Dietary Polyunsaturated Fat that Is Low in (n-3) and High in (n-6) Fatty Acids Alters the SNARE Protein Complex and Nitrosylation in Rat Hippocampus

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Abstract

Docosahexaenoic acid [DHA, 22:6(n-3)] is enriched in brain membrane phospholipids and is important to brain development and function through its influence on neurite outgrowth and neurotransmitter secretion. Fusion of intracellular vesicles with the plasma membrane involving SNARE [soluble N-ethylmaleimide-sensitive fusion (NSF) protein attachment protein receptor] protein assembly, membrane fusion, and then disassembly are events common in membrane extension and neurotransmitter release. We determined whether feeding an (n-3) fatty acid–deficient diet, known to reduce brain phospholipid DHA, alters SNARE protein and SNARE complex expression or protein nitrosylation in the hippocampus of rats. Female rats were fed diets with 1.3 or 0.02% energy (n-3) α-linolenic acid from 2 wk before gestation then throughout gestation and lactation (n = 8/diet), and the male offspring were weaned to the maternal diet. Hippocampus phospholipid fatty acids and SNARE proteins were determined in male offspring at 90 d of age. Hippocampus phospholipid DHA was lower and (n-6) docosapentaneoic acid [DPA, 22:5(n-6)] was higher in the (n-3) fatty acid–deficient rats compared with the control group (P < 0.05). Multiplex Western blots using antibodies to syntaxin, synaptosome-associated protein of 25kDa (SNAP-25), and complex II, showed higher ternary SNARE complexes but no differences in syntaxin, SNAP-25, or complex II expression in hippocampus of the (n-3) fatty acid–deficient rats compared with the control group (P < 0.05). S-nitrosylation of syntaxin was also significantly lower in the (n-3) fatty acid–deficient rats than in the control group. These studies suggest that altered SNARE complex binding or disassembly could be important in explaining the diverse cellular events associated with altered tissue DHA.

Introduction

Dietary deficiency of (n-3) fatty acids in rats results in decreased docosahexaenoic acid [DHA, 22:6(n-3)] and an increase in (n-6) fatty acids, particularly docosapentaneoic acid [DPA, 22:5(n-6)] in brain phospholipids, which, at the functional level, is accompanied by impairments in behavioral tasks of learning (1,2). In humans, low blood lipid concentrations of DHA are associated with lower visual and neural system maturation in infants and with several depressive and age-related cognitive disorders in adults (3–7). Dietary deficiency of (n-3) fatty acids also reduces DHA in brain growth cone membranes (the leading edge of the growing neurite), alters normal neurogenesis, decreases neurite outgrowth, reduces the complexity of dendritic arborizations (8–12), and alters the release of several neurotransmitters, including dopamine and acetylcholine in rats (13–15). The reasons for the seemingly diverse effects of dietary (n-3) fatty acid deficiency on brain development and function, however, are as yet unclear.

Fusion of intracellular vesicular storage material with the cystolic surface of the plasma membrane involving soluble N-ethylmaleimide-sensitive fusion (NSF) protein attachment protein receptor (SNARE) proteins is an essential feature in membrane extension and in the exocytosis of neurotransmitters from their intracellular storage vesicles (16–20). In the central nervous system, neurotransmitter secretion is initiated by pairing a synaptic vesicle bound SNARE (known as vesicular associated membrane protein-2, or VAMP-2) with its target membrane (t)-SNARE, consisting of syntaxin-1 and synaptosome-associated protein of 25kDa (SNAP-25) on the intracellular surface of the plasma membrane to form a ternary complex that bridges the 2 membranes and brings them into close approximation (16–20). Neurotransmitter exocytosis is coupled to the influx of Ca2+ into the neuron through a process possibly involving displacement of...
complexin from the primed SNARE complex (21,22). Next, dissociation of the SNARE complex assembly is important to enable the renewal of the exocytosis cycle. In addition, recent studies indicate that reversible S-nitrosylation of cysteine residues in NSF is important in the regulation of intracellular transport and functions to decrease exocytosis by inhibiting SNARE complex disassembly (23–25). S-nitrosylation of other SNARE proteins has been reported, including syntaxin and SNAP-25, but their physiological importance is not yet understood (26).

