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Authors
Igarashi, Miki
Santos, Rommel A
Cohen-Cory, Susana

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Impact of Maternal n-3 Polyunsaturated Fatty Acid Deficiency on Dendritic Arbor Morphology and Connectivity of Developing *Xenopus laevis* Central Neurons *In Vivo*

Miki Igarashi, Rommel A. Santos, and Susana Cohen-Cory
Department of Neurobiology and Behavior, University of California Irvine, Irvine, California 92697

Docosahexaenoic acid (DHA, 22:6n-3) is an essential component of the nervous system, and maternal n-3 polyunsaturated fatty acids (PUFAs) are an important source for brain development. Here, the impact of DHA on developing central neurons was examined using an accessible *in vivo* model. *Xenopus laevis* embryos from adult female frogs fed n-3 PUFA-adequate or deficient diets were analyzed every 10 weeks for up to 60 weeks, when frogs were then switched to a fish oil-supplemented diet. Lipid analysis showed that DHA was significantly reduced both in oocytes and tadpoles 40 weeks after deprivation, and brain DHA was reduced by 57% at 60 weeks. *In vivo* imaging of single optic tectal neurons coexpressing tdTomato and PSD-95-GFP revealed that neurons were morphologically simpler in tadpoles from frogs fed the deficient diet compared with the adequate diet. Tectal neurons had significantly fewer dendrite branches and shorter dendritic arbor over a 48 h imaging period. Postsynaptic cluster number and density were lower in neurons deprived of n-3 PUFA. Moreover, changes in neuronal morphology correlated with a 40% decrease in the levels of BDNF mRNA and mature protein in the brain, but not in TrkB. Importantly, switching to a fish oil-supplemented diet induced a recovery in DHA content in the frog embryos within 20 weeks and diminished the deprivation effects observed on tectal neurons of Stage 45 tadpoles. Consequently, our results indicate that DHA impacts dendrite maturation and synaptic connectivity in the developing brain, and it may be involved in neurotrophic support by BDNF.

Key words: **dendrite; DHA; in vivo imaging; maternal diet; tectal neuron; *Xenopus laevis***

Introduction

Docosahexaenoic acid (DHA, 22:6n-3) is an n-3 polyunsaturated fatty acid (n-3 PUFA) and an essential component of the CNS of humans as well as most animal species (Salem et al., 2001). During embryonic development, DHA accumulates in the CNS, and it can be supplied from the mother through the diet together with its precursors, α-linoleic acid (ALA, 18:3n-3) and eicosapentaenoic acid (EPA, 20:5n-3) (Gil-Sánchez et al., 2010). Further, DHA and its metabolites are known to help in maintaining proper neuronal function (Gómez-Pinilla, 2008; Wu et al., 2008; Bazan et al., 2011). Epidemiological studies have suggested that an imbalance in n-3 PUFA levels is associated with childhood cognitive disorders, such as attention-deflect hyperactivity disorder and autism (Schuchardt et al., 2010). In addition, clinical trials have reported that DHA supplementation improves later cognitive development in infants (Willatts et al., 1998a, b; Birch et al., 2000). Therefore, n-3 PUFA levels are thought to be critical for neuronal development in early embryonic and postnatal life.

The importance of n-3 PUFA in neuronal development has been demonstrated by analyzing effects on adult animal models or by using neurons isolated from their normal environment. Findings indicate that n-3 PUFA, especially DHA, can induce biological and morphological and synaptic changes, and this may lead to behavioral and functional changes (Yoshida et al., 1997; Ahmad et al., 2002a, 2002b; Calderon and Kim, 2004; Igarashi et al., 2007b; Cansev et al., 2009; Cao et al., 2009). Despite this knowledge, however, the function of n-3 PUFA during early neuronal development in the intact brain remained to be elucidated.

Early, dynamic events that guide neural circuit formation have been studied and visualized *in vivo* using imaging techniques in accessible, nonmammalian animal models, such as *Xenopus* (Cline, 2001; Haas et al., 2001; Cohen-Cory and Lom, 2004). This species seldom has been used in nutritional studies even though the maternal diet can modify nutrients in yolk that are transferred to the progeny within the oocyte (Fort et al., 1999), and development can be easily studied from the time of fertilization to well after functional neural circuits form. Here, we combined nutritional studies with *in vivo* imaging techniques in

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Correspondence should be addressed to Dr. Susana Cohen-Cory, Department of Neurobiology and Behavior, University of California Irvine, 2205 McGaugh Hall, Irvine, CA 92697-4550. E-mail: scohenco@uci.edu.

M. Igarashi's current address: Department of Anatomy and Neurobiology, University of California Irvine, 3216 Gillespie Neuroscience Research Facility, Irvine, CA 92697.

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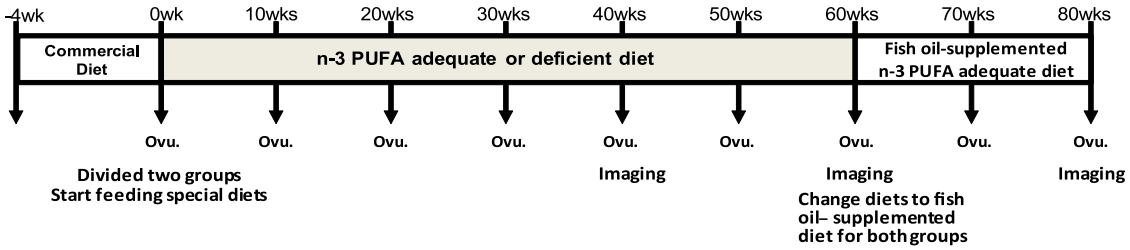

Figure 1. Experimental plan. Detailed experimental plan is stated in Materials and Methods. Briefly, after 4 week acclimatization, frogs were divided into two groups, n-3 PUFA-adequate or n-3 PUFA-deficient diets were fed to frogs for 60 weeks, and then both groups were fed fish oil-supplemented n-3 PUFA-adequate diet for an additional 20 weeks up to week 80. Ovulation (Ovu.) was induced every 10 weeks after the first ovulation, and neuron imaging was performed at 40, 60, and 80 weeks.

Xenopus laevis to understand effects of n-3 PUFA deficiency on neuronal development. Semipurified diets with modified n-3 PUFA content were used to feed adult female frogs for 80 weeks to change n-3 PUFA levels in their progeny and therefore in the embryonic brain. Changes in n-3 PUFA levels were followed every 10 weeks in oocytes and tadpoles obtained from frogs fed the special diets. The dynamic behavior of tectal neurons in Stage 45 tadpoles was followed over a 48 h imaging period to correlate changes in morphological and synaptic differentiation of neurons with changes in DHA levels caused by the n-3 PUFA deficiency. Our results revealed that maternal n-3 PUFA impacts the branching and synaptic connectivity of neurons in the developing brain and that these changes correlate with a decrease in BDNF in the brain.

Materials and Methods

Experimental design and adult frog feeding schedule. Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine (Animal Welfare Assurance Number A3416-01).

The study design is shown in Figure 1. Twelve female frogs were obtained from Nasco and acclimatized for 1 month in a recirculating water housing system. Frogs were fed a commercial diet (Advanced protocol *Xenopus* diet, PMI Nutrition International, LabDiet) during the acclimatization period. After acclimatization, frogs were induced to ovulate (first ovulation, week 0) and immediately after frogs were divided into two groups (6 frogs/group). Each group of adult female frogs was then fed either an n-3 PUFA-adequate or n-3 PUFA-deficient diet three times per week. Sixty weeks after the change in diet, both groups were switched to a fish oil-supplemented n-3 PUFA-adequate diet for 20 additional weeks (up to 80 weeks). The health condition of individual frogs was monitored daily and recorded three times per week at the time of feeding, and body weight was measured every 10 weeks. After the first ovulation and switch in diet, frogs were induced to ovulate every 10 weeks and *in vitro* fertilization of eggs was performed as described below. Fertilized embryos and swimming tadpoles from each spawning were used for analysis and imaging as described below.

Diets. A total of four diets were used in this study. A commercial *Xenopus* diet (Advanced protocol *Xenopus* diet) was fed during the acclimatization period. This diet was designed for amphibians and carnivorous reptiles, contains 57.4% protein, 15.8% fat, and 3.0% carbohydrate, and was prepared following the manufacturer's instructions. Three special diets were used during the experimental period. In rodents, semipurified diets with modified n-3 PUFA content have been widely used to alter n-3 PUFA levels, including DHA in the organism (Moriguchi et al., 2001; Igarashi et al., 2007a). Semipurified diets formulated for rodent research (Reeves et al., 1993) have been successfully used to study the impact of heavy metal deficiency on early developmental process in *X. laevis* (Fort et al., 1999, 2000, 2002). The dietary compositions of the n-3 PUFA-adequate and n-3 PUFA-deficient diets as well as of a fish oil-supplemented n-3 PUFA-adequate diet are shown in Table 1. These diets were created based on the AIN-93G formulation (Reeves et al., 1993) with modifications. The diets contained 20% protein, 10% fat, and 60% carbohydrates, and some dietary oils were mixed to modify the fatty acid composition in each diet. The n-3 PUFA-adequate diet contained hydrogenated soybean oil, safflower oil, and flaxseed oil. The n-3 PUFA-deficient diet contained hydrogenated soybean oil and safflower oil. The fish oil-supplemented n-3 PUFA-adequate diet contained hydrogenated soybean oil, safflower oil, flaxseed oil, and tuna oil (tuna oil-5% EPA and 25% DHA). These diets were prepared by Dyets. The tuna oil was obtained from Jedwards International. The diets were kept at −20°C until feeding and were mixed with 1.5% agar solution (1:1 w/v) to prepare a gel food mixture before feeding.

Table 1. Composition of special diets[a]

Components	n-3 PUFA-adequate diet	n-3 PUFA-deficient diet (g/100 g diet)	Fish oil-supplemented n-3 PUFA-adequate diet
Protein (20%)			
Casein	20	20	20
Carbohydrate (60%)			
Cornstarch	36.7486	36.7486	36.7486
Sucrose	10	10	10
Maltose dextrin	13.2	13.2	13.2
Fat (10%)			
Hydrogenated soybean oil	6.0	6.62	4.90
Safflower oil	3.23	3.38	3.13
Flaxseed oil	0.77	0	0.77
Tuna oil	0	0	1.2
Additives (10%)			
Cellulose	5.0	5.0	5.0
Mineral mix	3.5	3.5	3.5
Vitamin mix	1.0	1.0	1.0
L-Cystine	0.3	0.3	0.3
Choline bitartrate	0.25	0.25	0.25
TBHQ	0.0014	0.0014	0.0014

[a]TBHQ, Tertiary-butylhydroquinone (antioxidant).

