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Dietary n-3 polyunsaturated fatty acid depletion activates caspases and decreases NMDA receptors in the brain of a transgenic mouse model of Alzheimer's disease

Frédéric Calon,[1,3,†] Giselle P. Lim,[1,3] Takashi Morihara,[1,3] Fusheng Yang,[1,3] Oliver Ubeda,[1,3] Norman Salem Jr,[4] Sally A. Frautschy[1,2,3] and Greg M. Cole[1,2,3,*]

[1]Department of Medicine, University of California, Los Angeles, California, USA
[2]Department of Neurology, University of California, Los Angeles, California, USA
[3]Greater Los Angeles Veterans Affairs Healthcare System, Geriatric Research, Education and Clinical Center, Sepulveda, California, USA
[4]Section of Nutritional Neuroscience, Laboratory of Membrane Biochemistry and Biophysics, Division of Intramural Clinical and Biological Research, National Institute on Alcohol Abuse and Alcoholism, NIH, Rockville, Maryland, USA

Abstract

Epidemiological data indicate that low n-3 polyunsaturated fatty acids (PFA) intake is a readily manipulated dietary risk factor for Alzheimer's disease (AD). Studies in animals confirm the deleterious effect of n-3 PFA depletion on cognition and on dendritic scaffold proteins. Here, we show that in transgenic mice overexpressing the human AD gene *APPswe* (Tg2576), safflower oil-induced n-3 PFA deficiency caused a decrease in *N*-methyl-D-aspartate (NMDA) receptor subunits, NR2A and NR2B, in the cortex and hippocampus with no loss of the presynaptic markers, synaptophysin and synaptosomal-associated protein 25 (SNAP-25). n-3 PFA depletion also decreased the NR1 subunit in the hippocampus and Ca(2+)/calmodulin-dependent protein kinase (CaMKII) in the cortex of Tg2576 mice. These effects of dietary n-3 PFA deficiency were greatly amplified in Tg2576 mice compared to nontransgenic mice. Loss of the NR2B receptor subunit was not explained by changes in mRNA expression, but correlated with p85α phosphatidylinositol 3-kinase levels. Most interestingly, n-3 PFA deficiency dramatically increased levels of protein fragments, corresponding to caspase/calpain-cleaved fodrin and gelsolin in Tg2576 mice. This effect was minimal in nontransgenic mice suggesting that n-3 PFA depletion potentiated caspase activation in the Tg2576 mouse model of AD. Dietary supplementation with docosahexaenoic acid (DHA; 22 : 6n-3) partly protected from NMDA receptor subunit loss and accumulation of fodrin and gelsolin fragments but fully prevented CaMKII decrease. The marked effect of dietary n-3 PFA on NMDA receptors and caspase/calpain activation in the cortex of an animal model of AD provide new insights into how dietary essential fatty acids may influence cognition and AD risk.

Introduction

Alzheimer disease (AD) is the most common neurodegenerative disorder and the first cause of dementia in the elderly (Cummings & Cole, 2002). β-amyloid peptides (Aβ) deposition (neuritic plaques), neurofibrillary tangles, synaptic deficit and extensive neurodegeneration are the main neuropathological hallmarks of the disease (Selkoe, 2001; Cummings & Cole, 2002; Ingelsson *et al*., 2004). The discovery of mutations causing AD, the development of transgenic mouse lines reproducing many pathological features of AD and positive family history all strongly support a genetic basis for AD (St George-Hyslop, 2000; Ashford & Mortimer, 2002; Selkoe & Podlisny, 2002). However, environmental factors appear to modify genetic risk (Raiha *et al*., 1997; Selkoe, 2001; Ashford & Mortimer, 2002; Grant *et al*., 2002). For example, monozygotic twins can be discordant for AD and, for those who eventually become concordant, the onset of AD can be delayed up to 15 years (Raiha *et al*., 1997; Jarvenpaa *et al*., 2003). From a therapeutic standpoint, environmental factors are easier to manipulate than genetic factors. Therefore, a better knowledge of the mechanisms by which environmental factors drive an inherited susceptibility to full scale AD will improve our understanding of AD and help to develop new preventive approaches.

Converging epidemiological data suggest that low dietary intake of n-3 polyunsaturated fatty acids (PFA) is a candidate risk factor for AD. n-3 PFA such as α-linolenic acid (LNA, 18 : 3n-3), eicosapentaenoic acid (EPA, 20 : 5n-3) and docosahexaenoic acid (DHA, 22 : 6n-3), are essential fatty acids obtained from dietary sources (Mostofsky *et al*., 2001). n-3 PFA under-consumption is prevalent in Western societies (Simopoulos, 2002) and epidemiological studies suggest that people exposed to low levels of dietary n-3 PFA are more likely to develop AD dementia (Kalmijn, 2000; Barberger-Gateau *et al*., 2002; Grant *et al*., 2002; Otsuka *et al*., 2002; Morris *et al*., 2003). Accordingly, most studies (but not all) indicate that patients with AD or cognitive decline have lower blood levels of circulating n-3 PFA compared to normal individuals (Kyle *et al*., 1999; Conquer *et al*., 2000; Heude *et al*., 2003; Laurin *et al*., 2003; Tully *et al*., 2003).

Sufficient dietary intake of n-3 PFA is required for optimal cognitive performance in several animal species (Fiennes *et al*., 1973;

Correspondence: Dr Greg M. Cole, at *present address below.
E-mail: gmcole@ucla.edu

**Present address*: Greater Los Angeles Healthcare System, Veterans Administration Medical Center, 16111 Plummer St., Bldg 7, Room A102, North Hills, CA 91343, USA.

†*Present address*: Molecular Endocrinology and Oncology Research Center, Laval University Medical Center (CHUL), Québec, Qc, and Faculty of Pharmacy, Laval University, Québec, Qc, Canada.

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Wainwright *et al*., 1997; Ikemoto *et al*., 2001; Salem *et al*., 2001; Catalan *et al*., 2002; Hashimoto *et al*., 2002) and in an animal model of AD (Calon *et al*., 2004). However, the mechanism of action of n-3 PFA on cognition is unclear. Recent studies point out n-3 PFA dietary restriction combined with human mutant amyloid precursor protein (*APPswe*) overexpression in transgenic (Tg2576) mice lead to cognitive deficits and massive loss of the postsynaptic proteins, developmentally regulated brain protein (drebrin) and postsynaptic density-95 (PSD-95) (Calon *et al*., 2004). Treatment with DHA protected against these effects possibly through reduction of oxidative damage, activation of cell survival pathway and inhibition of caspases (Calon *et al*., 2004). This suggests that, through regulation of these pathological processes, n-3 PFA can impact synapse function and cognition, and, consequently, AD pathogenesis.