The (n-3) fatty acids are present in the diet predominately as α-linolenic acid [(ALA) 18:3(n-3)], found in vegetable oils, with smaller amounts of eicosapentaenoic acid [(EPA) 20:5(n-3)] and DHA in animal tissue lipids (1). Unlike rodents, the conversion of ALA to DHA is very slow in humans (27–29). Further, interventions to increase the intake of ALA, or lower competitive inhibition of high dietary LA on the desaturation of ALA to DHA, lead to an increase in blood lipid EPA, but have little or no effect in increasing DHA in humans (30,31). A further understanding of the effects of low DHA, particularly on neural function, is thus important. In this study we sought to determine whether feeding a diet deficient in (n-3) fatty acids, which we previously found reduced DHA in brain growth cone and synaptosomal phospholipids and altered neurogenesis and neurotransmitter levels (9,11), also alters the expression of SNARE proteins, ternary SNARE complex formation, or SNARE protein S-nitrosylation. We provide new evidence that the reduction in the usual high proportion of DHA in hippocampus phospholipids is associated with increased ternary SNARE complexes and decreased S-nitrosylation of SNARE protein.

Materials and Methods

Animals and diets. Female Long-Evans rats (Charles River Laboratories) were housed in a humidity and temperature-controlled animal facility with a 12 h:12 h light:dark cycle (light on from 0700 to 1900) and free access to food and water. The rats were fed 1 of 2 semipurified diets that contained 16% energy from fat and differed only in the composition of component fatty acids (9,11). The diets contained, per kg, 70 g oil, 200 g vitamin-free casein, 200 g sucrose, 400 g cornstarch, 50 g nonnutritive cellulose, 10 g multivitamin mix (Teklad Test Diets), and 35 g mineral multimineral mix (Bernhart-Tomarelli salt mix, General Biochemicals), with an additional 78 mg Mn 2+, 60 μg Se 2+, 1.0 g choline chloride, and 3.0 gL-methionine/ kg complete diet (9,11). One diet contained 1.2% energy from ALA and 3.2% energy from linoleic acid [LA, 18:2(n-6)] from canola oil and the other diet had 0.03% energy ALA and 11.8% energy from LA from safflower oil [n-3 deficient]; n = 8/diet. The diets also differed in oleic acid [18:1(n-9)] with 9.6 and 2.8% energy from 18:1(n-9) in the (n-3) deficient and control diet, respectively. The diets were fed from 2 wk prior to mating throughout gestation and lactation. The litters were reduced to 8 pups at birth, and the male offspring were weaned to their mother’s diet at 21 d of age. All procedures involving animals were approved and carried out in accordance with the Animal Care Committee of the University of British Columbia and conformed to the guidelines of the Canadian Council on Animal Care Guidelines.

Tissue preparation and biochemical analyses. At 90 d of age, one male rat from each of n = 8 litters/diet group was anaesthetized (ketamine 120 mg/kg plus rompun 20 mg/kg) and decapitated. The brains were removed, dissected free of surrounding tissue, weighed, and the hippocampus dissected on ice. The tissue was divided for analyses of lipid and SNARE protein expression and then immediately frozen in liquid nitrogen and transferred to storage at −80°C. For lipid analyses, total lipids were extracted, the phospholipids separated, and their component fatty acids analyzed by GLC (9,11,32). Because of the small quantity of tissue available, phospholipids were not resolved into individual classes prior to analyses of fatty acids.

Electrophoresis and Western blot analyses. Frozen samples were rapidly thawed and homogenized in 16-fold Nitrogo Assay Buffer (PerkinElmers Life and Analytical Sciences) and protein was determined using the DC Assay (Bio-Rad Laboratories) with bovine serum albumin as the standard. Samples were diluted to 1 g/L or 4 g/L for the determination of SNARE proteins or S-nitrosylated-proteins, respectively. Because (n-6) and (n-3) fatty acids influence gene expression and (n-3) fatty acids alter neurogenesis (1,2), this limits the inclusion of structural or other proteins as an internal standard against which to estimate SNARE protein expression. To overcome this, we resolved standardized amounts of protein in all experiments.