Fatty acid contents (μmol/g) in the diets used in this study are shown in Table 2. Total lipids extracted from 20 mg of each diet formulation ($n = 4$) were transmethylated, and the resulting fatty acid methyl esters were analyzed by gas chromatography as described below. The n-3 PUFA-adequate diet contained ALA at 12.5 μmol/g diet, whereas the n-3 PUFA-deficient diet contained ALA at 0.2 μmol/g. The fish oil-supplemented n-3 PUFA-adequate diet had ALA at 12.3 μmol/g, EPA at 2.0 μmol/g, docosapentaenoic acid (DPA, 22:5n-3) at 0.3 μmol/g, and DHA at 5.1 μmol/g. The commercial *Xenopus* diet contained ALA at 3.4 μmol/g, EPA at 21.7 μmol/g, DPA at 3.8 μmol/g, and DHA at 23.3 μmol/g. The commercial diet was rich in longer chain n-3 PUFA compared with the fish oil-supplemented n-3 PUFA-adequate diet.

In vitro *fertilization and embryo rearing.* Adult female *X. laevis* frogs were induced to ovulate every 10 weeks by priming with human chorionic gonadotropin (Chorulon, Intervet). Embryos were obtained by *in vitro* fertilization of oocytes with sperm obtained from male frogs (from Nasco) fed the commercial *Xenopus diet*. Embryos were raised in rearing solution (60 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$ 10 mM HEPES, pH 7.4, and 40 mg/L gentamycin) and were

Table 2. Fatty acid concentrations in diets[a]

Fatty acid	Commercial diet	n-3 PUFA-adequate diet (μmol/g food)	n-3 PUFA-deficient diet	Fish oil-supplemented n-3 PUFA-adequate diet
14:0	23.6 ± 0.5	0.8 ± 0.0	0.7 ± 0.0	2.0 ± 0.1
14:1n-5	2.4 ± 0.00.1	0.2 ± 0.0	0.2 ± 0.0	0.7 ± 0.1
16:0	65.7 ± 1.8	28.4 ± 3.6	27.9 ± 1.4	30.6 ± 2.8
16:1n-7	24.3 ± 1.6	0.2 ± 0.0	0.2 ± 0.0	1.5 ± 0.2
18:0	15.8 ± 0.4	137.9 ± 25.4	142.4 ± 9.9	113.8 ± 9.2
18:1n-9	29.3 ± 0.7	28.0 ± 0.5	22.7 ± 0.7	24.8 ± 1.9
18:1n-7	8.1 ± 0.2	ND	ND	1.3 ± 0.1
18:2n-6	11.8 ± 0.3	85.4 ± 1.3	80.8 ± 2.5	52.5 ± 4.4
18:3n-6	0.7 ± 0.0	1.5 ± 0.2	1.3 ± 0.1	1.4 ± 0.1
18:3n-3	3.4 ± 0.1	12.5 ± 0.4	0.2 ± 0.0	12.3 ± 2.2
20:3n-6	0.4 ± 0.0	ND	ND	0.9 ± 0.1
20:4n-6	2.8 ± 0.1	ND	ND	0.6 ± 0.0
20:5n-3	21.7 ± 0.5	ND	ND	2.0 ± 0.1
22:4n-6	2.0 ± 0.1	ND	ND	0.4 ± 0.0
22:5n-6	1.3 ± 0.0	ND	ND	0.4 ± 0.0
22:5n-3	3.8 ± 0.1	ND	ND	0.3 ± 0.0
22:6n-3	23.3 ± 0.6	ND	ND	5.1 ± 0.4
Total	240.6 ± 6.0	294.9 ± 28.7	276.4 ± 13.7	250.8 ± 16.6
Saturated	105.1 ± 2.7	167.1 ± 29.1	171.0 ± 11.3	146.4 ± 10.4
Monounsaturated	64.1 ± 1.6	28.4 ± 0.5	23.0 ± 0.7	28.3 ± 2.1
n-6 PUFA	19.1 ± 0.5	86.9 ± 1.3	82.1 ± 2.6	56.2 ± 4.6
n-3 PUFA	52.3 ± 1.2	12.5 ± 0.4	0.2 ± 0.0	19.8 ± 1.9
n-6/n-3	0.37	6.97	364	2.8

[a]Data are mean ± SEM ($n = 4$). ND, Not detected.

anesthetized during experimental manipulations with 0.05% tricane methanesulfonate (Finquel; Argent Laboratories). Staging was done according to Nieuwkoop and Faber (1994) at Hubrecht-Laboratorium (Embryologisch Instituut). For the assessment of oocyte quality and embryonic development, oocytes (>200) were laid into a Petri dish and total oocyte numbers were counted, including the number of necrotic and abnormally shaped eggs before fertilization. The proportion of fertilized oocytes (Stage 2, animal side up) per total number of laid eggs, as well as embryo survival at Stage 3 (4 cell stage), Stages 8 and 12 (late gastrula), Stage 22 (late neurula), and Stage 45 (swimming tadpole) were counted for each spawning from each frog. The proportion of embryos with abnormal or arrested development at these stages was also determined.

In vivo *labeling of tectal neurons and imaging.* Neuronal precursor cells in brain primordium of Stage 20–22, anesthetized *Xenopus* embryos were cotransfected with tdTomato (Clontech Laboratories) and PSD95-GFP (a gift from Dr D. Bredt, Janssen Pharmaceutical Company) expression plasmids (tdTomato: PSD95-GFP = 0.5:1.5) in DOTAP (total 1 μg plasmid/μl + 3 μl DOTAP) (Roche Applied Science) to colabel tectal neurons. Embryos were raised until Stage 45 in rearing solution plus 0.001% phenylthiocarbamide to prevent melanocyte pigmentation. Only tadpoles with single tectal neurons labeled with tdTomato showing specific, punctate PSD95-GFP labeling in their terminals were selected for imaging. Thin optical sections (1.5 μm) through the entire extent of dendritic arbor in the embryo were collected with Nikon PCM2000 laser-scanning confocal microscope equipped with Argon and HeNe lasers as described previously (Alsina et al., 2001; Sanchez et al., 2006). Dendritic arbor morphology and dynamics were followed in the double-labeled neurons at 0, 24, and 48 h by confocal microscopy, and embryos were maintained at 16°C in a 12 h/12 h light/dark cycle between imaging. The effects of dietary changes on dendritic arbor and postsynaptic site dynamics were examined by comparing 3D reconstructions of individual tectal neurons in tadpoles from frogs fed the individual diets. All analyses were performed from raw confocal images with no postacquisition manipulation or thresholding with the aid of the MetaMorph software (Molecular Devices). Total arbor length was measured from binarized images of the digitally reconstructed dendritic arbors and was converted to micrometers using MetaMorph. The number of total, new, and stable branches for each observation period were measured. The number of first-, second-, third-, and fourth-order branches in each individual dendritic arbor was also counted, and Dendritic Complexity Index (DCI) was calculated as described previously (Marshak et al., 2007). The distribution of PSD95-GFP puncta in the dendritic arbor was characterized by analyzing pixel-by-pixel overlaps of individual optical sections obtained at the two wavelengths. PSD95-GFP puncta numbers were manually counted in the entire neuron, and puncta density was calculated by dividing total puncta number by total dendritic arbor length for each neuron. The number of PSD95-GFP postsynaptic specializations per branch and the location of each postsynaptic cluster along the dendritic arbor was also determined.

Stage 45 tadpoles from each spawning from each adult frog in a feeding group were used for the morphological analysis of neurons, with one neuron analyzed per tadpole and at least 2–7 tadpoles analyzed per frog ($n = 6$ frogs for n-3 PUFA-adequate group and $n = 5$ frogs for n-3 PUFA-deficient group). To determine effects on morphological parameters only, 7 neurons from the adequate group and 11 neurons from n-3 PUFA-deficient group were first analyzed at 40 weeks. A total of 24 neurons from the adequate group and 25 neurons from the n-3 PUFA-deficient group were then used to analyze morphological and synaptic parameters at 60 weeks. At 80 weeks, 19 neurons in the adequate group and 13 neurons in the deficient group were used to analyze morphological and synaptic parameters following the second change in diet to the fish oil-supplemented diets.

Fatty acid analysis. Fatty acid contents were analyzed in the diets, oocytes, whole tadpoles, and tadpole brains (Stage 45). Briefly, an adequate amount of sample was homogenized with 0.5 M KCl, and then total lipids were extracted with chloroform/methanol (2:1, v/v) (Folch et al., 1957). Before extraction, appropriate quantities of internal standard (di-17:0 ChoGpl, Avanti Polar Lipids) were added to the samples. Total lipid extracts were transmethylated with 1% (v/v) H_2SO_4 in anhydrous methanol for 3 h at 70°C, and then fatty acid methyl esters were analyzed using a gas chromatography (6890N, Agilent Technologies) equipped with an SP-2330-fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (Supelco) and a flame ionization detector (Igarashi et al., 2007b). Fatty acid amounts were calculated by proportional comparison of peak areas of endogenous fatty acids to the area of the 17:0 as internal standard.

Total protein analysis. Oocytes (Stage 1, before fertilization) and whole tadpoles at Stage 45 were homogenized in PBS, and small portions of homogenates were used to analyze protein using Bio-Rad protein assay kit (Bio-Rad). Protein amounts were calculated based on standard curve prepared with BSA solution (Thermo Fisher Scientific).

Total lipid analysis. Oocytes (Stage 1, before fertilization) and whole tadpoles at Stage 45 were homogenized in 0.5 M KCl, and total lipids were extracted with chloroform/methanol (2:1, v/v) as above (Folch et al., 1957). Extracts were dried with nitrogen stream, and residues were weighted as total lipids.

Western blot analysis. Brain samples were homogenized in RIPA buffer containing protease inhibitors (BD Biosciences), and the samples were centrifuged at 10,000 rpm for 30 min at 4°C. Supernatants were used for Western blotting, and protein concentrations were determined as described above. Up to 20 μg of protein was loaded on SDS polyacrylamide gel, and then proteins were electrophoretically transferred to a PVDF membrane (EMD Millipore). The blots were preincubated in blocking buffer followed by incubation with specific primary antibodies: BDNF (R&D Systems), PSD-95 (EMD Millipore), TrkB (*Xenopus*-custom made, Bethyl Laboratories), and β-actin (GeneTex) overnight followed by a secondary antibody conjugated with HRP (Thermo Fisher Scientific). Protein detection was performed with Chemiluminescense Reagent Puls (PerkinElmer), and the luminescence was exposed on x-ray film (Kodak). Optical densities were analyzed using ImageJ, and the density of experimental proteins was normalized to β-actin expression.