The purpose of this paper was to use the *APPswe* Tg2576 mouse model for memory loss and Aβ deposition in AD (Hsiao *et al*., 1996; Lim *et al*., 2005) and normal aged C57Bl/6 mice to evaluate the potential mechanisms of action of n-3 PFA on synaptic proteins and cell death pathways. Specifically, we investigated the effect of n-3 depletion on presynaptic markers and key postsynaptic proteins essential for cognition [*N*-methyl-D-aspartate (NMDA) receptor complex and CaMKII]. We further investigated the role of n-3 PFA deficiency on specific protease activation by evaluating accumulation of caspase-cleaved fodrin and gelsolin in the brain of Tg2576 mice.

Materials and methods

Materials

Unless otherwise noted, reagents were obtained from Sigma (St-Louis, MO). Antibodies were purchased from: anti-NR2A (MAB5216), anti-NR2B (MAB5220), anti-neuronal nuclei (NeuN), anti-fodrin #1 (MAB1622), anti-fodrin #2 (polyclonal; AB992), synaptophysin (MAB368), and actin (Chemicon international, Temecula, CA), anti-GFAP (Sigma, St-Louis, MO), anti-synaptosomal-associated protein 25 (SNAP-25) (Sternberger Monoclonals, Lutherville, MD), anti-total BAD (Cell Signalling, Beverly, MA), anti-NR1 (Advanced Immunochemical, Long Beach, CA), anti-CaMKII (Stressgen, Victoria, BC, Canada), and anti-14-3-3 (Santa Cruz Biotechnology, Santa Cruz, CA). The monoclonal fodrin antibody targets full-length α-fodrin (epitope in the C-terminal region) and was previously used to detect caspase and calpain cleavage fodrin fragments (Janicke *et al*., 1998). Affinity purified anti-caspase-cleaved gelsolin rabbit polyclonal antibody recognizes PDQTD at the 3′-end sequence of the caspase-3 cleaved gelsolin N-terminal fragment and is a generous gift from Dr Toshi Azuma, Harvard Medical School.

Animals and diets

Use of animals was approved by the Greater LA VA IACUC and UCLA Chancellor's Animal Research Committee. Male and female Tg2576 Tg(+) and Tg(–) mice at least 17-months old from 12-litters were randomly split among three treatment groups as described previously (Calon *et al*., 2004). Mice were fed the next 3–5 following months with either control diet (Control Diet, PMI 5015, PMI International LabDiet, St. Louis, MO), safflower oil-based diet depleted of n-3 PFA (Low DHA Diet) (TD 00522, Harlan Teklad, Madison, WI) or the low DHA diet to which 0.6% (w/w) DHA (Martek Bioscience, Columbia, MD) was added (High DHA Diet, TD01200) (see Table 1). The three diets were similarly supplemented in minerals and vitamins. Animals were killed at 22.5 months of age (ketamine/xylazine anaesthesia) and perfused with 0.9% normal saline followed by Hepes buffer (pH 7.2)-containing protease inhibitors. Brain regions were dissected from one hemisphere as previously described (Lim *et al*., 2001). Unless otherwise noted, biochemical measurements were performed on the residual cortex (cortex region without frontal, entorhinal, or piriform areas). Frontal cortex was used for fatty acid analysis.

TABLE 1. Dietary polyunsaturated fatty acid (PFA) content

Dietary treatment	18:2n-6 (LA) (% w/w)	18:3n-3 (LNA) (% w/w)	20:4n-6 (ARA) (% w/w)	22:6n-3 (DHA) (% w/w)	n-6/n-3 (approx.)
Control	2.45	0.18	0.03	0.09	9 : 1
Low n-3 PFA	4.86	0.06	0.0003	0.0002	82 : 1
Low n-3 PFA + DHA	2.75	0.03	0.0007	0.6	4 : 1

LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; n-3 PFA, omega-3 polyunsaturated fatty acid.

Preparation of tissue samples

Tissue samples were homogenized in ten volumes of Tris-buffered saline (TBS) containing a cocktail of protease inhibitors (20 μg/mL each of pepstatin A, aprotinin, phosphoramidon, and leupeptin, 0.5 mM 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride (AEBSF), 1 mM EGTA, 5 μM fenvalerate, and 5 μM cantharidin). Samples were sonicated briefly (2 × 10 s) and centrifuged at 100 000 ***g*** for 20 min at 4 °C to generate a TBS-soluble fraction (cytosol fraction). The TBS-insoluble pellet was sonicated in ten volumes of lysis buffer (150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, 1% Triton X-100, 0.5% SDS, and 0.5% deoxycholate) containing the same protease inhibitor cocktail. The resulting homogenate was centrifuged at 100 000 ***g*** for 20 min at 4 °C to produce a lysis buffer-soluble fraction (membrane fraction).

In-vitro *caspase digestion*

Brain cortex from Tg(–) control mice were homogenized in 7.5 volumes of an incubation/homogenization buffer containing 50 mM Hepes, pH 7.2, 50 mM NaCl, 0.1% Chaps, 2 mM EDTA, 5% Glycerol, and 5 mM dithiothreitol. Samples were sonicated briefly (3 × 10 s) and centrifuged at 10 000 ***g*** for 20 min at 4 °C to generate a supernatant fraction for digestion assays. Active human recombinant caspase-3 (2.5–12.5 unit/mg protein depending of the experiment) (Biovision, Mountain View, CA), with or without caspase inhibitor (DVED, 150 μM, and/or ZVAD, 500 μM, Biovision) was added and incubation was performed at 37 °C from 0 to 120 min in the incubation/homogenization buffer. After incubation, Laemmli's (up to 1×) was added to the samples, which were then boiled for 5 min and electrophoresed as described below. Brain protein digested with caspase was used as a standard for identification of 120-kDa and 150-kDa caspase-cleaved fodrin breakdown products (CCFBDP) on immunoblots.

Immunoblotting

Samples (30 μg protein) were electrophoresed on 10% acrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon, Millipore, MA) before blocking in 10% nonfat dry milk and 0.1% gelatin in PBS for 1.5 h. Blots were immunoblotted with appropriate primary and secondary antibody and chemiluminescence (ECL, Amersham/Pharmacia Biotech, Piscataway, NJ or Supersignal,

Pierce, Rockford, IL). Band intensities were scanned and quantified with densitometric software (Molecular Analyst II, Bio-Rad, Hercules, CA). Immunoblot data were normalized to total protein load by quantification of all the sample in a single assay before loading and confirmation of equal loading by image analysis of scanned Coomassie blue stained gels after blotting. For hippocampal samples, data were further normalized to optical density of total Coomassie blue gel colouration per lane.

