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Gene and protein expression profiling of the *fat-1* mouse brain

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ABSTRACT

Polyunsaturated fatty acids (PUFAs) are essential structural components of all cell membranes and, more so, of the central nervous system. Several studies revealed that n-3 PUFAs possess anti-inflammatory actions and are useful in the treatment of dyslipidemia. These actions explain the beneficial actions of n-3 PUFAs in the management of cardiovascular diseases, inflammatory conditions, neuronal dysfunction, and cancer. But, the exact molecular targets of these beneficial actions of n-3 PUFAs are not known. Mice engineered to carry a *fat-1* gene from *Caenorhabditis elegans* add a double bond into an unsaturated fatty acid hydrocarbon chain and convert n-6 to n-3 fatty acids. This results in an abundance of n-3 eicosapentaenoic acid and docosapentaenoic acid specifically in the brain and a reduction in n-6 fatty acids of these mice that can be used to evaluate the actions of n-3 PUFAs. Gene expression profile, RT-PCR and protein microarray studies in the hippocampus and whole brain of wild-type and *fat-1* transgenic mice revealed that genes and proteins concerned with inflammation, apoptosis, neurotransmission, and neuronal growth and synapse formation are specifically modulated in *fat-1* mice. These results may explain as to why n-3 PUFAs are of benefit in the prevention and treatment of diseases such as Alzheimer's disease, schizophrenia and other diseases associated with neuronal dysfunction, low-grade systemic inflammatory conditions, and bronchial asthma. Based on these data, it is evident that n-3 PUFAs act to modulate specific genes and formation of their protein products and thus, bring about their various beneficial actions.

1. Introduction

Fat forms an important component of the body. Sixty percent of the brain and all cell membranes consist of fat. The fluidity of cell membranes depends on their fatty acid content: higher the saturated fatty acids content more the rigidity of the cell membrane, whereas unsaturated fatty acids render cell membrane more fluid. If the cell membrane is rigid, it will not react in an optimal way to external stimuli including hormones, growth factors, and drugs. If the saturated fatty acids in the cell membrane are replaced by polyunsaturated fatty acids (PUFAs), its ability to react to the external stimuli will be increased such that optimal responses to hormones and growth factors could be seen. This is one of the reasons why n-3 PUFAs can increase the sensitivity of the cell to hormones and growth factors. Furthermore, the improved fluidity and so the plasticity of the cell membrane can influence the enzyme activity, the number and function of receptors, and the permeability of the cell membrane to ions [1–3].

There are two types of PUFAs: n-3 and n-6 and both are essential for life and cannot be produced in the body and hence, must be supplied in the food. The number and location of double bonds seems to be crucial for the function of PUFAs. In general, n-6 PUFAs form precursor to pro-inflammatory eicosanoids whereas those derived from n-3 are less pro-inflammatory and hence, have more favorable effects in the body [1–3]. Traditionally, to obtain the beneficial actions of n-3 PUFAs, it is recommended that the supplementation of these fatty acids is given or consumption of food that is rich in these fatty acids is encouraged. An approach to eliminate the need of n-3 fatty acid supplementation is to produce n-3 fatty acids endogenously, ideally from n-6 so that the pro-inflammatory actions of the later are less and the beneficial actions of n-3 are augmented. Mammals cannot convert n-6 to n-3 fatty acids since they lack the gene for this purpose. Using transgenic approach, Kang et al. [4] and Kang [5] heterologously

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expressed the *C. elegans fat-1* gene in mice. The resultant mice not only showed enhanced concentrations of n-3 α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPA), but also significantly reduced n-6 linoleic (LA) and arachidonic (AA) acids in muscle, red blood cells, heart, brain, liver, kidney, lung, and spleen tissues such that the ratio of n-6 to n-3 dropped from 20 to 50 to almost 1. Despite such a drastic change in the ratio of n-6 to n-3 the transgenic mice were found to be normal and healthy. Thus, the *fat-1* transgenic mouse model is ideal to study the effects of tissue n-6/n-3 ratio in the body. Despite the fact that an increase in the tissue levels of n-3 fatty acids has several beneficial actions, the exact molecular mechanism is still elusive.

We hypothesized that increase in tissue concentrations of n-3 fatty acids may alter gene expression and the formation of their specific proteins to bring about their health benefits. Our previous findings suggested that diet rich in n-3 PUFA induced specific gene expression changes in the brain related to different cellular functions [6–8]. Additionally, altered expression of numerous genes due to perinatal omega-3 PUFA deficiency have been already known including overexpression of ZnT3 gene, that caused abnormal zinc metabolism in the brain [9].