Quantitative RT-PCR. Total RNA was isolated using TRIzol (Invitrogen) from brains of Stage 45 tadpoles and was further purified using a RNeasy mini kit (QIAGEN). First-strand complementary DNA was prepared from 2 μg purified total RNA using High Capacity cDNA Reverse Transcription Kit (Invitrogen). mRNA levels of total BDNF and GAPDH were measured with iQTM SYBR Green Supermix (Bio-Rad) using MX3000OTM Real-Time PCR systems (Stratagene, Agilent Technolo-

Table 3. Oocyte quality and embryo development at 0, 60, and 80 weeks[a]

			60 weeks		80 weeks	
Stage[b]	Description	0 weeks ($n = 3$)	Adequate ($n = 4$)	Deficient ($n = 3$)	Adequate ($n = 4$)	Deficient ($n = 3$)
1	Normal shape, prefertilization	99.6 ± 0.1	99.9 ± 0.1	99.4 ± 0.3	100.0 ± 0.0	99.9 ± 0.1
1	Successful fertilization	97.5 ± 1.0	80.4 ± 7.4	73.2 ± 13.2	83.9 ± 7.4	75.0 ± 4.9
3	Embryo survival	92.0 ± 1.8	69.1 ± 12.7	66.2 ± 13.7	75.1 ± 9.5	63.4 ± 7.6
8	Embryo survival	89.7 ± 1.0	68.8 ± 12.7	64.0 ± 14.7	71.8 ± 9.0	61.4 ± 6.6
12	Embryo survival	87.8 ± 1.5	67.2 ± 13.1	63.4 ± 14.4	69.7 ± 8.7	59.4 ± 5.7
22	Embryo survival	85.4 ± 2.3	67.1 ± 13.1	62.5 ± 14.4	67.7 ± 8.1	57.8 ± 5.0
45	Tadpole survival with normal morphology	82.8 ± 2.7	63.3 ± 11.5	62.5 ± 14.1	61.3 ± 6.5	53.8 ± 4.2
	Tadpole survival with malformations[c]	1.7 ± 0.5	2.2 ± 0.8	1.2 ± 0.74	3.7 ± 1.2	1.8 ± 0.9

[a]Data are mean ± SEM (percentage of total oocyte numbers laid at Stage 1). There was no significant difference among the five groups at any stage. Analysis was performed by one-way ANOVA.

[b]Representative embryonic stages were determined by the method of Nieuwkoop and Faber (1994).

[c]Gut malformation, edema, and tailfin flexure were mostly found.

Table 4. Protein and total lipid contents in oocytes and Stage 45 tadpoles from frogs fed special diets at 0, 60, and 80 weeks[a]

		60 weeks		80 weeks	
Parameters	0 week	Adequate	Deficient	Adequate	Deficient
Protein					
Oocytes (mg/oocyte)	0.18 ± 0.01 ($n = 4$)	0.18 ± 0.13 ($n = 6$)	0.17 ± 0.01 ($n = 5$)	0.17 ± 0.01 ($n = 6$)	0.19 ± 0.01 ($n = 4$)
Tadpoles (mg/embryo)	0.11 ± 0.01 ($n = 6$)	0.12 ± 0.01 ($n = 6$)	0.13 ± 0.00 ($n = 5$)	0.12 ± 0.00 ($n = 6$)	0.12 ± 0.01 ($n = 4$)
Total lipids					
Oocytes (mg/oocyte)	0.30 ± 0.04 ($n = 4$)	0.34 ± 0.04 ($n = 5$)	0.31 ± 0.03 ($n = 3$)	0.31 ± 0.04 ($n = 5$)	0.34 ± 0.04 ($n = 4$)
Tadpoles (mg/embryo)	0.36 ± 0.07 ($n = 4$)	0.36 ± 0.04 ($n = 6$)	0.33 ± 0.03 ($n = 4$)	0.34 ± 0.03 ($n = 5$)	0.36 ± 0.03 ($n = 4$)

[a]Data are mean ± SEM. There was no significant difference for protein and total lipids among the five groups for either oocytes or tadpoles. Analysis was performed by one-way ANOVA.

gies). Specific primers used were as follows: BDNF, 5′-GTGGAGAGCTGAGTGTGTGTGAC-3′ (sense) and 5′-GAAAGCCCGCACGTAAGACTGAG-3′ (antisense); GAPDH, 5′-TTAAGACTGCATCAGAGGGCCCAA-3′ (sense) and 5′-AAGATCCACAACACGGTGGCTGTA-3′ (antisense). Data were analyzed with comparative cycle threshold. Data were expressed as the level of the target gene in animals fed the deficient diet, normalized to the endogenous control (GAPDH), and relative to the level in animals fed the adequate diet.

Immunohistochemistry. Stage 45 tadpoles were killed with tricaine methanesulfonate and fixed in 4% PFA in phosphate buffer, pH 7.5, overnight, cryoprotected in 30% sucrose, and embedded in OCT compound. Thirty-micrometer cryostat sections were obtained and immunostained with an anti-neurofilament-associated protein antibody (3A10, 1:2000 dilution) and an Alexa-488 anti-mouse secondary antibody (1:500 dilution; Invitrogen). The 3A10 antibody developed by Dr. T.M. Jessell and Dr. J. Dodd was used to immunostain presynaptic axon terminals and was obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). Sections were counterstained with DAPI and imaged with a Nikon E800 epifluorescent microscope equipped with a high-sensitivity/high-resolution Zyla SCMOS camera (Andor).

Statistical analysis. Statistical analysis was performed using SigmaPlot (version 11.0, Systat Software). Data are expressed as the mean ± SEM. Unpaired *t* test was used to compare two groups. One-way and two-way ANOVAs following Tukey's and Dunett's *post hoc* tests were applied for multiple comparison. $p \leq 0.05$ was used as a cutoff for statistical significance.

Results

Health condition of adult frogs over 80 week feeding

Special diets formulated for rodents were fed to female frogs over an 80 week period to modify fatty acid composition in oocytes and tadpoles. Adult female frogs were carefully monitored over the 80 weeks to observe whether the change in diet could cause any change in health. No health problems were observed in frogs fed the special diets compared with frogs fed a frog-formulated commercial diet at any point over the 80 week study. Moreover, body weight remained unchanged in frogs in the n-3 PUFA-adequate and -deficient diet groups over the 80 week feeding period compared with their body weight at week 0 ($p > 0.05$ for comparison between adequate and deficient groups at same time point by one-way ANOVA with Tukey's test), and from the average body weight of frogs fed frog-formulated commercial food. For example, the average body weight at 60 weeks was in 137 ± 3 g for the n-3 PUFA-adequate diet group ($n = 6$ frogs) and 158 ± 9 g in n-3 PUFA-deficient group ($n = 5$ frogs), respectively.

Oocyte quality and embryo development over 80 week feeding

We examined whether the maternal n-3 PUFA levels affect oocyte quality and/or embryo development from spawnings obtained every 10 weeks. Analysis of unfertilized oocytes (Stage 1), fertilized embryos (Stage 2, animal side up), 4 cell Stage (Stage 3), mid-blastula (Stage 8), gastrula (Stage 12), and neurula-tailbud (Stage 22) embryos, as well as swimming tadpoles (Stage 45) was performed. Assessment data at 0, 60, and 80 weeks is shown in Table 3. There was no significant difference in oocyte necrosis, fertilization rate, abnormal gastrulation, and embryo viability (Stages 8, 12, 22, and 45) among the groups. Furthermore, total protein and total lipids were unchanged (Table 4). Mean values for total protein and total lipids in embryos and tadpoles were 0.17–0.19 mg in oocyte and 0.11–0.13 mg in Stage 45 tadpoles, respectively. These observations therefore indicate that a change in diet does not impact *Xenopus* oocyte quality and embryonic development whether purified diets are formulated to include or exclude n-3 PUFAs, or are supplemented with fish oil.

DHA amount is altered in oocytes by n-3 PUFA-deficient diet

Because commercial frog diets are normally supplemented with n-3 PUFAs, we determined the time required to change maternal n-3 PUFA levels by the formulated diets by comparing embryonic fatty acid levels between n-3 PUFA-deficient diet and the n-3 PUFA-adequate diet (adequate diet supplemented with oils that

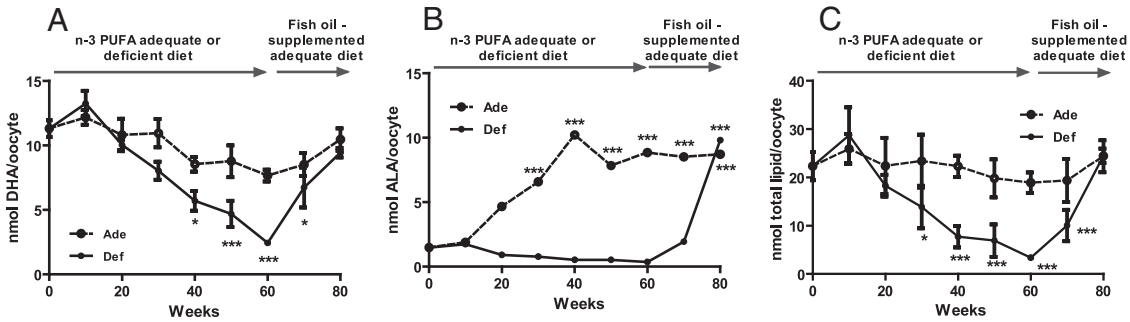

Figure 2. n-3 PUFA contents in oocytes from female frogs fed special diets over 80 weeks. DHA (***A***), ALA (***B***), and total n-3 PUFA (***C***) contents in oocytes from frogs fed special diets over the 80 week study. Data are mean ± SEM. *$p < 0.05$, compared with week 0 (before change in diet) (one-way ANOVA followed by Dunnett' multiple comparison test). ***$p < 0.001$, compared with week 0 (before change in diet) (one-way ANOVA followed by Dunnett' multiple comparison test). $n = 4–6$ samples per time point. Ade, Adequate diet; Def, deficient diet.

provide ALA as n-3 PUFA source only; see Materials and Methods). The amount of n-3 PUFA in oocytes was therefore measured every 10 weeks over the 80 week feeding period to determine the impact and timing of n-3 PUFA deprivation on embryonic DHA, ALA, and total n-3 PUFA levels (Fig. 2*A–C*). As expected, ALA content increased over time in oocytes from frogs fed the n-3 PUFA-adequate diet, with ALA reaching plateau levels 40 weeks after the change in diet (Fig. 2*B*). Although slightly lower at 40 weeks, total n-3 PUFA and DHA contents were not significantly different in oocytes from frogs fed the n-3 PUFA-adequate diet at any time point following the switch from commercial diet, even after further supplementing the n-3 PUFA-adequate diet with fish oil at week 60 (Fig. 2 *A*, *C*; week 0; control). In contrast, total n-3 PUFA and DHA were reduced over time in the n-3 PUFA-deficient group, reaching significance 40 weeks after the change in diet. DHA content was significantly reduced in oocytes from frogs fed the deficient diet at 40 weeks ($p < 0.05$), 50 weeks ($p < 0.001$), and 60 weeks ($p < 0.001$) compared with oocytes obtained from these same frogs at week 0 (Fig. 2*A*). These results indicate that, within 40 weeks after the initial switch in diet, the dietary n-3 PUFA condition had an effect on the maternal fatty acid contents, thus impacting embryonic DHA content within the same time frame.