Fatty acid measurement

Fatty acid analysis in frontal cortex was performed using Folch's extraction method and gas chromatography with flame ionization detection, as previously described (Moriguchi *et al*., 2000). Fatty acid data in diets from Harlan were analysed by Cornell University, diagnostic laboratory nutritional and environmental analytical services (Ithaca, NY).

Quantitative real-time RT-PCR (QPCR) of NR2B mRNA

Total RNA was isolated from brain using the RNAqueous kit (Ambion, Austin, TX) as per manufacturer's instructions and treated with DNase. RNA (0.7 μg) was reverse transcribed using dT primers using the Retroscript kit (Ambion), then aliquoted. Analysis of RNA levels was performed using QPCR with the Sybr Green Supermix reagent (Bio-Rad) containing a fluorescein internal standard and a MyIQ real-time thermocycler (Bio-Rad), followed by dissociation curve analysis that verified amplification of a single PCR species. Thermocyling parameters were standard for the MyIQ instrument: 95° for 3 min, and 40 cycles of 55° for 45 s and 95° for 1 min. Each sample was tested in triplicate. A standard curve was run to allow relative comparisons of sample values. The standard curve was made using cDNA (using dT primers) from brain tissue RNA reverse transcribed at two times the concentration of RNA (1.4 μg) compared to that used for reverse transcription of the sample RNA; standard curves typically had an *R*-squared >0.99. The primers for NR2B were designed using the Primer Express software (Applied Biosystems, Foster City, CA) using the mouse NR2B sequence (NM_008171). The primer sequences were: forward 5′-TGTGCCAGGTCGTTTCCA-3′ (300 nM); reverse 5′-ACTAGAA AGTTTCTCATAAACATGTCCATT-3′ (900 nM), whereas the probe sequence was 5′-*FAM*-AGGACATTTGTATAGGGAACCAGTCCAA CCC-*BHQ*-3′ (200 nM). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were also measured for each cDNA sample as an internal housekeeping gene control using the probes and primers from the TaqMan Rodent GAPDH Control Reagents (Applied Biosystems). Levels of NR2B mRNA were normalized to levels of GAPDH mRNA for each sample.

Statistical analysis

Statistical comparisons of data were performed using an ANOVA followed by *posthoc* pair-wise comparisons with Fisher's probability of least significant difference test (PLSD). Square root transformation to establish homogeneity of variance was used for NR2B data in mice. Coefficients of correlation and significance of the degree of linear relationship between parameters were determined with a simple regression model.

Results

Effect of diets on brain fatty acids

In Tg(–) mice, safflower oil-induced n-3 PFA deficiency (Table 1) increased levels of docosapentaenoic acid (DPA, 22 : 5n-6) and decreased eicosapentaenoic acid (EPA, 20 : 5n-3) in the frontal cortex (Table 2). In contrast, DHA-enriched diet (low n-3 PFA + DHA) decreased levels of arachidonic acid (ARA, 20 : 4n-6), docosatetraenoic acid (DTA, 22 : 4-n-6) and DPA while increasing EPA and DHA concentration in the frontal cortex (Table 2). The EPA increase after DHA supplementation suggests conversion of DHA to EPA. Interestingly, dietary n-3 PFA restriction decreased the cortical DHA content in Tg(+) mice but not in the Tg(–) animals (Table 2). The effects of safflower oil-induced n-3 PFA deficiency on both n-3 and n-6 PFA levels were exacerbated in Tg2576 mice compared to Tg(–) resulting in a significantly larger increase in the n-6/n-3 ratio (Table 2). Notably, the low n-3 PFA diet resulted in lower levels of DHA and higher concentrations of DPA in Tg(+) compared to Tg(–) mice (Table 2). These data suggest that alterations in dietary intake of n-3 PFA are more strongly translated into changes of brain PFA concentration in Tg2576 mice compared to control Tg(–) mice. Our results suggest that the *APPswe* transgene overexpression confers an increased susceptibility to n-3 PFA depletion.

Synaptic markers

Dietary n-3 PFA restriction caused a decrease of NR2A and NR2B subunits of the NMDA receptor in the cortex of Tg(–) (Table 3) and, more strikingly, in Tg(+) mice (Fig. 1). Similar effects were observed

TABLE 2. Polyunsaturated fatty acid (PFA) frontal cortex content

		n-6 PFA				n-3 PFA		Ratio
	N	18:2n-6(1)	20:4n-6(2)	22:4n-6(3)	22:5n-6(4)	20:5n-3(5)	22:6n-3(6)	n-6/n-3
Tg(–)								
Control	13	0.63 ± 0.02	9.6 ± 0.1	2.83 ± 0.07	0.52 ± 0.02	0.020 ± 0.003	19.2 ± 0.3	0.70 ± 0.01
Low n-3 PFA	6	0.80 ± 0.03*	9.8 ± 0.1	3.00 ± 0.12	1.43 ± 0.12**	0.002 ± 0.002	18.0 ± 0.5	0.84 ± 0.02**
Low n-3 PFA + DHA	7	0.93 ± 0.09	8.4 ± 0.2††	2.13 ± 0.10††	0.29 ± 0.05††	0.088 ± 0.027††	20.5 ± 0.5††	0.57 ± 0.02††
Tg(+)								
Control	8	0.73 ± 0.05	9.5 ± 0.1	2.62 ± 0.08	0.59 ± 0.03	0.019 ± 0.006	19.5 ± 0.5	0.69 ± 0.02
Low n-3 PFA	6	0.90 ± 0.06*	9.9 ± 0.2	3.01 ± 0.12**	1.81 ± 0.20**‡‡	0.009 ± 0.009	16.4 ± 0.8**‡	0.96 ± 0.06**‡‡
Low n-3 PFA + DHA	6	1.01 ± 0.04	7.9 ± 0.2††	1.89 ± 0.12††	0.22 ± 0.02††	0.118 ± 0.009††	21.3 ± 0.6††	0.51 ± 0.02††

Values are expressed as mean percentage of total frontal cortex fatty acid ± SEM; within same transgene, $*P<0.05$, $**P<0.01$ vs. control diet, and $††P<0.01$ vs. low n-3 PFA diet; between transgene; $‡P<0.05$, $‡‡P<0.01$ vs. Tg(–) Low n-3 PFA diet (1) linoleic acid (LA), (2) arachidonic acid (ARA), (3) docosatetraenoic acid (DTA), (4) docosapentaenoic acid (DPA), (5) eicosapentaenoic acid (EPA), (6) docosahexaenoic acid (DHA).