Since n-3 fatty acids have been reported to be useful in the prevention and treatment of several neurological conditions such as Alzheimer's disease and schizophrenia [10–12], we investigated the changes in gene expression in the hippocampus of wild-type and *fat-1* transgenic mice at the mRNA level on a DNA microarray platform. To evaluate the significance of the changes in gene expression observed, we performed quantitative real-time polymerase chain reaction (QRT-PCR) of 28 genes which showed significant changes and proteome analysis by using protein microarray technology to follow changes in protein expression/modification in response to the *fat-1* gene presence.

2. Materials and methods

2.1. Samples and RNA preparation

Wide-type and *fat-1* transgenic male mice (18 weeks old), housed in a room maintained at 12 h light–dark cycles and a constant temperature of 22±2 °C, were fed with standard laboratory chow. Total RNAs were isolated from hippocampus from each animal (40 mg tissue each) (6 wide type and 6 transgenic animals) with NucleoSpin RNA purification kit (Macherey-Nagel, Dürren, Germany) according to the manufacturer's instructions. The quantity and the quality of all RNA preparations were assessed by gel electrophoresis and spectrophotometry (NanoDrop, Rockland, DE, USA). Total RNA was used for microarray analysis as well as for reverse transcription quantitative PCR. For microarray analysis 3 pools were generated from each type of animal (2 animals/pool).

2.2. Analysis of FA composition

Lipids were extracted with chloroform/methanol (2:1, V/V) from individual animals and were analyzed by HPLC samples according to the method described by Folch et al. [13]. Methyl esters of the total phospholipid FAs were prepared by transesterification in absolute methanol containing 5% HCl at 80 °C for 2.5 h. The methyl esters were separated on an FFAP column (0.25 mm i.d. capillary column 30 m long from Supelco, Bellefonte, PA) in a Hewlett-Packard Model 6890 gas chromatograph. The measurements were made in duplicates and the averages of the two measurements were used. Peaks were identified with the aid of authentic standards from Sigma. The reported data are means ± SEMs of $N = 3$ independent experiments.

2.3. Quantitative real-time PCR

QRT-PCR was performed on a RotorGene 3000 instrument (Corbett Research, Sydney, Australia) with gene-specific primers and SybrGreen protocol as described [14,15]. In brief, 2 μg of total RNA from each sample was reverse transcribed in the presence of poly(dT) sequences in total volume of 20 μl. After dilution of the mix with 80 μl of water, 2 μl of this mix was used as template in the QRT-PCR. Reactions were performed in a total volume of 20 μl containing 0.2 mM of dNTP, 1 × PCR reaction buffer (ABGene, Epsom, UK), 5 pmole of each primer, 4 mM of $MgCl_2$, 1 × SYBR Green I (Molecular Probes, Eugene, Oregon) at final concentration, and 0.5 units of thermostart Taq DNA polymerase (ABGene). The amplification was carried out with the following cycling parameters: 600 s heat start at 95 °C, 45 cycles of denaturation at 95 °C for 25 s, annealing at 60 °C for 25 s and extension at 72 °C for 20 s. Fluorescent signals were gathered after each extension step at 72 °C. Curves were analyzed by the RotorGene software using dynamic tube and slope correction methods with ignoring data from cycles close to baseline. Primers were designed by using the PrimerExpress software (Applied Biosystems). Relative expression ratios were normalized to the geometric mean of two housekeeping genes, GAPDH and hypoxanthine phosphorybosyltransferase. Expression ratios were calculated using the Pfaffl method [16]. All the PCRs were performed four times in separate runs from the two time points.

2.4. DNA microarray protocols

2.4.1. Microarray probe preparation and hybridization

For probe preparation, 2 μg of total RNA was reverse transcribed using poly-dT primed Genisphere Expression Array 900 Detection system (Genisphere, Hatfield, PA, USA) in 20 μl total volume using 20 Unit RNAsin (Fermentas, Vilnius, Lithuania), 1 × first strand buffer and 200 Units of RNAse H (−) point mutant M-MLV reverse transcriptase (Fermentas) [16]. All the other probe preparation steps were done according to the manufacturer's instructions (Genisphere). Both the first step cDNA hybridization and the second step capture reagent hybridization were carried out in a Ventana hybridization station (Ventana Discovery, Tucson, AZ, USA) by using the "antibody" protocol. First hybridization was performed at 40 °C for 6 h in "FGL2" hybridization buffer (10 × Denhart solution, 0.25 M sodium phosphate buffer pH 7.0, 1 mM EDTA, 1 × SSC, 0.5% SDS), then 2.5 μl of each Cy5 and Cy3 capture reagents were added to the slides in 200 μl "Chiphyb" hybridization buffer (Ventana) and incubated at 42 °C for 2 h. After hybridization, the slides were washed in 0.2 × SSC twice at RT for 10 min, then dried and scanned.