To determine whether the DHA deficiency caused by the n-3 PUFA-deficient diet could be reversed, we switch to a fish oil-supplemented n-3 PUFA-adequate diet that was fed to the frogs 60 weeks after feeding the deficient diet. Fish oil supplementation rapidly reversed and significantly increased total n-3 PUFA levels and DHA content in oocytes obtained from frogs fed the n3-PUFA-deficient diet within 10 weeks when again changing the diet for an additional 20 weeks (until week 80) (Fig. 2*A*). In contrast, supplementing the n-3 PUFA-adequate diet with fish oil for 20 weeks did not further increase n-3 PUFA or DHA contents in oocytes from frogs fed the adequate diet for the initial 60 weeks. Therefore, the DHA deprivation in oocytes by the maternal diet can be reversed with DHA supplementation. Moreover, having ALA as the only n-3 PUFA source in the maternal diet was sufficient to provide the required fatty acid precursors to be converted to DHA in the maternal body and to accumulate in the yolk.

n-3 PUFA-deficient diet reduces all of n-3 PUFAs in tadpole brains at 60 weeks

To further determine the effects of the maternal n-3 PUFA deficiency on fatty acids in the progeny, we compared changes in fatty acid contents in oocytes, Stage 45 tadpoles, and tadpole brains 60 weeks after the change in diet (Table 5). DHA content was significantly reduced in oocytes (−68%, $p < 0.001$) and whole tadpoles (−67%, $p < 0.001$) from females fed the deficient diet compared with the adequate diet (Fig. 2). Importantly, the n-3 PUFA-deficient diet induced a significant decrease in DHA and total n-3 PUFA content in the brain of Stage 45 tadpoles by 60 weeks (−57%, $p < 0.05$; Table 5). Total n-3 PUFA and the fatty acids 18:3n-3, 20:5n-3, and 22:5n-3 were all decreased in oocytes, tadpoles, and tadpole brains from frogs fed the deficient diet. In contrast, the n-6 fatty acids, 20:4n-6, 22:4n-6, and 22:5n-6, were increased in oocytes, tadpoles, and tadpole brains from frogs fed the deficient diet, but not 18:2n-6, 18:3n-6, 20:3n-6 and total n-6 PUFAs. Saturated fatty acids and monounsaturated fatty acids were not affected by the deficient diet. These findings demonstrate that maternal n-3 PUFA deficiency induces significant reduction of all n-3 PUFA in embryos and in the tadpole brain, and also modifies n-6 PUFA metabolism but does not affect saturated and monounsaturated fatty acids.

n-3 PUFA deprivation influences dendrite length and total branch number in central neurons of n-3 PUFA-deficient tadpoles

To examine the effect of maternal n-3 PUFA deprivation on developing central neurons, we visualized the morphology and postsynaptic maturation of individual optic tectal neurons by coexpressing tdTomato together with PSD95-GFP in single neurons in live *Xenopus* tadpoles. Neurons were imaged at Stage 45, a developmental period when they actively branch and make functional synaptic connections with presynaptic retinal axons. Representative *in vivo* confocal microscopy images of tectal neurons from both dietary groups are shown in Figure 3. For the n-3 PUFA-adequate group, the complexity of the dendritic arbor was similar for neurons of Stage 45 tadpoles from fertilizations performed at 20, 40, and 60 weeks after the change in diet (adequate, Fig. 3*A–C*). Dendritic arbor morphology was similar in neurons from tadpoles in the n-3 PUFA-deficient group to those of neurons from tadpoles in the adequate group at 20 weeks (Fig. 3*A*). However, both at 40 weeks (Fig. 3*B*) and 60 weeks (Fig. 3*C*) after the change in diet, neurons remained simpler in morphology in tadpoles from the n-3 PUFA-deficient group compared with the n-3 PUFA-adequate group. Therefore, coincident with the decrease in embryonic DHA levels, n-3 PUFA deprivation seemed to alter the morphological differentiation of developing neurons in the tadpole brain.

To further evaluate the effects of altered n-3 PUFA brain levels on neuronal morphology, we performed a quantitative analysis of total arbor length and dendritic branch number in neurons of tadpoles obtained at 40 and 60 weeks after the change in diet (Fig. 4). Both at 40 and 60 weeks after the change in diet, total branch

Table 5. Fatty acid concentrations in oocytes, tadpoles, and brains of tadpoles from frogs fed n-3 PUFA-adequate and -deficient diets for 60 weeks[a]

	Oocyte (nmol/oocyte)		Tadpole (nmol/tadpole)		Brain (pmol/brain)	
Fatty acid	Adequate	Deficient	Adequate	Deficient	Adequate[b]	Deficient
16:0	57 ± 2	57 ± 4	45 ± 3	53 ± 3	896 ± 59	822 ± 93
16:1n-7	20 ± 4	30 ± 6	12 ± 2	17 ± 4	70 ± 14	86 ± 13
18:0	12 ± 1	12 ± 1	10 ± 1	12 ± 1	459 ± 30	456 ± 45
18:1n-9	64 ± 5	63 ± 4	43 ± 4	40 ± 5	1488 ± 154	1264 ± 194
18:1n-7	5.3 ± 0.4	5.7 ± 0.5	4.6 ± 0.5	3.9 ± 0.6	112 ± 10	88 ± 12
18:2n-6	101 ± 10	96 ± 6	64 ± 9	54 ± 7	705 ± 115	609 ± 113
18:3n-6	2.6 ± 0.5	2.2 ± 0.2	1.7 ± 0.3	1.3 ± 0.1	39 ± 9	37 ± 14
18:3n-3	8.8 ± 1.1	0.35 ± 0.02***	4.9 ± 1.0	0.17 ± 0.02**	32 ± 6	1.8 ± 0.4**
20:3n-6	7.8 ± 0.8	6.3 ± 0.6	4.9 ± 0.6	3.7 ± 0.4	75 ± 18	95 ± 29
20:4n-6	11 ± 1	14 ± 1*	7.6 ± 0.7	8.8 ± 1.1	214 ± 51	239 ± 82
20:5n-3	1.8 ± 0.2	0.40 ± 0.02***	1.2 ± 0.2	0.20 ± 0.01***	30 ± 7	6.3 ± 2.2*
22:4n-6	0.55 ± 0.05	1.0 ± 0.0***	0.30 ± 0.04	0.46 ± 0.06*	5.8 ± 0.6	7.5 ± 0.4
22:5n-6	0.42 ± 0.05	1.9 ± 0.3***	0.24 ± 0.04	1.0 ± 0.2**	7.1 ± 1.7	29 ± 6**
22:5n-3	0.62 ± 0.06	0.21 ± 0.03***	0.40 ± 0.05	0.18 ± 0.03**	13 ± 2	6.4 ± 1.5*
22:6n-3	7.7 ± 0.5	2.4 ± 0.1***	4.5 ± 0.4	1.5 ± 0.2***	130 ± 23	56 ± 6*
Total	299 ± 22	292 ± 15	203 ± 20	197 ± 21	4275 ± 399	3803 ± 548
Saturated	69 ± 3	69 ± 4.3	55 ± 3	65 ± 4	1355 ± 86	1278 ± 136
Monounsaturated	89 ± 9	99 ± 9	59 ± 6	61 ± 10	1669 ± 160	1438 ± 211
n-6 PUFA	123 ± 12	121 ± 7	78 ± 11	69 ± 8	1046 ± 176	1017 ± 214
n-3 PUFA	17 ± 1	3.4 ± 0.1***	11 ± 1	2.1 ± 0.2***	204 ± 35	70 ± 8**
n-6/n-3	7.2	35	7.2	35	5.1	14

[a]Data are mean ± SEM.

[b]$N = 6$ ($n = 5$) for the adequate group and $n = 5$ for the deficient group.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; unpaired t test.

number was similar in neurons from tadpoles in the n-3 PUFA-adequate group to those of control tadpoles from frogs fed the commercial diet (control 14.66 ± 0.639 branches $n = 87$ neurons, vs adequate at 40 weeks 10.57 ± 0.428 branches $n = 7$ neurons, $p = 0.74$, vs adequate at 60 weeks 12.58 ± 1.27 branches $n = 24$ neurons, $p = 0.1389$; data not shown graphically), thus indicating that the change from a commercial to a formulated diet with adequate n-3 PUFA content does not influence dendritic differentiation of tectal neurons. In contrast, total branch number was significantly lower in neurons from n-3 PUFA-deficient Stage 45 tadpoles compared with those from the adequate group at 40 weeks (adequate 10.57 ± 0.428 branches $n = 7$ neurons, deficient 6.18 ± 0.882 branches $n = 11$ neurons, $p = 0.002$; data not shown graphically) and 60 weeks after the change in diet (adequate 12.58 ± 1.27 $n = 24$, deficient 7.16 ± 0.857 $n = 25$, $p = 0.005$; Fig. 4*A*). *In vivo* imaging showed that neurons became more complex over time by dynamic remodeling of branches, with average total branch number increasing significantly within 48 h in neurons from the adequate group (from 12.6 ± 1.3 branches to 18.6 ± 1.9 branches, $p = 0.005$; Fig. 4*A*). As observed for tectal neurons from n-3 PUFA-deficient tadpoles at Stage 45, total dendrite branch number was significantly lower than that of neurons from the adequate group at both 24 h ($p < 0.001$) and 48 h ($p < 0.001$) after the first imaging session (Fig. 4*A*). Moreover, total branch number did not change significantly for neurons in the n-3 PUFA-deficient group within 48 h of imaging (from 7.2 ± 1.3 to 10.8 ± 1.3 branches, $p = 0.127$; Fig. 4*A*). Similarly to branch number, total dendrite arbor length was significantly lower in neurons from n-3 PUFA-deficient tadpoles compared with those from the adequate group at Stage 45 (adequate 285 ± 20 μm; deficient 169 ± 21 μm; $p < 0.001$; Fig. 4*B*), and this difference was maintained 24 and 48 h after the first imaging (Fig. 4*B*), even as neurons in both groups significantly increased their total arbor length over time (adequate at 48 h, 418 ± 21 μm, $p < 0.001$ compared with 0 h; deficient at 48 h, 267 ± 21 μm, $p = 0.007$ compared with 0 h).