TABLE 3. Synaptic markers in the cortex of Tg(–) mice

Tg(–)	*N*	NR2A	NR2B	NR1	Synaptophysin
Control	13	100 ± 3	100 ± 20	100 ± 11	100 ± 7
Low n-3 PFA	6	47 ± 7**	37 ± 10**	97 ± 10	98 ± 2
Low n-3 PFA + DHA	7	58 ± 13*	53 ± 19**	73 ± 15	101 ± 3

Values are expressed as mean percentage of Tg(–) mice on control diet ± SEM, $^{**}P < 0.05$, $^{**}P < 0.01$ vs. Tg(–) control diet.

with CaMKII (Fig. 1) whereas low n-3 PFA diet did not alter the NR1 subunit, synaptophysin or SNAP-25 in Tg(–) and Tg(+) animals (Fig. 1, and Table 3). Addition of DHA to the diet had a preventive effect on the loss of NR2A, NR2B and CaMKII (Fig. 1), suggesting that DHA deprivation contributed in part to the effect of the low n-3 PFA diet.

In the hippocampus, the effects of the low n-3 PFA diets on NR2A and NR2B subunits of the NMDA receptor were similar to the cortex but less striking (Fig. 2). Another regional difference is that the rescue effect of DHA was sometimes blunted in the hippocampus, particularly for NR2A, and NR1 was also lost after n-3 PFA restriction (Fig. 2). Moreover, the alterations of CaMKII levels were non-significant due to higher intragroup variability (Fig. 2). In contrast, we detected no change of synaptic markers NR2B, NR1, PSD-95, CaMKII, synaptophysin and SNAP-25 in homogenates from the thalamus/striatum regions of the same animals by immunoblotting (not shown). This suggests that the effect of *APPswe* transgene overexpression and/or n-3 PFA depletion on synaptic markers is restricted to brain structures where significant amyloid pathology is found.

Index of caspase and calpain activation

To determine if activation of caspases accompanied the decrease in NMDA receptor subunits, experiments to measure levels of caspase-cleaved fodrin breakdown products were undertaken. Caspase cleavage of fodrin produces a 120-kDa fragment whereas both caspase and calpain can generate the 150-kDa fragment (Siman *et al.*, 1984; Wang, 2000). Figure 3A illustrates the production *in vitro* of a 120-kDa and 150-kDa fodrin fragment as a function of time, which was blocked by addition of caspase inhibitors. Figure 3B and E show the modest increase of 120-kDa and 150-kDa CCFBDP observed in Tg(+) compared to Tg(–) mice. Dietary n-3 PFA restriction massively increased 120-kDa and 150-kDa CCFBDP in Tg(+) animals (Fig. 3C and E) whereas it decreased levels of intact fodrin (240/235 kDa; Fig. 3D and E). The effect of n-3 PFA deficiency on intact fodrin was fully countered by DHA (Fig. 3D and E) whereas the protective effect of DHA on the accumulation of both CCFBDP was partial (Fig. 3C and E). In contrast, analysis of Coomassie blue gels revealed no evidence of generalized proteolysis (not shown), suggesting that protease degradation of fodrin was specific, consistent with our published data showing increased drebrin in the soluble fraction and unchanged glial fibrillary acidic protein (GFAP) and synaptophysin in lysis fractions from n-3 PFA-depleted Tg(+) mice (Calon *et al.*, 2004).

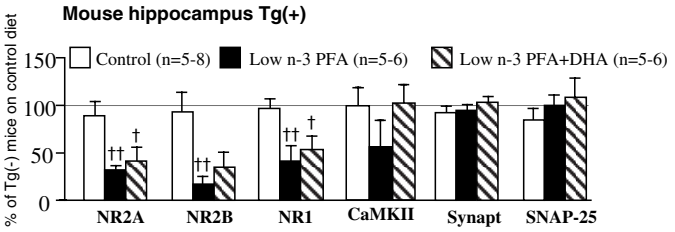

FIG. 2. Omega-3 polyunsaturated fatty acids (n-3 PFA) depletion induced a loss of NR2A, NR2B and NR1 in Tg2576 mice with no effect on synaptophysin and synaptophysin, synaptosomal-associated protein 25 (SNAP-25) in the hippocampus. Dietary supplementation with docosahexaenoic acid (DHA) did not protect against these changes. Values shown were normalized to total protein content and are expressed in % of Tg-mice on control diet as the mean ± SEM. †$P < 0.05$, ††$P < 0.01$ vs. Tg(+) Ctrl.

To substantiate evidence of caspase activation, caspase-cleaved gelsolin was measured using a cleavage site-specific antibody. Immunoblot studies show that dietary n-3 PFA restriction also induced a significant increase in caspase-cleaved gelsolin in the cortex of Tg2576 mice (Fig. 4). The pro-apoptotic caspase regulatory protein, Bad, was also significantly increased with DHA-depletion and restored to normal levels by DHA (Fig. 4). However, while DHA reduced the percentage of caspase-cleaved vs. total fodrin (Fig. 3E), addition of DHA to the diet did not significantly reduce the caspase-cleaved gelsolin fragment (Fig. 4).

NR2B mRNA

To determine whether alteration in NR2B protein reflected changes at the transcription levels, NR2B mRNA cortical content was assessed using QPCR. Dietary n-3 PFA restriction had no significant effect on NR2B mRNA whereas addition of DHA slightly reduced NR2B mRNA levels in the cortex (Fig. 5). The absence of a parallel alteration between the protein and mRNA level argues against a decreased transcription rate as an explanation of the massive decrease in NR2B after low n-3 PFA intake in Tg2576 mice.