Commercially available mouse-specific oligonucleotide microarrays were used in the study (38.5 K Mouse Array- Illumina MEEBO oligos (25,000 genes and 35,302 70mer oligonucleotide probes)) www.microarrays.com) (Microarrays Inc., Nashville, TN, USA).

2.4.2. Scanning and data analysis

Each array was scanned under a green laser (543 nm for Cy3 labeling) or a red laser (633 nm for Cy5 labeling) using a ScanArray Lite (GSI Lumonics, Billerica, MA) scanning confocal fluorescent scanner with 10 μm resolution (Laser power: 85% for Cy5 and 90% for Cy3, Gain: 80% for Cy5 and 75% for Cy3) [14]. Scanned output files were analyzed using the GenePix Pro5.0 software (Axon Instruments Inc., Foster City, CA). Each spot was

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defined by automatic positioning of a grid of circles over the image. For each channel the median values of feature and local background pixel intensities were determined [16]. The background corrected expression data (background signals were subtracted for each channel) was filtered for flagged spots and weak signal. Technical replicates on the same array were averaged. Data were excluded in cases where technical replicates were significantly different. Normalization was performed using the print-tip LOWESS method [16]. Next we used the one-sample *t*-test in order to determine the genes to be regarded as regulated in response treatment. A color-flip experiment was done for replication. Color-flip data were normalized and logarithm was taken from each expression ratio to fulfill the *t*-test's requirement for a normal distribution. Genes for which the mean of log-ratios across the biological replicates was equal to zero at a significance level $\alpha = 0.05$ are considered to have an unchanged expression. On the other hand, genes having a *p*-value smaller than α and the average-fold change (increase or decrease) of the four data points were at least 2.0-fold was considered as regulated genes.

The distant regulatory elements of co-expressed genes were analyzed by using the web-based promoter analysis software DIRE [17].

2.4.3. Protein microarray protocols

Protein microarray studies were performed using Panorama AB Microarray Cell Signaling Kit (Sigma, Cat. No. CSAA1, Budapest, Hungary), according to the manufacturer's instructions. Briefly, proteins were extracted from freshly removed cerebellum of transgenic and control mice, each extract was then labeled with Cy3 and Cy5 monofunctional reactive dyes (Amersham Biosciences), respectively. The free dye was removed from the labeled samples by applying on SigmaSpin column. The Cy3 and Cy5 labeled samples at equal protein concentrations were diluted in 5 ml of incubation buffer and incubated on the microarray for 45 min. Then the microarray was washed 3 times for 5 min with Protein Array Wash Buffer (Whatman Inc., Sanford, ME). Incubation and the washing procedures were done at room temperature in dark on a platform shaker. The microarray was scanned in both the Cy3 and Cy5 channels using ScanArray LITE (GSI Lumonics, Billerica, MA) confocal fluorescent scanner with 10 μm resolution. Scanned output file was analyzed using GenePix Pro 6.0 software (Axon Instruments Inc., Foster City, CA). The background corrected expression data were filtered for flagged spots and weak signals, and then they were normalized to sum fluorescence intensities. A color-flip experiment was done for replication. Average values were calculated from signals generated from each experiment. We used −1.40 and 1.40-fold change cut-off values for down-regulation and up-regulation, respectively.

2.4.4. Gene ontology analysis

To determine biological significance of differentially expressed genes, functional classification was performed using gene ontology/pathway analysis using open-access software [18] and as described in our previous report [19]. Gene ontology analysis gives relative representation of up-regulated and down-regulated genes in each function. To determine pathways associated with differentially expressed genes, pathway analysis was performed.