To further quantify the morphological effects of the change in the diet, we calculated average branch length using the measured branch number and total branch length for each individual neuron (Fig. 4*C*). No difference was found in average branch length for neurons between groups at the initial observation period (adequate 25.4 ± 2.2 μm/branch, deficient 26.8 ± 1.9 μm/branch, $p = 0.581$,) or 24 or 48 h after the first imaging. Therefore, these results indicated that n-3 PUFA deprivation interfered with dendrite branch elaboration but not with the lengthening of dendrites.

n-3 PUFA-deprived tectal neurons develop morphologically simpler dendritic arbors

Next, we assessed whether n-3 PUFA deprivation affects overall dendritic arbor complexity by determining the proportion of branches of first, second, third, and fourth order for each tectal neuron. Neurons in n-3 PUFA-deficient tadpoles had fewer secondary and tertiary branches at 0, 24, and 48 h (Fig. 5*A–C*) even though neurons increased their proportion of secondary and tertiary branches within 48 h (Fig. 5*C*). The proportion of fourth order branches was lower at all time points than in neurons in tadpoles from the adequate group. As an additional measure of dendritic arbor complexity, we also calculated a DCI using individual branch order numbers as described in Figure 5 (see also Marshak et al., 2007). DCI values were significantly lower in the n-3 PUFA-deficient group compared with the adequate group at every observation time point (Fig. 5*D*). We further compared differences in arbor complexity between neurons in tadpoles from frogs fed the n-3 PUFA-deficient diet 48 h after the initial imaging (Fig. 5*C*; Stage 47) with those from frogs fed the adequate diet at the initial observation interval (Fig. 5*A*; Stage 45) as there was no significantly difference in total branch number in neurons in the deficient group at 48 h from those in the adequate group at 0 h (Fig. 4*A*; $p = 0.3414$). The proportion of first, second, third, and fourth order branches as well as DCI values (Fig. 5*D*; deficient at 48 h, DCI value 2.22 ± 0.064; adequate at 0 h, DCI value 2.388 ± 0.058, $p = 0.065$) were not significantly different be-

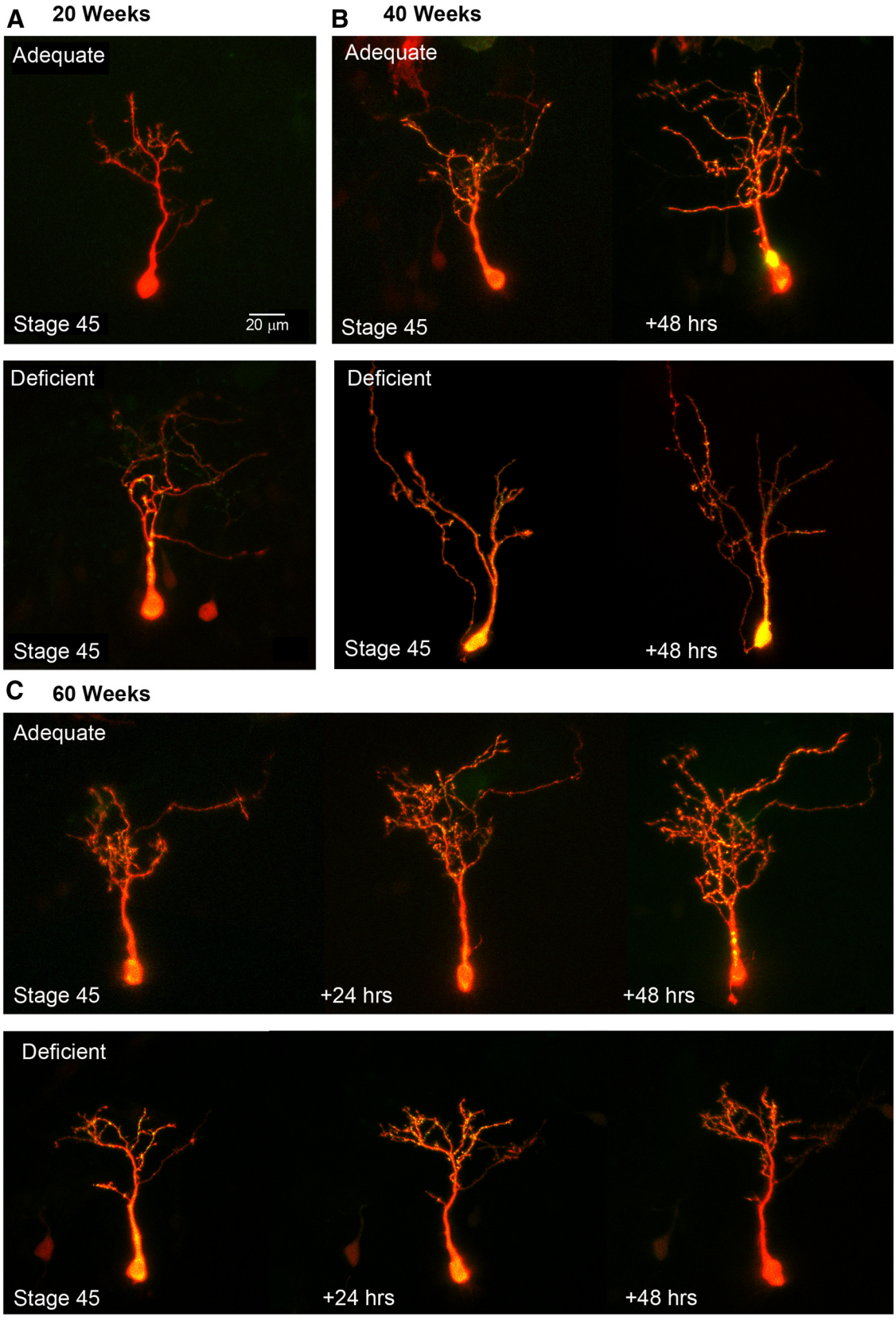

Figure 3. Morphology of tectal neurons in tadpoles from frogs fed n-3 PUFA-adequate or n-3 PUFA-deficient diets. Representative tectal neurons expressing tdTomato (red) together with GFP-PSD-95 (green) in tadpoles from frogs fed n-3 PUFA-adequate and -deficient diets at (***A***) 20, (***B***) 40, and (***C***) 60 weeks. Neuron imaging was performed at 0 h (Stage 45), 24 and 48 h later as indicated. Scale bar, 20 μm.

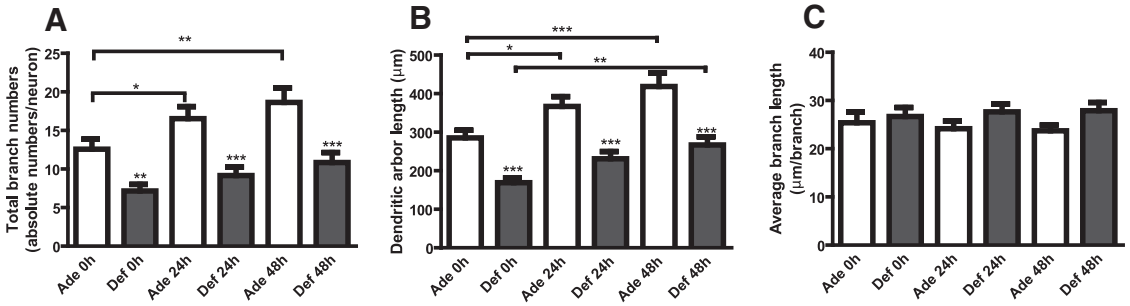

Figure 4. Tectal neurons have fewer branches in n-3 PUFA-deprived brain. ***A***, Total branch number, (***B***) total dendritic length, and (***C***) average branch length were analyzed in tectal neurons in tadpoles from frogs fed the n-3 PUFA-adequate (Ade) or deficient (Def) diets for 60 weeks. Data are mean ± SEM. $N = 24$ neurons for the adequate, and $n = 25$ neurons for the deficient group, with one neuron analyzed per tadpole. Tadpoles analyzed were obtained from 6 and 5 frogs, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; asterisks above gray bars indicate comparison with time-matched adequate group. (Tukey test).

tween neurons in the deficient group at 48 h and those in the adequate group at 0 h. Together, these results indicate that maternal n-3 PUFA deprivation results in the development of morphologically simpler dendritic arbors and suggest a delay in neuronal maturation in n-3 PUFA-deficient tadpoles.

n-3 PUFA deprivation influences neuronal morphology by interfering with new branch addition

Developing neurons increase their complexity over time by adding new branches and eliminating a portion of the existing branches while stabilizing some branches (Niell et al., 2004; Sanchez et al., 2006). To determine whether maternal n-3 PUFA deprivation impacts dendritic complexity by altering the dynamic branching of developing tectal neurons, we analyzed addition and stability of individual branches over the 48 h observation period. Quantitative analysis revealed that the absolute number of branches added every 24 h was significantly lower for neurons from n-3 PUFA-deficient tadpoles than for neurons from tadpoles in the adequate group. Because tectal neurons in n-3 PUFA-deficient tadpoles had fewer branches overall, significantly fewer branches were then stabilized (Fig. 6*A*,*B*). Relative to the proportion of total branches, however, the number of branches added and stabilized (expressed as percentage) was not different from neurons in tadpoles at the same stage from the adequate group at 60 weeks (Fig. 6*C*,*D*). Consequently, the n-3 PUFA deprivation caused neurons to remain simple by interfering with new branch addition rather than by influencing branch formation and elimination rates.