Hippocampal protein, 7 μg, was resolved on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, alternating treatments across the lanes, and was transferred to a polyvinylidene difluoride membrane (Millipore Bioscience Division). Samples were blocked with 1% BSA in Tris buffered saline overnight, then incubated with anti syntaxin (SP6, 1:100), anti-SNAP-25 (SP12, 1:200), anticomplexin II (LP27, 1:200) monoclonal antibodies (provided by W. G. Honer), or anti-SNAP (Synaptic Systems, 1:100) at room temperature. The levels of SNARE proteins present as SNARE complexes or monomer proteins was assessed by multiplex Western blot with antibodies to syntaxin, SNAP-25, and complexin II. Total expression of SNARE proteins was assessed by Western blot by boiling the samples at 100°C, for 5 min to dissociate SNARE complexes prior to loading on the SDS-polyacrylamide gels. Membranes were incubated for 40 min in secondary goat anti-mouse or anti-rabbit HRP-conjugated antibodies (1:2500–5000, Jackson ImmunoRes Laboratories), visualized with chemiluminescence detection (ECL Plus, GE Healthcare-Biosciences), and the blots were exposed to Kodak Biomax Light film (GE Healthcare-Biosciences). Immunoreactivity was quantified by densitometry analysis using a photo-image scanner and NIH Scion Image program as the densitometry software.

Biotin-switch method for detecting S-nitrosylated synaptic protein. S-nitrosylation of SNAP-25, syntaxin and NSF was assessed using the Nitrogo Assay kit (PerkinElmers Life and Analytical Sciences) based on the methods of Jaffrey et al. (33). In brief, samples were centrifuged at 759 × g for 10 min at 4°C, the supernatant discarded, CHAPS detergent added (1% wt:v) and the sample incubated 10 min on ice. The biotin switch assay was initiated by addition of 3 vol blocking buffer containing the thiol-specific methylthiolating agent methylmethanethiosulfonate to block free thiols on proteins, then incubated for 1 h at 50°C. Then, methylmethanethiosulfonate was removed completely by acetone precipitation at −20°C, and the sample reconstituted in solubization buffer. The nitrosothiol bonds were selectively decomposed to thiols with freshly prepared 50 mmol/L ascorbate, and the newly formed thiols reacted with N-[6-(biotinamido)hexyl]-3′-(2-pyridyldithio) propionamide (biotin-HPDP) for 1 h at room temperature. To identify specific S-nitrosylated proteins, biotinylated proteins were purified on streptavidin-agarose (Upstate Biotechnology), and eluted with sample buffer containing fresh 2-mercaptoethanol. Proteins derived from the acetone precipitation and biotinylated proteins, representing the S-nitrosylated fraction, were boiled and loaded onto 12% SDS polyacrylamide gels, transferred to a polyvinylidene difluoride membrane, and incubated with specific antibody, as discussed above.

Statistical analyses. Values in the text are means ± SEM. Statistical analyses were performed using student’s t test or nonparametric Mann-Whitney tests. Differences of P < 0.05 were considered significant.

Results

Effects of dietary (n-6) and (n-3) fatty acids on hippocampal fatty acids. Rats consuming the diets containing 1.2% (control) or 0.03% [(n-3) fatty acid deficient] ALA had no significant effect on body weight (372 ± 15.6 g and 406 ± 18.6 g) or on brain weight (1.86 ± 0.004 g and 1.92 ± 0.027 g), respectively, when measured at 90 d of age. The concentration of DHA in the hippocampus phospholipids of rats fed the (n-3) fatty acids and SNARE protein.
fatty acid–deficient diet was 42% of the concentration in the control group (Table 1). This marked reduction in DHA was accompanied by significantly higher 22:4(n-6) and a >200-fold increase in DPA in the hippocampus phospholipids in the (n-3) fatty acid–deficient rats compared with those of the control group \( (P < 0.001) \). Over 85% of the reciprocal replacement of (n-6) fatty acids for DHA in the hippocampus of rats fed (n-3) fatty acid–deficient diet compared with those fed the control diet was explained by the increase in DPA.