3. Results

Mice engineered to carry a *fat-1* gene from *Caenorhabditis elegans* can add a double bond into an unsaturated fatty acid hydrocarbon chain and convert n-6 to n-3 fatty acids as shown in Fig. 1. In the present study, we measured the fatty acid composition in the brain of transgenic (*fat-1*) and wild-type mice kept on a normal chow (the diet used was D04 from Safe, Augy, France; containing 3.1% of lipids). The results are shown in Table 1 and were compared to the literature data where 10% n-6 diet was applied [4]. We found that significant changes in the n-6 to n-3 ratio were not present under normal nutrition conditions in comparison to the changes seen in the fatty acid composition when these *fat-1* mice were fed 10% n-6 diet. However, it should be noted here that the fatty acid data of the present study are for the hippocampus region of the brain whereas the data given in Ref. [4] are for the brain that could account for some of the differences in the fatty acid composition noted. Despite this difference in the data, it can be suggested that the changes in the fatty acid composition in the brain of transgenic mice (*fat-1*) are evident only when the animals are fed diet rich in n-6 fat, implying that *fat-1* gene is active and able to convert n-6 to n-3 fatty acids due to the presence of *fat-1* gene. However, the concentration of EPA and DPA, which are present in low quantities in normal brain tissues, increased significantly in transgenic (*fat-1*) mice kept on a normal diet (see Table 2). These results indicate that *fat-1*gene from *C. elegans* is active only when the diet is rich in n-6 fatty acids. Since no significant changes in the n-6 to n-3 ratio were noted under normal nutrition conditions (when fed normal chow) in comparison to the changes seen in the fatty acid composition when these *fat-1* mice were fed 10% n-6 diet, indicates that only when dietary n-6 fatty acids exceed certain limit the transgenic mice are able to convert n-6 fatty acids to relevant n-3 fatty acids. These results also imply that certain percentage of n-6 fatty acids are essential for various tissues that could be the reason for no significant change in the n-6 to n-3 ratio under normal nutrition conditions and only when the dietary n-6 fatty acids exceed this minimal percentage *fat-1* gene is able to act and convert n-6 to n-3 fatty acids in the tissues and especially so in the brain. This is supported by the fact that brain is rich not only in n-3 EPA and DHA but also in n-6 AA. In general, several products of AA such as prostaglandins, thromboxanes, and leukotrienes possess pro-inflammatory actions [2,3]. In view of this, AA is considered harmful. But, AA also forms precursor to anti-inflammatory compounds such a lipoxins [2,3], and is essential for the formation of SNAP25 (synaptosomal-associated protein of 25 kDa), a syntaxin partner implicated in neurite outgrowth, that interacts with syntaxin 3 only in the presence of AA to form the binary syntaxin 3-SNAP 25 complex. AA stimulated syntaxin 3 to form the ternary SNARE complex (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), which is needed for the fusion of plasmalemmal precursor vesicles into the cell surface membrane that leads to membrane fusion that facilitates neurite outgrowth [20]. Thus, AA when present in optimal amounts could be of significant benefit in the prevention and treatment of neurological conditions such as Alzheimer's disease [10,11].

Further on we investigated the changes in gene expression in the hippocampus of wild-type and *fat-1* transgenic mice at the mRNA level on a DNA-microarray platform (see Fig. 2). The results of this study are given as supplemental data (www.brc.hu/~chiplab/Fat1.xls). We observed specific changes at transcription level in the full-genome mouse study. Of all the genes investigated, 4.2% of the genes showed significant changes in *fat-1* transgenic mice: 2.2% overexpression, and 2% repression. Among the down-regulated genes activating transcription factor 4, protein phosphatase 1-3f, hydroxysteroid dehydrogenase-2, holocarboxylase synthetase, and serotonin receptor 3a showed the most dramatic repression. Among the up-regulated genes stearoyl-Coenzyme A desaturase 2, potassium voltage-gated channel Q1, solute carrier family 5 (iodide transporter) 8, prostaglandin D2 synthase, aminophospholipid transporter-like 8A2, and cadherin 4 showed more than 4-fold induction.