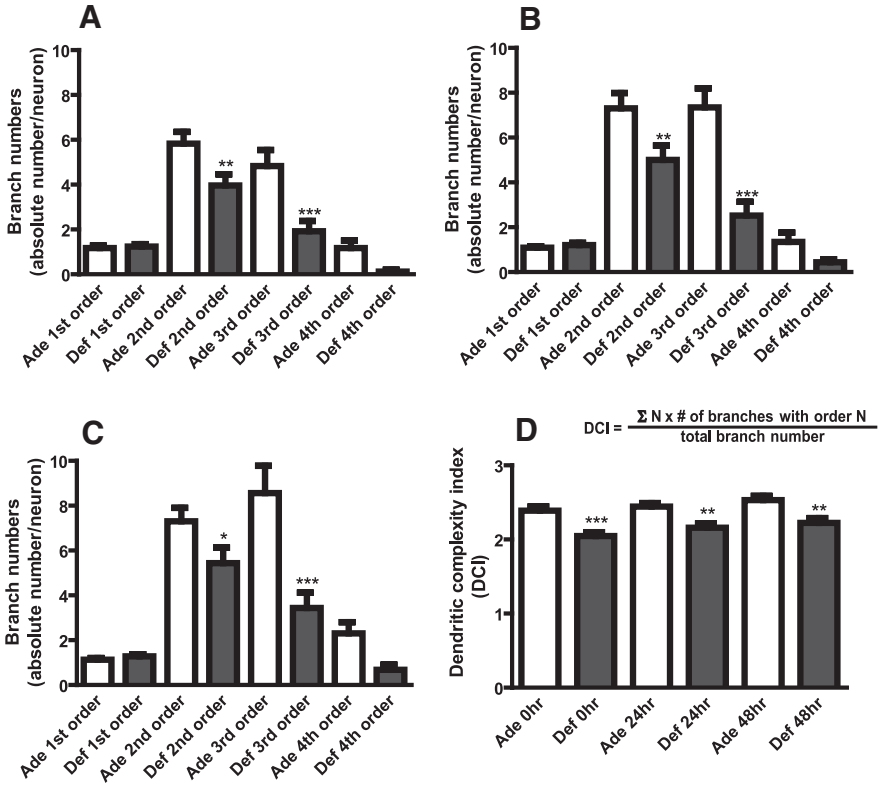

Figure 5. Dendritic arbors are morphologically simpler in n-3 PUFA-deprived brain. Branch order was analyzed in tectal neurons at (***A***) Stage 45, and (***B***) 24 h and (***C***) 48 h after the first imaging. ***D***, DCI was calculated at Stage 45 as defined by the equation (Marshak et al., 2007). Data are mean ± SEM. $N = 24$ neurons for the adequate (Ade), and $n = 25$ neurons for the deficient (Def) group, with one neuron analyzed per tadpole. Tadpoles analyzed were obtained from 6 and 5 frogs, respectively. $^{*}p < 0.05$, compared with the adequate group (unpaired *t* test). $^{**}p < 0.01$, compared with the adequate group (unpaired *t* test). $^{***}p < 0.001$, compared with the adequate group (unpaired *t* test).

n-3 PUFA deprivation interferes with postsynaptic maturation and connectivity

Synaptogenesis occurs as dendrites branch and differentiate, and developing neurons establish new branches and synapses in dynamic way. Therefore, to examine the effect of maternal n-3 PUFA deficiency on synaptic differentiation, we also analyzed PSD95-GFP-labeled postsynaptic specializations in neurons from tadpoles from frogs fed special diets. *In vivo* imaging showed fewer PSD95-GFP postsynaptic clusters overall in tectal neurons coexpressing tdTomato in tadpoles from frogs fed the n-3 PUFA-deficient diet for 60 weeks (Fig. 3). Quantitative analysis revealed that postsynaptic site number was significantly lower in tectal neurons in the n-3 PUFA-deficient tadpoles compared with neurons in the adequate group (adequate, 19.0 ± 4.3 postsynaptic clusters/neuron; deficient 8.1 ± 1.7 postsynaptic clusters/neuron, $p = 0.0328$; Fig. 7*A*). Moreover, postsynaptic cluster density was also significantly lower in the deficient group at 0 and 48 h (adequate at 0 h 0.67 ± 0.06 clusters/10 μm; defi-

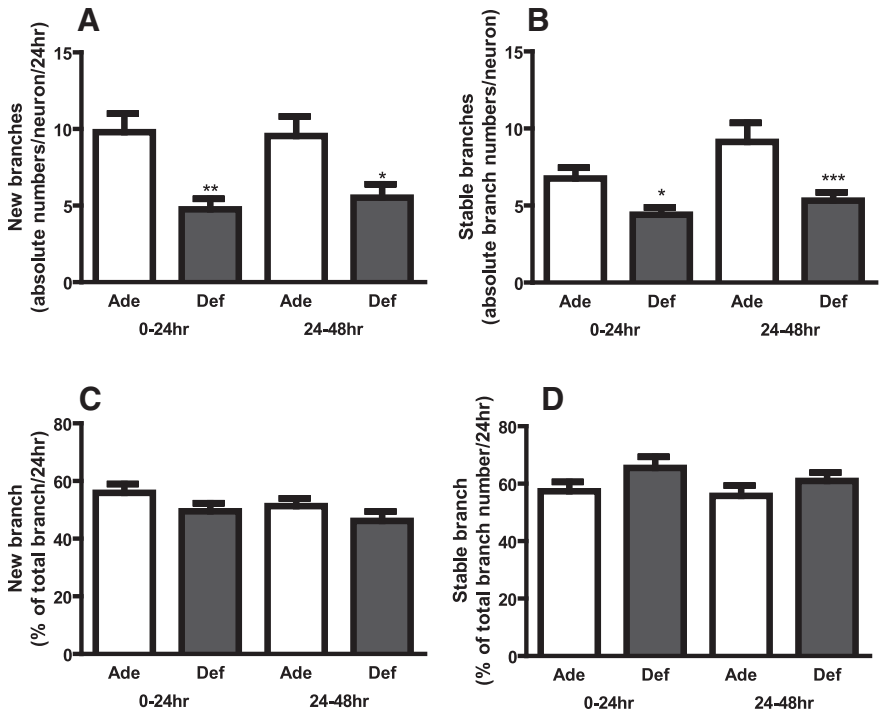

Figure 6. New dendrite branch addition is altered in neurons from tadpoles with n-3 PUFA deficiency. The number of new branches and of stable branches was counted every 24 h to determine dendrite dynamics. The absolute number of (***A***) new branches added and (***B***) stable branches were reduced in the n-3 PUFA-deficient (Def) group. However, as percentage of total, the number of new branches (***C***) and stable branches (***D***) was not changed. Data are mean ± SEM. $N = 24$ for the adequate (Ade) and $n = 25$ for the deficient group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

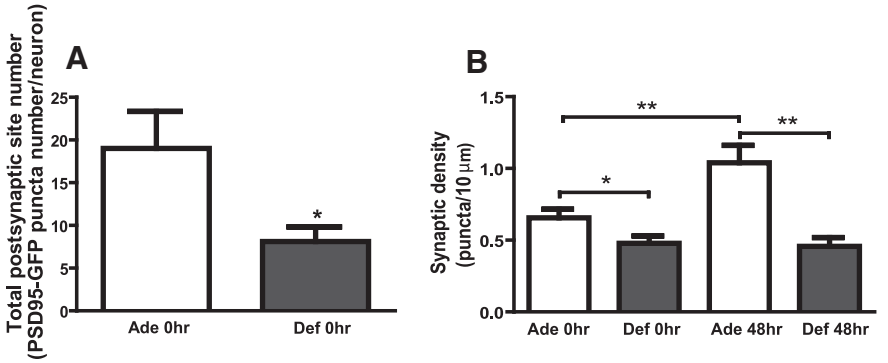

Figure 7. Postsynaptic site number and density are decreased in n-3 PUFA-deprived tectal neurons. ***A***, The total number and (***B***) density of PSD95-GFP puncta were determined for tectal neurons in tadpoles at Stage 45 (0 h) and 48 h later (Stage 47; 48 h) from frogs fed the n-3 PUFA-adequate (Ade) or deficient (Def) diets for 60 weeks. ***B***, Postsynaptic site density was calculated using total synapse number and total dendrite arbor length (Fig. 4*A*) for each individual neuron. Data are mean ± SEM. $N = 9$ for the adequate and deficient groups. *$p < 0.05$ with unpaired *t* test. **$p < 0.01$.

cient at 0 h 0.48 ± 0.05 clusters/10 μm, $p = 0.0405$; adequate at 48 h 1.04 ± 0.12 clusters/10 μm, deficient at 48 h 0.45 ± 0.06, clusters/10 μm, $p = 0.0018$; Fig. 7*B*) even as the morphologically simpler dendritic arbors of tectal neurons in tadpoles with n-3 PUFA deficiency added a few new branches (Fig. 4*A*). To further determine whether the changes in PSD95-GFP postsynaptic specialization number reflect a change in postsynaptic differentiation, we examined endogenous PDS-95 expression by Western blot analysis. Endogenous PSD-95 protein levels were significantly reduced in the tadpole brain by maternal n-3 PUFA deprivation (Fig. 8). Together, these results indicate that, in addition to influencing dendritic branching, maternal n-3 PUFA deprivation impacts the synaptic connectivity of developing neurons in the tadpole brain.

n-3 PUFA deprivation decreases mature BDNF expression in tadpole brain

Work from our laboratory demonstrated that the neurotrophin BDNF influences retinotectal synaptic connectivity by acting directly presynaptically on RGC axons branching in the optic tectum (Alsina et al., 2001; Hu et al., 2005; Marshak et al., 2007). Furthermore, the changes in presynaptic site number elicited by alterations in BDNF signaling induce changes not only in postsynaptic site number on tectal neurons but also influence dendrite arbor length and synapse ultrastructure (Sanchez et al., 2006; Marshak et al., 2007; Nikolakopoulou et al., 2010). Therefore, to determine whether morphological deficits in developing neurons deprived of n-3 PUFAs can be correlated with alterations in BDNF signaling, we measured the levels of mature and pro-BDNF protein as well as TrkB protein levels in brains of tadpoles from frogs fed n-3 PUFA-adequate or -deficient diets over 60 weeks (Fig. 8). Western blot analysis showed that the relative levels of mature BDNF protein were lower in brains from n-3 PUFA-deficient tadpoles (36% reduction, $p = 0.038$ compared with the adequate group), whereas pro-BDNF and total TrkB protein levels were unchanged. Furthermore, quantitative PCR analysis also showed that total BDNF mRNA levels were lower in brains from n-3 PUFA-deficient tadpoles compared with those in the adequate group (45% reduction, $p = 0.049$; Fig. 8). These results indicate that n-3 PUFA deprivation-induced changes in BDNF expression may impact the morphological and synaptic differentiation of central neurons in the living brain.

DHA feeding reverses the morphological changes in tectal neurons caused by n-3 PUFA deprivation

To further determine whether morphological and synaptic changes in neurons are due to changes in maternally derived DHA levels and whether these effects are reversible, we supplemented the adult frog diet with fish oil 60 weeks after the initial change in diet and examined neuronal morphology in Stage 45 tadpoles from fertilizations performed at 80 weeks (20 weeks fish oil supplementation). Imaging individual neurons coexpressing tdTomato and PSD95-GFP in Stage 45 tadpoles from frogs fed the n-3 PUFA-adequate or n-3 PUFA-deficient diet supplemented with fish oil (Fig. 9*A*,*B*) showed that total branch number (Fig. 9*C*), total dendritic arbor length (Fig. 9*D*), and dendritic arbor complexity (DCI values, Fig. 9*E*) were not significantly different among these two groups. Furthermore, BDNF mRNA and protein levels were not significantly different among tadpoles

from frogs fed the n-3 PUFA-adequate or n-3 PUFA-deficient diets supplemented with fish oil (Fig. 9*F*,*G*). Therefore, feeding a fish oil-supplemented maternal diet, that is enriched in DHA, was able to reverse the deficit n-3 PUFA content in frog oocytes and tadpoles as well as the associated morphological and synaptic connectivity defects on differentiating tectal neurons caused by n-3 PUFA deprivation. Moreover, the recovery of morphology by dietary fish oil supplementation was associated with the restoration of BDNF levels in the tadpole brain.