**SNARE protein and complex expression.** The expression of the ternary SNARE complex, assessed using multiplex Western blots with antibodies to syntaxin, SNAP-25, and complexin II, and the levels of monomer SNARE proteins in the hippocampus of rats fed the control or (n-3) fatty acid–deficient diet, are shown in Figures 1 and 2. The expression of ternary SNARE complex was higher in the hippocampus of rats fed the (n-3) fatty acid–deficient diet compared with those fed the control diet \( (P = 0.017) \), although monomer syntaxin, SNAP-25, and complexin II expression did not differ between the groups (Fig. 2). Similarly, the expression of syntaxin, SNAP-25, or complexin II did not differ among hippocampus samples in which SNARE complexes were dissociated prior to the Western blot analysis (Fig. 1 and 2). To address whether differences in the levels of the ternary SNARE complexes could involve differences in protein expression or protein loading, we assessed the total expression of syntaxin, SNAP-25, and complexin II as the sum of the protein band densities on the Western blots of dissociated and undissociated (monomer plus SNARE complex) protein samples. The sum of the densities for the dissociated and undissociated proteins were 11,550 ± 1040 and 11,720 ± 1195 arbitrary units for the (n-3) fatty acid–deficient group \( (P = 0.9) \) and 12,460 ± 1501 and 11,740 ± 991 arbitrary units for the control group \( (P = 0.7) \), respectively, suggesting that protein loading or the expression of the SNARE proteins did not differ.

**S-nitrosylated SNARe proteins.** The biotin switch Western blot analysis showed a lower expression of S-nitrosylated syntaxin and a lower \( S \)-nitrosylated to total syntaxin ratio in the hippocampus of rats fed the (n-3) fatty acid–deficient diet (157 ± 24.3) than in those fed the control diet (77.4 ± 45.6), with ratios of 0.043 ± 0.007 and 0.018 ± 0.009 arbitrary units, respectively \( (n = 81/group; P < 0.05) \). The expression of \( S \)-nitrosylated SNAP-25 relative to total SNAP-25, however, did not differ between the groups (data not shown). The levels of \( S \)-nitrosylated NSF were below the limit of quantitation in our assay, and NSF expression in the hippocampus did not differ between the (n-3) fatty acid–deficient rats and the controls (data not shown).

**Discussion**

We addressed whether dietary (n-3) fatty acid restriction, leading to reduced DHA in the hippocampus, was associated with changes in the expression of SNARe proteins or ternary SNARe complex formation. Our findings showed that, in vivo, dietary-induced changes in hippocampus (n-6) and (n-3) fatty acids were associated with increased SNARe ternary complex levels. Membrane fusion, in which 2 distinct lipid bilayer membranes merge into 1, is the common final step in the transport of proteins among intracellular compartments, the controlled release of hormones and neurotransmitters by exocytosis, and the addition of membrane material for neural membrane extension (16–20). Several in vivo and in vitro studies provided evidence that DHA influences diverse processes such as neurite extension, the complexity of dendritic arborizations, and release of neurotransmitters, including dopamine and acetylcholine (8–15). We therefore questioned whether changes in brain DHA influences SNARe protein expression or ternary complexes, thus providing a unifying mechanism through which neural tissue DHA is associated with multiple diverse cellular events.

To our knowledge, the results of our studies provide the first demonstration that dietary fat influences ternary SNARe complex expression. Specifically, we showed that decreased levels of DHA in hippocampus, with a concomitant increase in (n-6) fatty acids, results in increased SNARe complex expression, with no difference in the expression of syntaxin, SNAP-25, or complexin II. Consistent with our results, other investigators have shown that feeding a (n-3) fatty acid diet had no effect on SNAP-25 or synaptophysin in the brain cortex of aged mice (34). Additional studies indicating the ability of syntaxin 3 to partner with other SNAReS in PC12 cells that strictly require the binding of (n-3) or (n-6) fatty acids, a role fulfilled by DHA (35), also indicate the role of membrane fatty acids in modulating SNARe complex metabolism. Several mechanisms, however, may explain our results. Exocytosis of neurotransmitters from vesicles in the primed SNARe complex at the plasma membrane is coupled to the

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**TABLE 1** Major (n-6) and (n-3) fatty acids in hippocampus of rats fed the control or (n-3) fatty acid–deficient diet to 90 d of age

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>(n-3) Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2(n-6)</td>
<td>0.41±0.01</td>
<td>0.56±0.02*</td>
</tr>
<tr>
<td>20:4(n-6)</td>
<td>12.9±0.23</td>
<td>14.0±0.47</td>
</tr>
<tr>
<td>22:4(n-6)</td>
<td>3.61±0.07</td>
<td>4.98±0.17*</td>
</tr>
<tr>
<td>22:5(n-6)</td>
<td>0.34±0.03</td>
<td>8.06±0.23*</td>
</tr>
<tr>
<td>22:5(n-3)</td>
<td>0.15±0.01</td>
<td>0.01±0.01*</td>
</tr>
<tr>
<td>22:6(n-3)</td>
<td>16.3±0.40</td>
<td>6.88±0.21*</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 8. *Different from control group \( P < 0.05 \) (t test).
(n-3) Fatty acids and SNARE 1855

