in 18:0,22:6-PC as a function of lateral tension. We observed that 75% of the increase in chain length and reduction in area per molecule was the result of changes in the average conformation of the polyunsaturated chain (142).

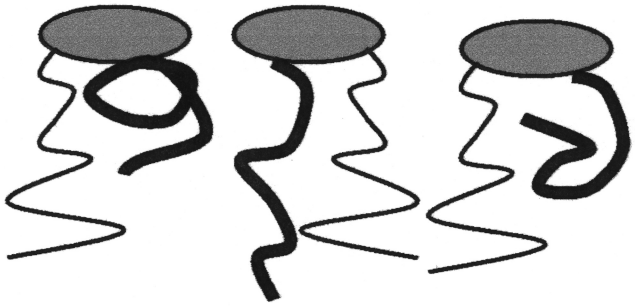

FIG. 9. A schematic that highlights the conformational freedom of docosahexaenoic acid (DHA) chains in mixed-chain lipids. DHA converts rapidly between looped and extended conformations. The center of mass of the DHA chain is closer to the lipid–water interface.

CONFORMATION AND FLEXIBILITY OF THE DHA CHAIN

The alteration of elasticity of neural membranes according to the number of double bonds per fatty acid is one possible role of lipid polyunsaturation. Membranes contain a matrix of lipid molecules with hydrophilic ("water-loving") headgroups (shown in gray motif) and hydrophobic ("water-rejection") fatty acid chains in black (Fig. 9). Membranes respond to external perturbations like elastic rubber bands. With NMR spectroscopy and X-ray diffraction, the elastic deformation of membranes under tension, including the changes in structure and motions of lipid hydrocarbon chains, could be measured. The results suggest that polyunsaturated chains in membranes prefer flexible, looped, and helical structures with rapid transitions among a large number of conformers. This provides increased flexibility to receptor-rich neural membranes that contain high concentrations of DHA. A common perception of polyunsaturated chains is that they are stiff and inflexible due to the presence of motionally restricted double bonds. In contrast, our NMR studies indicate exceptionally high deformability of DHA chains in biomembranes.

DIFFERENCE IN MEMBRANE PROPERTIES BETWEEN DHA AND DPA LIPIDS

Although there is ample evidence that membrane biophysical properties control membrane protein function to a significant extent, the idea that the loss of a single double bond from DHA to DPAn-6 in lipids is sufficient to alter membrane biophysical properties has been met with some skepticism. We compared deuterated *sn*-1 chain order parameter profiles in mixed-chain 18:0,22:6-PC and 18:0,22:5-PC by ^{2}H NMR order parameter experiments.

Saturated chain order of the first half of the chain near the lipid–water interface was high and almost constant (order parameter plateau), whereas order of the second half of the chain decreased with a steep gradient toward the terminal methyl chain end. This order profile has been linked to the probability of gauche-*trans* isomerization in chains (higher at the terminal methyl end).

Changes in hydrocarbon chain length and area per molecule are reflected in order parameter changes. An increase of average chain order by $\Delta S = +0.002$, which can be easily resolved, corresponds to an increase of average bilayer thickness of 0.1 Å and a decrease of area per molecule of 0.2 Å^2. Compared with monounsaturated bilayers, the order of *sn*-1 chains that are paired with polyunsaturated chains in the *sn*-2 position is lower, mostly for the second half of the chain, with a maximal decrease by $\Delta S = -0.018$ near carbon atom number 13 for 18:0,22:6-PC (149). We propose that this decrease reflects formation of short looped chain conformations (*vide infra*) with a higher density of polyunsaturated chain segments near the lipid–water interface. As a result of this redistribution of DHA chain density, the lower segments of the saturated chain have more freedom for movement and lower chain order parameters in the second half of the chain. The surprising observation has been that the loss of a single double bond from DPAn-6 to DHA in 18:0,22:5-PC results in order parameters that are much closer to those of a matrix with monounsaturated oleic acid chains in position *sn*-2.

In summary, assigned order parameters and relaxation times related to molecular motions were measured using novel MAS NMR approaches and classical ^{2}H NMR order parameter studies on chain perdeuterated, polyunsaturated lipids. In comparison to saturated chains, DHA order parameters were low, reflecting both a change in bond geometry and an increase in chain dynamics. The loss of a single double bond near the terminal methyl group of hydrocarbon chains has a significant influence on lipid matrix properties; therefore, membranes rich in DHA have unique properties. We speculate that the differences are of importance for the function of receptor proteins in retinal and synaptosomal membranes.

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