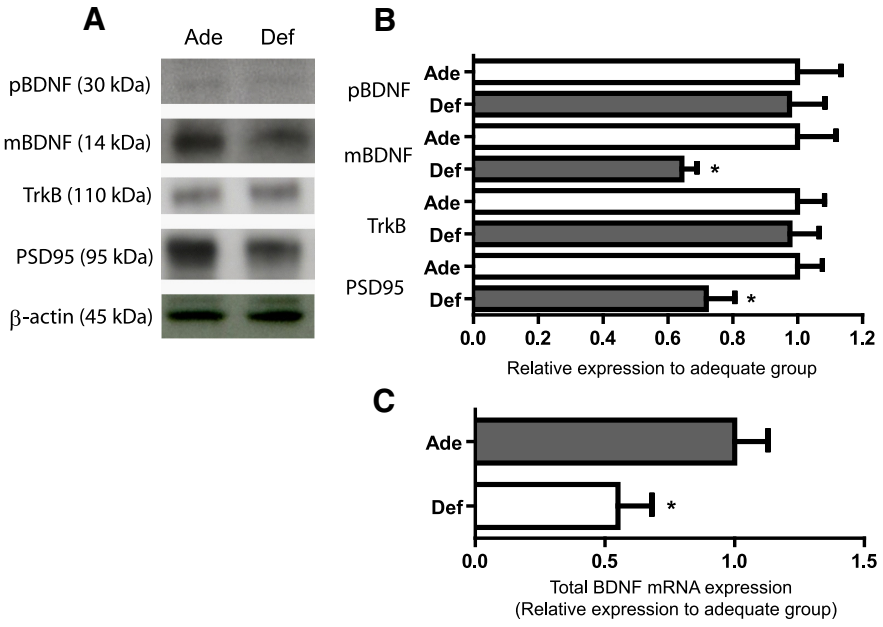

Figure 8. Reduction of mature BDNF and PSD-95 protein levels in the tadpole brain. ***A***, Representative immunoblots and (***B***) relative expression levels of pro-BDNF, mature-BDNF, TrkB, and PSD-95 and β-actin, and (***C***) relative total BDNF mRNA levels in tadpole brains from frogs fed n-3 PUFA-adequate (Ade) and deficient (Def) diets for 60 weeks. Tissues from Stage 45 tadpoles obtained from 6 frogs fed the adequate diet and 5 frogs fed the deficient diet were collected for Western blot and quantitative RT-PCR analyses. For Western blot, data were normalized to β-actin expression, which served as internal control for protein loading. Data are expressed as change in values relative to the adequate group. Data are mean ± SEM. *$p < 0.05$ (unpaired *t* test).

Maternal n-3 PUFA deficiency does not influence the anatomical organization of the tadpole visual circuit

To evaluate whether potential differences in projection patterns or brain sizes among tadpoles derived from mothers fed the deficient versus the adequate diet correlate with deficits in the morphological development of tectal neurons, we analyzed coronal sections of fixed Stage 45 tadpoles. Immunostaining with an antibody against a neurofilament-associated antigen expressed in axons showed no gross abnormalities or differences in projection patterns of axonal tracts in tadpoles from all groups analyzed ($n = 12$ tadpoles per condition). Retinal projections out of the eye, along the optic nerve and tract, as well as the targeting and organization of axons in the midbrain tectum were indistinguishable in tadpoles from mothers fed the n-3 PUFA-deficient for 60 weeks from those in the adequate group (Fig. 10) as well as from those from frogs fed the DHA-supplemented diets for an additional 20 weeks (week 80; data not shown). Moreover, this analysis further revealed that the size and gross anatomical organization of the retinal and brain tissues were unaffected by the diets, consistent with our additional measures of normal developmental timing presented in Table 3. Together, these results indicate that the time delay in neuronal maturation of tectal neurons, and the changes in expression of BDNF and synaptic proteins, were not due to gross anatomical changes in the visual circuit of developing tadpoles.

Discussion

This study provides novel *in vivo* evidence that highlights the significant impact that maternal n-3 PUFA deprivation exerts during neuronal development in the embryonic brain. By combining dietary manipulations in female *X. laevis* frogs and *in vivo* imaging of developing tadpoles, our results demonstrate that maternal n-3 PUFA deprivation significantly reduces n-3 PUFA content in developing embryos, and these changes correlate with significant deficits in the morphological differentiation and connectivity of central neurons. Moreover, we show that maternal n-3 PUFA supplementation allows for the full recovery of morphological and synaptic differentiation deficits in developing neurons caused by the deprivation. Consequently, our studies support a role for maternal n-3 PUFA levels in sustaining/nourishing developing neurons in the intact brain to maintain their normal connectivity and function.

X. laevis has been widely used as model to understand molecular and cellular mechanisms of development due to the accessibility and transparency of the embryos. However, *Xenopus* has seldom been used in nutritional studies because detailed information of nutritional requirements of developing and adult frogs is lacking. Yolk is rich in amino acids, fatty acids, cholesterol, phospholipids, phosphate and numerous positively charged ions, and yolk is stored inside large organelles called yolk platelets. In *Xenopus*, yolk platelets are distributed to all embryonic cells (which consume the yolk intracellularly) and to a yolk sac, where yolk platelets are digested and from where nutrients are distributed to the embryo proper (Jorgensen et al., 2009). *X. laevis* embryos and tadpoles are therefore limited in their nutritional resources to those contained in the yolk until they begin to feed at Stage 47. Previous studies demonstrated that the nutritional status of female frogs can modify the yolk's nutritional status, and that dietary changes can in turn affect oocyte quality and embryonic development. In this study, we used semipurified diets, which are based on published nutritional requirements for rodents, to modify lipid contents (see Tables 1 and 2) to determine the effects that maternal n-3 PUFA deficiency has during early neuronal development in the intact brain. Our results demonstrate that the special diets did not cause any harmful effects to frog health over the 80 weeks of feeding. Throughout the study, frogs fed the deficient diet showed no changes in weight or health, and the quality of their embryos, their development, and protein and total lipids levels were not affected compared with those of frogs fed the adequate diet. The semipurified diets formulated for rodents successfully altered the nutritional status of *X. laevis* embryos, in agreement with Fort et al. (1999, 2000, 2002). The n3-PUFA-deficient diet modified fatty acid contents in oocytes and tadpoles from female frogs over eight sequential ovulations (Fig. 2; Tables 3, 4, and 5), and changes in fatty acid content in oocytes

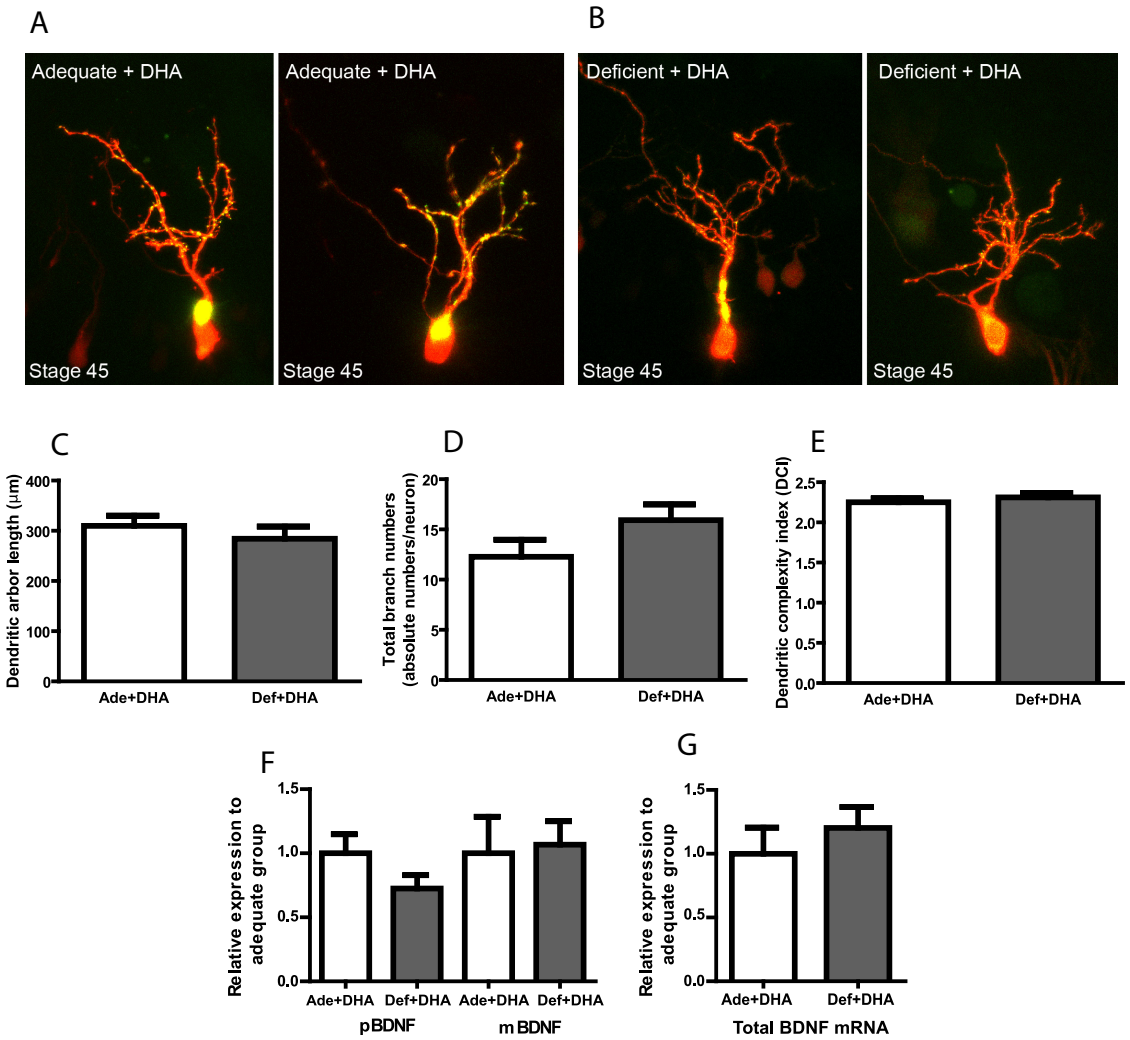

Figure 9. Maternal DHA supplementation reverses morphological deficits in neurons and in BDNF expression caused by n-3 PUFA deprivation. Representative tectal neurons in Stage 45 tadpoles from frogs fed the (***A***) adequate and (***B***) deficient diet for 60 weeks and then switched to a fish oil-contained n-3 PUFA-adequate diet for 20 weeks. Sample neurons were from two different tadpoles, each obtained from oocytes from two different frogs in each group (***A***, ***B***). ***C***, Total branch numbers, (***D***) total dendrite arbor length, and (***E***) dendritic arbor complexity index (DCI) were measured. $N = 19$ neurons for the adequate group (Ade), and $n = 13$ neurons for the deficient group (Def) in tadpoles from 5 frogs per group. Relative expression levels of (***F***) pro-BDNF and mature-BDNF, and of (***G***) total BDNF mRNA, measured in brains of Stage 45 tadpoles from frogs at week 80 ($n = 4$ or 5). Data are mean ± SEM.

translated into decreased DHA and fatty acid content in the brains of tadpoles.