influx of Ca^2+ into the neuron, binding of Ca^2+ to the Ca^2+
sensor synaptotagmin, and displacement of complexin from the
SNARE complex (21,22,36). The latter processes are believed to
involve the spatial coordination of membrane protein interac-
tions involving the formation of lipid rafts (37–39). However,
although the low affinity of cholesterol for DHA-rich phosphol-
lipids is important in facilitating the formation of lipid rafts (40),
our results showed an increase in SNARE protein complexes in
the hippocampus of rats fed the (n-3) fatty acid–deficient diet. In
contrast, dietary (n-3) fatty acid deficiency resulted in decreased
DHA turnover and altered phospholipase A2 expression in
the brain of rats (41), and DHA also appeared to be important
in neuronal Ca^2+ ion channel activity (42). G-protein–coupled
signaling efficiency was reduced in retina membranes of rats fed
the (n-3) fatty acid–deficient diet (43). Interestingly, G-protein
coupled receptors were also involved in the control of exocytosis
from ternary SNARE complexes (44), raising the possibility that
the increased SNARE ternary complex levels in the hippocam-
us of (n-3) fatty acid–deficient rats in the present study may
have involved decreased SNARE complex dissociation and
reduced membrane phospholipid turnover and signaling path-
ways. Alternatively, other studies have indicated a specific inter-
action between DHA-containing lipids and Meta-III rhodopsin
(45). Whether interactions occur between DHA-containing
lipids and other membrane proteins, such as SNAREs, is not
known. We also note that altered expression or posttranslational
modifications of SNARE proteins, which we did not examine,
could explain the increased SNARE complex expression in the
hippocampus of rats fed an (n-3) fatty acid diet.

Although nitric oxide (NO), generated by conversion of
L-arginine to L-citrulline via the NO synthases, is a major con-
tributor to central nervous system inflammatory and degenera-
tive disorders through its excessive production (46), reversible
S-nitrosylation of proteins plays an important role in regulating
intracellular protein trafficking and influencing neurotrans-
mission, inflammation, and vasodilation (23–25). Because the
S-nitrosylation of cysteine residues in NSF reduces NSF trans-
port to the plasma membrane and reverses the disassembly of the
SNARE complex cycle (23–25), we attempted to assess the
S-nitrosylation of NSF in hippocampus proteins. However, NSF
was present in relatively low abundance, and the S-nitrosylated
fraction was below the sensitivity of our biotin-switch assay.
However, our results, showing lower S-nitrosylated syntaxin in
the hippocampus of rats fed the (n-3) fatty acid–deficient diet,
suggests that changes in hippocampus fatty acids influence
protein S-nitrosylation, although the mechanism of the effect is
not known.

In summary, the role of dietary components in brain devel-
opment and in healthy brain aging is an area of intense scientific
inquiry and is of considerable importance to human health.
Epidemiological and intervention studies have shown a positive
association among dietary intakes, blood concentrations of DHA,
and cognitive and motor skill development in infants (1–5).
Recent studies also point to a role of DHA in several depressive
disorders and age-related neurodegenerative diseases in adults
(4,6,47,48). Our study provides novel results indicating that,
in vivo, a decrease in hippocampus DHA is associated with
increased ternary SNARE complex, which could reflect either
impaired disassembly or increased neurotransmitter vesicle traf-
ficking. Changes in SNARE protein function may possibly ex-
plain altered neurotransmitter secretion and deficits in tests of
learning behavior found in rodents fed an (n-3) fatty acid–
deficient diet (1,2,13–15). We propose that a unifying mecha-
ism for DHA in diverse cellular events involving secretion of
intracellular vesicular components, such as neurotransmitters,
hormones, and membrane lipids, may be through the role of
DHA in modifying the expression, through posttranslational
modifications, or through the activity of SNARE proteins.

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Literature Cited

42. McNamara RK, Hahn CG, Jandacek R, Rider T, Tso P, Stanford KE, Richert M. Selective deficits in the omega-3 fatty acid docosahexaenoic acid in the postmortem orbital frontal cortex of patients with major depressive disorder. Biol Psychiatry. 2006;6;E-pub.