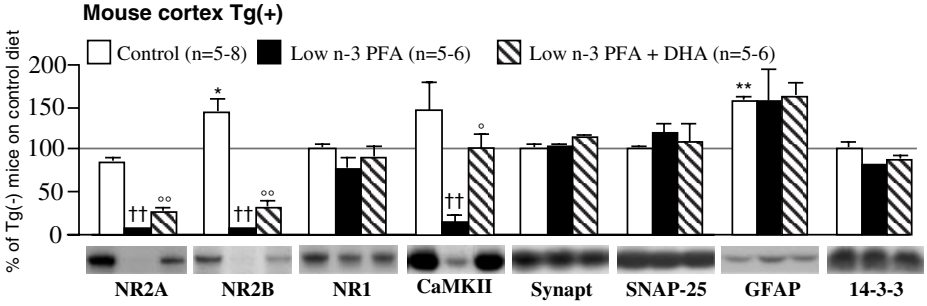

FIG. 1. Omega-3 polyunsaturated fatty acids (n-3 PFA) depletion induced a massive cortical loss of NR2A, NR2B and Ca(2+)/calmodulin-dependent protein kinase II (CaMKII) in Tg2576 mice with no effect on NR1, synaptophysin, synaptosomal-associated protein 25 (SNAP-25), glial fibrillary acidic protein (GFAP) and 14-3-3 protein. Dietary supplementation with docosahexaenoic acid (DHA) exerted a protective effect against these changes. Values shown are in % of Tg-mice on control diet as the mean ± SEM. ★$P < 0.05$, ★★$P < 0.01$ vs. Tg(–) Ctrl , ††$P < 0.01$ vs. Tg(+) Ctrl, and $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, vs. Tg(+) low n-3 PFA.

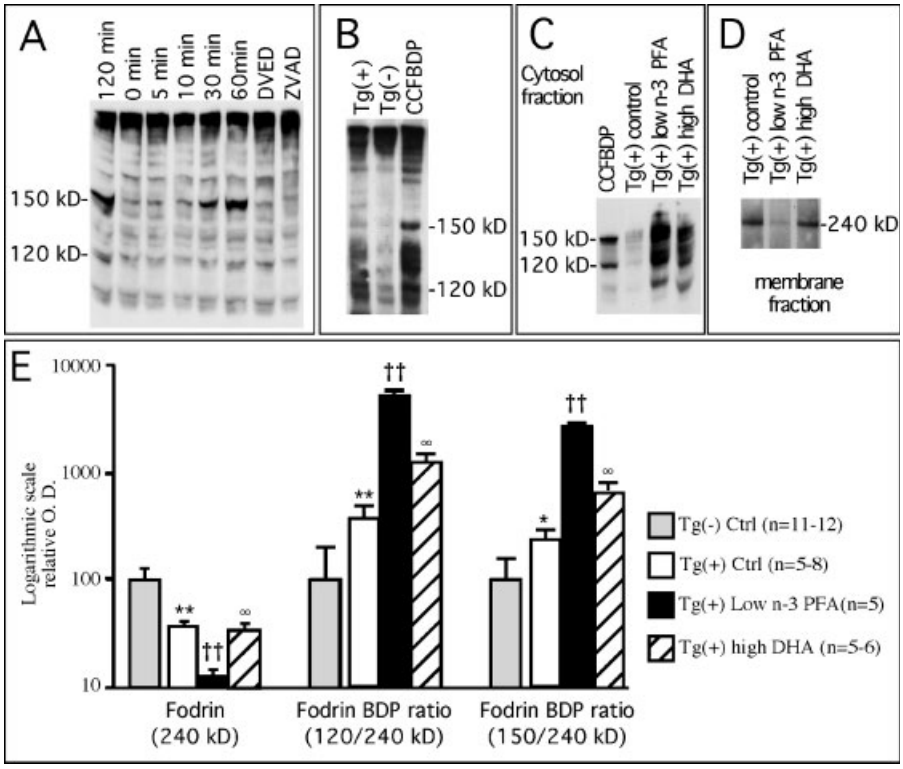

FIG. 3. Omega-3 polyunsaturated fatty acids (n-3 PFA) depletion induced a decrease in fodrin (240 kD) and a concomitant increase in fodrin breakdown products (BDP) produced by caspase (120 kD and 150 kD) detected with an anti-fodrin monoclonal antibody (MAB1622). (A) Digestion of normal mouse cortex with caspase-3 (2.5 U/mg of protein) produced a 150 kD and a 120 kD BDP of fodrin whereas caspase inhibitors DVED and ZVAD completely blocked digestion of fodrin after 60 min. (B) Representative example showing fodrin BDP (120 kD and 150 kD) in a Tg(+) mouse compared to a Tg(–) mouse. Samples were run along caspase-cleaved fodrin breakdown products (CCFBDP) produced by digesting a mouse cortex extract with caspase-3 (12.5 U/mg protein). (C) Immunoblot picture showing the important increase in fodrin BDP in a Tg(+) fed low n-3 PFA diet mouse cortex compared to a Tg(+) control and Tg(+) fed high DHA diet. Samples were run along caspase-cleaved fodrin breakdown products (CCFBDP) produced by digesting a mouse cortex extract with caspase-3 (12.5 U/mg protein). (D) Immunoblot example showing that n-3 PFA dietary restriction caused a loss of fodrin (240 kD) in the cortex of a Tg(+) mouse, which was protected by the DHA-enriched diet. (E) Histograms showing the effect of *APPswe* transgene as well as dietary n-3 PFA on fodrin (240 kD) and its 120 kD and 150 kD BDP in the cortex of mouse. Docosahexaenoic acid (DHA) partially protected against fodrin BDP accumulation. Values shown are expressed (mean ± SEM) as ratio over full-length fodrin in % of Tg(–) on control diet plotted on a logarithmic scale. ★$P < 0.05$, ★★$P < 0.01$ vs. Tg(–) Ctrl, ††$P < 0.01$ vs. Tg(+) Ctrl, and $^{\circ\circ}P < 0.01$, vs. Tg(+) low n-3 PFA. DVED/ZVAD, caspase inhibitors.

Correlative analysis

To substantiate links between NMDA receptor changes with markers of caspase activity, we show in Fig. 6 how Tg2576 mice with lower cortical levels of NR2A and NR2B protein levels show a correspondingly increased index of caspase activation (caspase-cleaved fodrin, gelsolin and actin) in the cortex. Moreover, the NR2B subunit content was also negatively correlated with levels of the caspase activator, Bad, in the cortex of Tg(+) animals (Fig. 6). NR2A and NR2B receptor levels were inversely correlated with cortical dinitrophenylhydrazine (DNPH) derivatized protein carbonyls ($r^2 = 0.333$, $P = 0.013$; $r^2 = 0.369$, $P = 0.019$; respectively) and very positively correlated with p85α subunit of phosphatidylinositol 3-kinase (p85) ($r^2 = 0.850$, $P < 0.001$; $r^2 = 0.540$, $P < 0.001$; respectively). Fractin, carbonyls and p85 values for correlation analysis were obtained from our previous study (Calon *et al*., 2004).