All animal species, including frogs, depend on plants and/or insects as nutritional sources of linoleic acid (LA) and ALA. LA and ALA are converted in the liver to their respective longer chain fatty acids, arachidonic acid (AA) and DHA, through a series of enzymatic steps (Gurr et al., 2002), and DHA and AA are also provided from the diet. Therefore, dietary n-3 PUFA is important to maintain normal n-3 PUFA content in all organs. In mammals, maternal consumption of n-3 PUFA during pregnancy and lactation has been correlated with both DHA levels in organs and with neuronal function in their progeny (Hoffman et al., 2009). In this study, DHA deprivation in *Xenopus* oocytes and tadpoles was successfully induced by a maternal n-3 PUFA-deficient diet, and DHA content was rapidly reversed by DHA supplementation (Fig. 2). In contrast to our studies, studies that used a n-6 PUFA-rich diet (beef liver) instead of an n-3 PUFA-rich diet (commercial fish food) (Rizzo et al., 1999) had failed to modify n-3 PUFA content *X. laevis* embryos. This underscores the advantage of using semipurified diets in which fatty acid content is specifically controlled, as in the present study, as a means to modify n-3 PUFA content in embryos and tadpoles.

DHA is important for proper neuronal development and function. Studies both in humans and in rodents have shown that DHA supplementation in pregnancy and during lactation improves learning ability and cognitive function of the progeny, whereas DHA deprivation during development induces cognitive deficits in animal models (Innis, 2008; Schuchardt et al., 2010). Studies in rodent animal models and cells in culture have also shown that DHA levels are associated with neurite growth, soma size, and with changes in levels of expression of synaptic proteins (Ahmad et al., 2002a, b; Calderon and Kim, 2004; Wu et al., 2008; Cao et al., 2009). However, how n-3 PUFA levels impact the morphological development and connectivity in central neurons in living brain remained to be elucidated. In this study, we were able to manipulate embryonic DHA levels by feeding female frogs n-3 PUFA-modified diets and to correlate these changes with dynamic changes in neuronal development and differentiation in their progeny throughout sequential ovulations over 80 weeks (Table 1). Our *in vivo* imaging studies revealed that maternal n-3

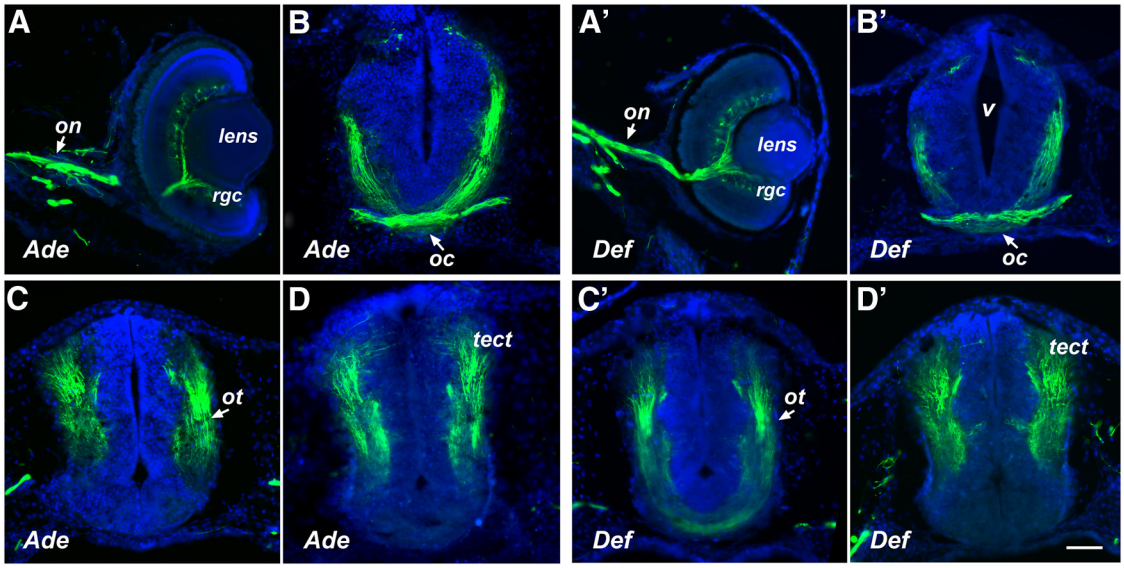

Figure 10. Maternal n-3 PUFA condition does not influence retinal axonal projections to the brain. Representative coronal sections of Stage 45 tadpoles from frogs fed the (***A–D***) n-3 PUFA-adequate (Ade) or (***A′–D′***) n-3 PUFA-deficient (Def) diet for 60 weeks and immunostained with a neurofilament-associated protein antibody (green) illustrate the normal projection patterns of retinal axons (***A***, ***A′***) out of the eye and along the optic nerve (on), and (***B***, ***B′***) at the level of the optic chiasm (oc). Projection of axonal fibers along (***C***, ***C′***) the optic tract (ot) and (***D***, ***D′***) the rostral tectum (tect) were also similar in Stage 45 tadpoles from both groups. Counterstaining with DAPI (blue) reveals normal size and cellular organization in the (***A***, ***A′***) retina, and (***B–D***, ***B′–D′***) brains of tadpoles from frogs fed the adequate (***A–D***) and deficient (***A′–D′***) diets. rgc, Retinal ganglion cell layer; v, ventricle. Dorsal is up, ventral is down. Scale bar, 50 μm.

PUFA deprivation impacts the morphological development of neurons by altering their dynamic branching and delaying their maturation (Fig. 3), and that maternal DHA supplementation is capable of inducing a fast recovery of the morphological deficits induced by n-3 PUFA-deficient maternal diets (Fig. 8).

Optic tectal neurons in Stage 45 tadpoles from frogs fed an n-3 PUFA-deficient diet for 40–60 weeks had fewer branches and remained simpler over time (Figs. 4 and 5) than those from tadpoles from mothers fed an n-3 PUFA-adequate diet. This change in morphology coincided with the time at which DHA levels significantly decreased in the tadpole brain. Coincident with a decrease in dendrite arbor growth, our dynamic studies also revealed that maternal n-3 PUFA deprivation caused a significant reduction in the number and density of postsynaptic sites in live developing neurons, visualized by the expression of PSD-95-GFP. The reduction in the number and density of postsynaptic sites in *Xenopus* central neurons remained even as neurons increased their complexity over 48 h, and is consistent with a decrease in endogenous PSD-95 protein levels in the tadpole brain (Figs. 7 and 8). This indicates that n-3 PUFA levels in the brain can impact synaptogenesis independently of its effects on dendrite branching. Studies have shown that DHA levels can impact neurite length and neurite number of hippocampal primary neurons in culture (Calderon and Kim, 2004; Cao et al., 2009) and that n-3 PUFA levels can influence synaptic differentiation and synaptic protein expression (Cao et al., 2009). Therefore, our *in vivo* observations in *Xenopus* are consistent with effects of n-3 PUFA deprivation in mammals. A potential molecular mechanism mediating these cellular effects may involve, at least in part, BDNF. In rodents, DHA deprivation has been shown to decrease BDNF expression, cAMP response element binding protein (CREB) transcription factor activity that regulates BDNF expression, and p38 mitogen-activated protein kinase (MAPK) activity which in turn activates CREB (Rao et al., 2007). In the *Xenopus* visual system, BDNF acts as neurotrophic factor that mediates synaptic differentiation and maturation of the retinotectal circuit through cell autonomous TrkB signaling on retinal ganglion cells (Sanchez et al., 2006; Marshak et al., 2007). In the current study, we demonstrate that n-3 PUFA deprivation reduced BDNF brain levels, but not of its receptor TrkB. These effects, together with the reduction in endogenous PSD-95 expression and in the density of PSD-95-GFP labeled postsynaptic specializations in individual neurons (Figs. 7 and 8), are therefore consistent with n-3 PUFA shaping the maturation and synaptic connectivity of developing neurons through modulation of BDNF (Yoshii and Constantine-Paton, 2007, 2010, 2014).

During development, neuronal connections are established through a coordinated growth and differentiation of presynaptic and postsynaptic arbors, where presynaptic input and growth can then influence dendritic arbor structure and growth (Cline, 2001; Sanchez et al., 2006; Cohen-Cory et al., 2010). Like BDNF, n-3 PUFA could act directly on retinal ganglion cells and then indirectly influence tectal neurons (Sanchez et al., 2006; Marshak et al., 2007; see also Rauskolb et al., 2010), or it may also act directly on the postsynaptic cells. DHA is a major structural lipid of retinal photoreceptor outer segment membranes, and its highly unsaturated nature supports the efficient activity of the visual cascade (SanGiovanni and Chew, 2005). It is therefore possible that the maternal n-3 PUFA deprivation may also affect early visual cascades important for normal vision and that this in turn impacts retinotectal development, connectivity, and function. Whereas the gross anatomical organization of the tadpole visual system was unaffected by the n-3 PUFA deprivation (Fig. 10), single-cell imaging indicates that DHA treatment can influence retinal axon branching in the *Xenopus* brain (M. Igarashi and S. Cohen-Cory, unpublished). This is consistent with observations that an n-3 PUFA-restricted diet alters the projection of ipsilateral retinal axons in the adult rat, resulting in abnormal wiring patterns and interfering with the closure of the critical period of plasticity (de Velasco et al., 2012), a process in which BDNF is involved (Hanover et al., 1999; Huang et al., 1999). This is also in agreement with recent observations that embryonic and postnatal n-3 PUFA deficiency impacts functional connectivity in adult monkeys at multiple levels along the visual pathway (Grayson et

al., 2014). The observation that supplementation of maternal diets with DHA rich fish oil can reverse deficits in morphology and connectivity of central neurons within the developing tadpole brain therefore emphasizes the significant interactions between fatty acid metabolism and neurotrophic function and further underscores the importance of proper maternal nutrition during gestational age for proper brain development and function among all vertebrate species.

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