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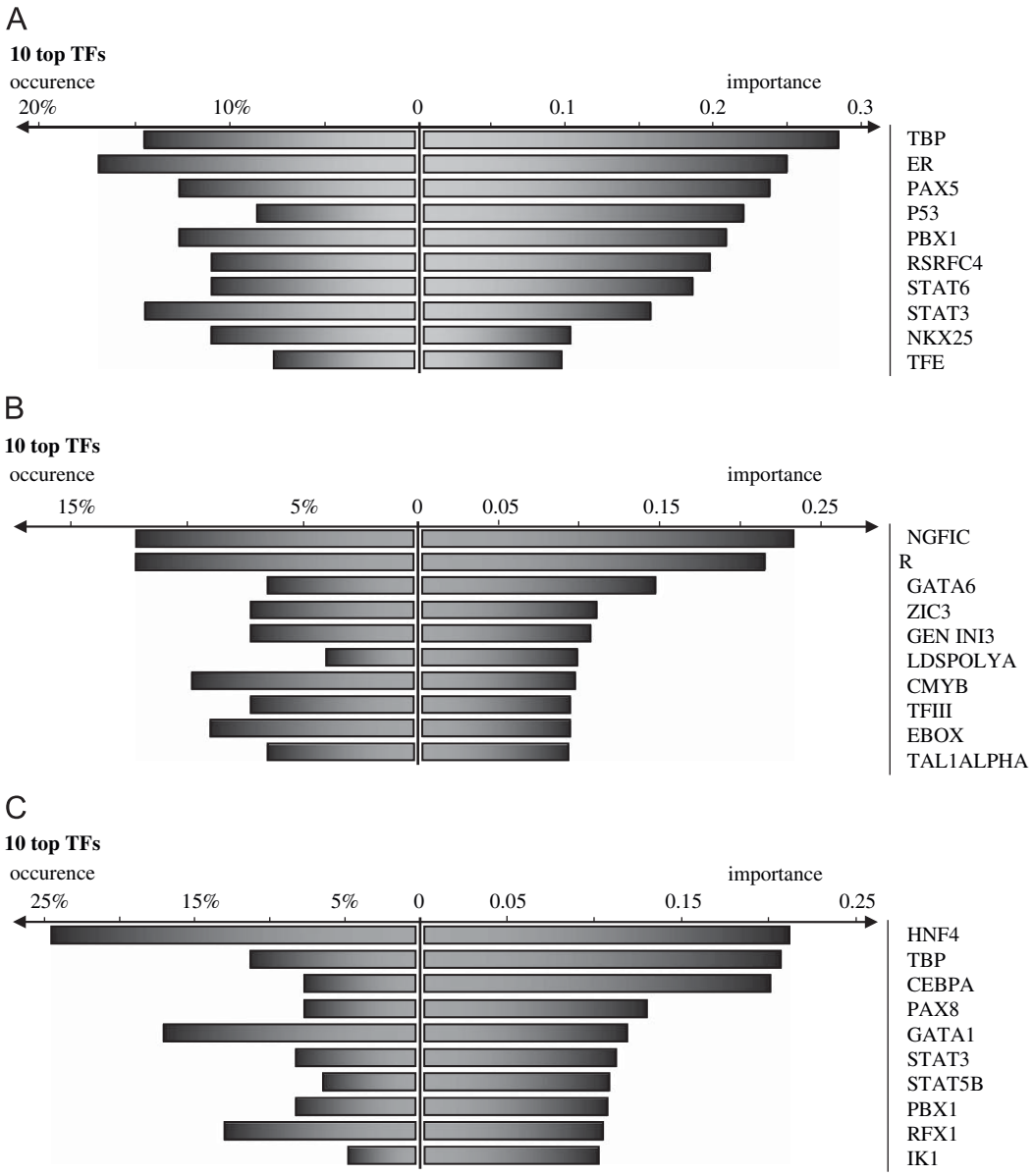

Fig. 1. Identification of the proximal promoters and distant regulatory elements of the genes that showed down-regulation (A), up-regulation (B) and genes with altered expression (C) in the *fat-1* transgenic mouse.

Table 1
Differences in the ratio between n-6/n-3 fatty acids in brain of transgenic (*fat-1*) and wild-type mice kept on normal chow and 10% n-6 diet (from Ref. [4]).

10% n-6 diet				Normal diet			
n-6/n-3		AA/(EPA+DPA+DHA)		n-6/n-3		AA/(EPA+DPA+DHA)	
WT	TG	WT	TG	WT	TG	WT	TG
3.90	0.80	3.60	0.70	0.70	0.65	0.48	0.45

WT = wild-type mice; TG = transgenic mice (*fat-1*).

We have also analyzed the distant regulatory elements of co-expressed genes exhibited changes in their expression (either up- or down-regulation) by using the web-based promoter analysis software [17]. The top 10 hits of the DNA-binding factors are listed in Fig. 1. Among these hepatocyte nuclear factor 4-alpha (HNF4), Paired box protein Pax-8, CEBPa, and two signal transducer and activator of transcription factors (STAT3 and STAT5B) are found most abundantly in the genes with altered expression. Among the up-regulated genes, the NGFIC regulatory element was found in most cases. Among the down-regulated genes, TBP, ER, and PAX5 were the most abundant regulatory element.