Discussion

The present results suggest that dietary n-3 PFA intake is an important regulator of caspase activation and NMDA receptor subunit proteins in the cerebral cortex. First, dietary n-3 PFA restriction led to massive loss of postsynaptic markers (NMDA receptor subunit and CaMKII in Tg2576 mice) without concomitant decreases of presynaptic markers. Second, low n-3 PFA intake led to caspase activation in the cortex of Tg2576 mice as shown by the detection of increased specific products of caspase cleavage of fodrin and gelsolin. Third, dietary DHA supplementation had a preventive effect on these changes. Therefore, it is suggested that these effects of dietary n-3 PFA may be relevant to the action of n-3 PFA on cognition previously reported in normal animals and Tg2576 mice as well as in AD.

NMDA receptor channels are heteromers composed of the key receptor subunit NMDAR1 (NR1) and one or more of the four NMDAR2 subunits: NR2A, NR2B, NR2C, and NR2D (Cull-Candy *et al*., 2001). NMDA receptors are ionotropic glutamate receptors playing a pivotal role in mediation of excitotoxic neuronal injury and are concentrated at postsynaptic sites where high levels of energy consumption and oxidative stress are found (Mattson & Duan, 1999; Attwell & Iadecola, 2002). NMDA receptors are part of a large protein complex called the 'hebbosome' (including drebrin, CaMKII, PSD-95, actin, and many other proteins), which is closely related to the actin cytoskeleton (Husi *et al*., 2000). NMDA receptors along with proteins associated with the hebbosome regulate dendrite morphology, synaptic plasticity and cognition (Migaud *et al*., 1998; Cull-Candy *et al*., 2001; Newcomer & Krystal, 2001). For example, overexpression of the NR2B subunit in a transgenic animal model

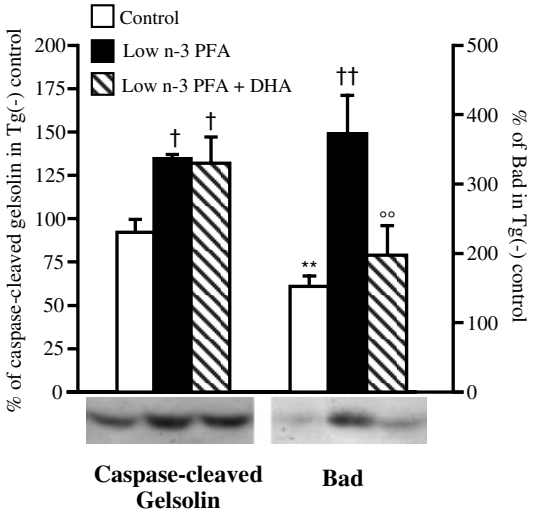

FIG. 4. Dietary n-3 PFA restriction increased caspase-cleaved gelsolin and Bad in the cortex of transgenic mice. DHA supplementation did not prevent the caspase-cleaved gelsolin increase but did protect against Bad increase. Values shown are in % of Tg-mice on control diet as the mean ± SEM. ★★$P < 0.01$ vs. Tg(–) Ctrl, †$P < 0.05$, ††$P < 0.001$ vs. Tg(+) Ctrl, and $^{○○}P < 0.01$, vs. Tg(+) low n-3 PFA.

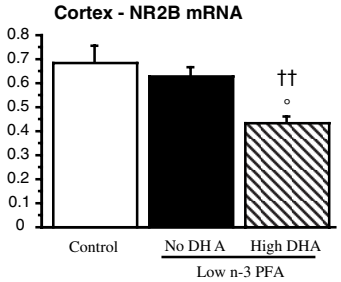

FIG. 5. Quantitative real-time RT-PCR measurements of NR2B subunit mRNA in cortex of Tg(+) mice fed with control diet or low n-3 PFA diet (with No DHA or High DHA). ††$P < 0.01$ vs. Tg(+) Ctrl, and $^{○}P < 0.05$, vs. Tg(+) Low n-3 PFA (No DHA).

enhances learning and memory, whereas blockade of NMDA receptor with antagonist or genetic inactivation of the NR2A or NR1 subunit produces cognitive deficits in mice (Tsien *et al*., 1996; Tang *et al*., 1999; Gainetdinov *et al*., 2001). As discussed below, deficits in NMDA subunits have been reported in AD brain, which potentially contribute to cognitive deficits. Significant reduction in the dietary intake of n-3 PFA is well known to produce cognitive deficits in normal animals after long-term depletion (Fiennes *et al*., 1973; Ikemoto *et al*., 2001; Salem *et al*., 2001; Catalan *et al*., 2002; Hashimoto *et al*., 2002) or after 3 months depletion in aged Tg2576 mice (Calon *et al*., 2004). Therefore, the massive loss of NMDA receptor subunit shown here is likely involved in the mechanism for the cognitive dysfunction caused by the combination of n-3 PFA depletion and *APPswe* overexpression.

Surprisingly, 3 months of dietary n-3 PFA deprivation was sufficient to significantly decrease brain DHA and the n-3/n-6 ratio in the Tg2576 mouse. The changes were small (−15%) but significantly higher than those observed in Tg(–) littermates (−6%; Table 2). This vulnerability of Tg(+) mice to n-3 PFA depletion suggests that overexpression of *APPswe* transgene exerts an effect on how the brain

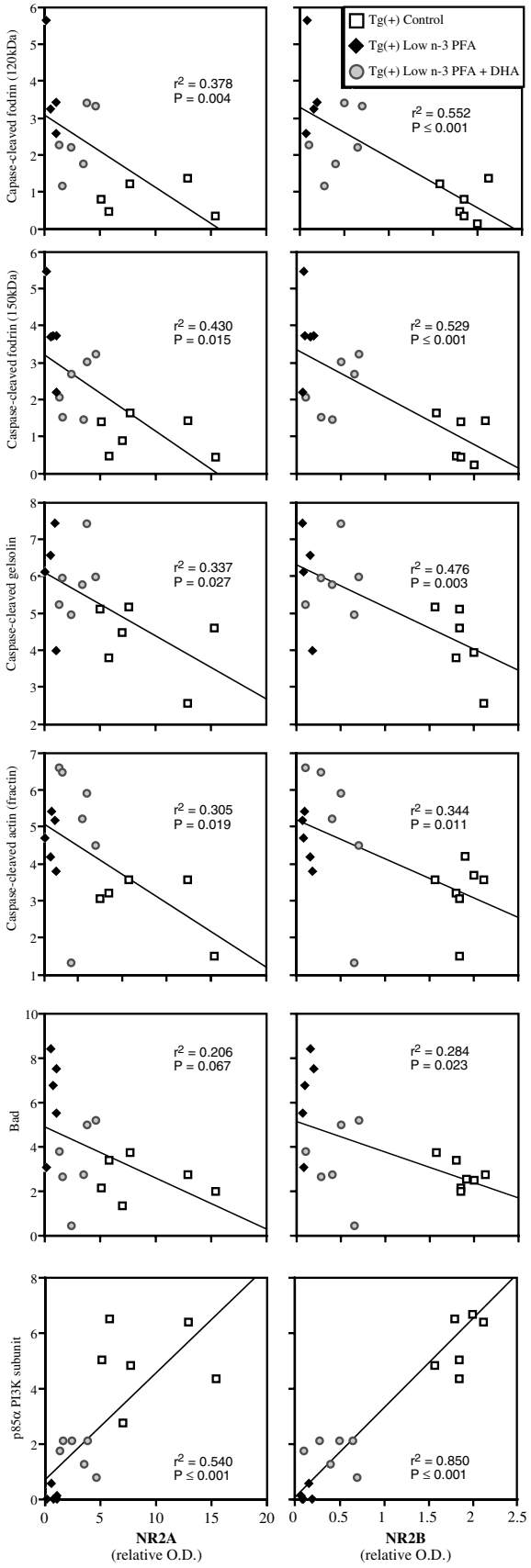

FIG. 6. Correlation analysis between NR2A or NR2B receptor levels and marker of caspase activation in the cortex of Tg(+) mice fed control diet, Low n-3 PFA diet or Low n-3 PFA + DHA diet.

handles n-3 PFA. Although the precise mechanism for this susceptibility is not known, oxidative stress secondary to Aβ pathology in the Tg2576 model may explain the rapid depletion in DHA pool in these animals (Lim *et al*., 2001; Pratico *et al*., 2001; Calon *et al*., 2004). Indeed, n-3 PFA, such as DHA, are particularly vulnerable to oxidation in AD (Nourooz-Zadeh *et al*., 1999; Montine *et al*., 2002; Yavin *et al*., 2002; Montine *et al*., 2004). If our observations with a human familial AD mutation in mice were transposable to humans, it would imply that individuals bearing a genetic risk for AD might be more susceptible to brain DHA depletion. Such information would suggest that optimization of the dietary consumption of n-3 PFA in older patients with a family history of AD may be beneficial.

A wealth of published evidence suggests that caspase activation occurs at some point during AD pathogenesis (Cole *et al*., 1999; Gervais *et al*., 1999; Mattson, 2000; Rossiter *et al*., 2000; Wang, 2000; Yuan & Yankner, 2000; Rohn *et al*., 2001; Su *et al*., 2001; Eckert *et al*., 2003; Gamblin *et al*., 2003; Guo *et al*., 2004; Rissman *et al*., 2004). However, according to the standard scheme of programmed cell death, caspase activation is the final act leading to apoptosis. To fit in the time frame of a slow progressive disease like AD, caspase activation would most probably be a chronic phenomenon that is kept at bay by cellular protective mechanisms. In other words, caspase activation in AD might be part of a slow and localized degenerative process that contrasts with the rapid classical apoptotic cascade (Cole *et al*., 1999; Marx, 2001; Rohn *et al*., 2001; Calon *et al*., 2004).

Our present data show an accumulation of caspase cleavage products in the brain of Tg2576 mice, which is accentuated by dietary n-3 PFA restriction. Indeed, caspases have been shown to produce 40-kDa gelsolin BDP as well as 150-kDa and 120-kDa fodrin BDP by cleavage of the full-length protein (Kothakota *et al*., 1997; Janicke *et al*., 1998; Wang, 2000). Although our results do not provide information on the time course of caspase activation, they argue that dietary PFA regulates a chronic form of caspase activation in Tg2576 mice that led to the cleavage of fodrin and gelsolin without killing brain cells. The present data are consistent with our previous observations showing caspase-cleaved actin in the brain of Tg2576 mice in the absence of neuron loss (Cole *et al*., 1999; Calon *et al*., 2004). Reduced fodrin cleavage following DHA-enriched diets underscores the potential neuroprotective action of this long n-3 PFA in the Tg2576 animal model of AD.

Although fodrin and gelsolin BDP accumulation occurred concomitantly in animals with reduced cortical NR2 subunits, it is not clear how n-3 PFA depletion led to a decrease in NMDA receptor subunit nor what role caspase activation plays in these massive decreases. A direct effect of n-3 PFA on NMDA receptor transcription appears unlikely because NR2B mRNA cortical content as assessed with QPCR (Fig. 5) did not show a pattern of change compatible with the alterations in proteins described in Figs 1 and 2. Although there is no published evidence that NR2 subunit is a caspase substrate, NR2B is a calpain substrate (Bi *et al*., 1998; Guttmann *et al*., 2001; Tomimatsu *et al*., 2002). Hence, calpain activation could also explain the observed 150-kDa fodrin fragments (Siman *et al*., 1984; Wang, 2000). While no lower molecular weight NR2B BDPs were detected, fragments may have been long degraded at the time of our analysis. Indeed, while we were unable to positively document it using two different antibodies to calpain-cleaved fodrin (not shown), calpains may play a role in the model because caspases and calpains are frequently coactivated and have many common substrates (Wang, 2000). Although caspase cleavage of NMDA receptor subunits has not been shown directly, it is still possible that activated caspases can cleave a component of membrano-cytoskeletal complex essential for anchoring the NMDAR to the hebbosome. One candidate could be fodrin (also known as α-spectrin), shown here to be cleaved by caspases and lost after n-3 PFA dietary restriction. Fodrin is known to regulate NMDA receptors, and a loss of function of fodrin after caspase cleavage might underlie NMDA subunit loss shown here (Siman *et al*., 1985; Janicke *et al*., 1998). Another caspase substrate that could release the NMDA receptor subunit after cleavage is actin (Calon *et al*., 2004). In such a scheme, actin cleavage by caspase could similarly release NMDA subunit from the membrane to be degraded. In summary, the involvement of caspase activation with NMDA receptor complex depletion may be mediated by direct interaction of caspase with subunits of the receptor or with its anchor.

Besides caspases regulation, n-3 PFA may have prevented NMDA receptor loss through a consequence of its protective effect against Aβ-pathology, which was reported recently in Tg2576 mice (Lim *et al*., 2005). Indeed, experimental evidence suggest that brain n-3 PFA depletion aggravates Aβ-driven oxidative stress (Hashimoto *et al*., 2002; Calon *et al*., 2004), which interferes with glutamatergic neurotransmission (Mark *et al*., 1997; Butterfield & Pocernich, 2003). Therefore, safflower oil-induced n-3 deficiency might have altered NMDA receptor content by potentiating oxidative damage in the cortex of Tg2576 animals. Because levels of p85α, NR2B and other markers were only partially restored by DHA repletion, it is likely that the transgene/high n-6 PFA diet had independent deleterious effects, possibly related to increased oxidative damage. Such a scenario is consistent with increased oxidative stress, Aβ pathology and loss of glutamatergic function observed in AD brain.

DHA is the most important n-3 PFA in the brain; it accounts for approximately 15% of total fatty acids in grey matter where it is enriched at synapses (Breckenridge *et al*., 1972; Salem *et al*., 2001). While EPA may have significant CNS effects, DHA supplementation in dietary n-3 PFA restricted mice re-established both brain EPA and DHA levels higher than in control diet fed animals. DHA addition importantly decreased long chain n-6 PFA such as ARA, but did not reverse the linoleic acid (LA; 18 : 2n-6) increase seen in the n-3 PFA restricted animal (Table 2). DHA fully protected against the effect of safflower oil-induced n-3 PFA deficiency on CaMKII loss and Bad expression and was partially effective in maintaining NMDA receptor content and the index of caspase activation, suggesting that DHA depletion *per se* was an important factor. Other characteristics of the soybean-based control diet, such as high linolenic acid or low linoleic acid levels, may also counteract Aβ-driven caspase activation and protect from NMDA receptor loss in Tg2576 on PMI5015 laboratory chow. DHA-mediated reduction of the levels of Bad such as we report here has been observed *in vitro* as a direct effect of neuroprotective enzymatic metabolites of DHA (Mukherjee *et al*., 2004).

Previous data suggest that n-3 PFA regulate insulin-like pathway signalling dependent on the phosphatidylinositol 3 (PI3)-kinase/pAKT cellular pathway *in vitro* (Akbar & Kim, 2002) and *in vivo* (Stein & Johnson, 2002; Calon *et al*., 2004; Zhao *et al*., 2004). Interestingly, a decrease in NR2A and NR2B receptors was reported in diabetic rats and corrected after insulin or gliclazide treatments (Delibas *et al*., 2004). Like Fyn kinase, PI3-kinase is known to directly phosphorylate NR2 subunits and NR2B directly binds and coimmunoprecipitates with p85α PI3-kinase after ischemia (Takagi *et al*., 2003). This direct interaction may help explain the especially tight ($r^2 = 0.86$) correlation observed here between p85α and NR2B (Fig. 6). Therefore, it is possible that high safflower oil/n-3 PFA depletion deactivates the PI3-kinase pathway with direct effects on NR2B and indirect effects mediated by caspases both leading to decreased NMDA receptor subunits.

One interpretation of our data is that the combination of *APPswe* overexpression and low n-3 PFA diet might have generated an animal

model closer to AD. Firstly, the loss of NR2A and NR2B seen here in the cortex Tg2576 animals fed low n-3 PFA diet may indeed better replicate the reduction of NMDA receptor subunits reported in most post-mortem studies in brain of AD patients using different methodologies (Greenamyre *et al*., 1987; Sze *et al*., 2001; Mishizen-Eberz *et al*., 2004) but not all studies (Geddes *et al*., 1986; Monaghan *et al*., 1987). This alteration in postsynaptic NMDA/PSD receptor complex may reflect a change in dendrite function relevant to the cognitive dysfunction afflicting AD patients (Cull-Candy *et al*., 2001; Newcomer & Krystal, 2001). Secondly, in terms of low n-3 PFA content, the safflower-based diet used here is not that different than common diets widespread in North America. The n-6/n-3 ratio of most laboratory rodent chow stands between 6 : 1 and 10 : 1. In humans, even though ratios between 1 : 1 and 4 : 1 are recommended (Kris-Etherton et al., 2000; Simopoulos, 2002), it is estimated that the average n-6/n-3 ratio of the North American diet ranges between 10 : 1 and 20 : 1 (much less desirable than rodent chow). The present n-6/n-3 ratio of over 80 : 1 of the low n-3 PFA might be closer to what many American AD patients actually eat compared to standard rodent chow. Thirdly, caspase activation (as indexed here with measurement of CCFBDP in the brain of Tg2576 mice fed low n-3 PFA diet) has been shown also in AD brain. Indeed, increased 120-kDa and 150-kDa CCFBDP in the cytosolic fraction along with decreased 240-kDa fodrin in the particulate fraction has been related to synapse loss in postmortem tissue from AD patients (Masliah *et al*., 1990). Using an antibody specific for the N-terminal peptide sequence of caspase-cleaved fodrin, Rohn *et al*. (2001) have also shown increased 120-kDa CCFBDP in AD brain sections. Other evidence of caspase activation in AD brain has been gathered from various groups (Yang *et al*., 1998; Chan *et al*., 1999; Cole *et al*., 1999; Gervais *et al*., 1999; Rossiter *et al*., 2000; Yuan & Yankner, 2000; Guo *et al*., 2004). Fourthly, the protein Bad shown here to be increased in Tg2576 mice fed low n-3 PFA diet is also elevated more than two-fold in AD (Kitamura *et al*., 1998).

Conclusion

Our results show that safflower oil-induced n-3 PFA deficiency greatly potentiates cortical caspase activation and decreases NMDA receptor complex in the brain of the Tg2576 mouse model of AD. These two effects were amplified by *APPswe* overexpression providing new insights on how dietary n-3 PFA might alter AD risk. Furthermore, our data suggest new mechanisms by which dietary n-3 PFA and Aβ pathology are linked to cognitive deficits in AD.

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Abbreviations

Aβ, β-amyloid peptides; AD, Alzheimer's disease; *APPswe*, human mutant amyloid precursor protein; ARA, arachidonic acid (20 : 4n-6); BDP, breakdown products; CaMKII, Ca(2+)/calmodulin-dependent protein kinase; CCFBDP, caspase-cleaved fodrin breakdown products; DHA, docosahexaenoic acid (22 : 6n-3); DPA, docosapentaenoic acid (22 : 5n-6); drebrin, developmentally regulated brain protein; DTA, docosatetraenoic acid (22 : 4-n-6); EPA, eicosapentaenoic acid (20 : 5n-3); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; LNA, α-linolenic acid (18 : 3n-3); NMDA, *N*-methyl-D-asparate; PI3, phosphatidylinositol 3; PSD, postsynaptic density; PFA, n-3 polyunsaturated fatty acids; QPCR, quantitative real-time RT-PCR; SNAP-25, synaptosomal-associated protein 25; TBS, Tris-buffered saline; Tg2576, transgenic mouse line overexpressing the human AD gene *APPswe*.

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