In order to confirm alteration at the transcription level, 28 of these genes were examined by QRT-PCR. These QRT-PCR results correlated well with the microarray data. These 28 genes are believed to play an important role in diseases such as Alzheimer's disease (*scd*, *apod*), schizophrenia (*apod*), inflammation (*pla2*), neuronal dysfunction, and asthma (*ptgds*) (Table 3).

In order to identify proteins that are differentially expressed in the *fat-1* transgenic mouse vs. wide-type mouse protein microarray study was performed. On the protein microarray (Panorama AB Microarray Cell Signaling Kit from Sigma) the top four functional classes of proteins which expression can be followed are the following: signal transduction, cytoskeleton, neurobiology, and apoptosis-related proteins. After preparation and differentially labeling of total protein extract from both of the wide-type and transgenic mouse brain we determined the down-regulated

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and up-regulated proteins which interacted with specific monoclonal and polyclonal antibodies on the protein microarray surface. The results can be seen in Table 4. Among the up-regulated proteins synuclein-alpha, estrogen receptor, and MAP Kinase-activated phospothreonine are the most important ones. Among the down-regulated proteins cytokeratin 19, 8, 12, caspase 4, and hsp70 showed more than 60% repression. It may be noted here that two different parts of the brain have been used for the microarray (hippocampus) and protein (cerebellum) analysis and hence, these results are not completely comparable though this might give an indication as to the general changes in the gene expression and protein synthesis in the brain.

In order to determine biological significance of differentially expressed genes, functional classification was performed using gene ontology/pathway analysis which revealed that genes involved in neurogenesis and neuronal development showed altered expression in the *fat-1* transgenic mouse (see Fig. 2).

Table 2
Brain total lipid fatty acid composition (molar percent).

Fatty acid	WT Mean ± SD	*Fat-1* mouse Mean ± SD
14:0	0.20 ± 0.00	0.15 ± 0.02
16:0	24.24 ± 0.416	24.50 ± 0.27
16:1	0.66 ± 0.03	0.66 ± 0.05
18:0	19.30 ± 0.07	19.52 ± 0.78
18:1	21.36 ± 0.29	20.78 ± 1.15
18:2 n-6	0.50 ± 0.03	0.65 ± 0.09
18:3 n-6	0.03 ± 0.04	0.06 ± 0.00
18:3 n-3	0.03 ± 0.04	0.01 ± 0.02
20:0	0.31 ± 0.02	0.23 ± 0.08
20:1 n-9	2.09 ± 0.20	2.00 ± 0.62
20:2 n-6	0.14 ± 0.02	0.16 ± 0.03
20:3 n-6	0.44 ± 0.03	0.55 ± 0.05
20:4 n-6	8.91 ± 0.08	8.73 ± 0.58
20:3 n-3	0.00	0.00
20:5 n-3	0.02 ± 0.02	0.18 ± 0.03
22:0	0.12 ± 0.01	0.09 ± 0.03
22:1 n-9	0.16 ± 0.02	0.14 ± 0.03
22:4 n-6	2.61 ± 0.05	2.41 ± 0.11
22:5 n-6	0.26 ± 0.04	0.12 ± 0.02
22:5 n-3	0.14 ± 0.02	0.38 ± 0.03
22:6 n-3	18.43 ± 0.42	18.67 ± 0.53
24:0	0.05 ± 0.07	0.02 ± 0.02
24:1 n-9	0.00	0.00
n-6	12.89	12.68
n-3	18.62	19.24
n-6/n-3	0.69 ± 0.48	0.65 ± 0.45

Values are expressed as Mean ± SD of 2 wild-type and 3 *fat-1* mice.

4. Discussion

It is known that modifications induced in the cell membrane by altering its fatty acid composition can affect a number of cellular functions, including carrier-mediated transport, the properties of certain membrane-bound enzymes, binding to the insulin and opiate receptors, phagocytosis, endocytosis, depolarization-dependent exocytosis, immunologic and chemotherapeutic cytotoxicity, prostaglandin production, and cell growth [21]. For instance, cell membrane enrichment with oleate and linoleate increased the rate of low-density lipoprotein degradation in mononuclear cells due to an increase in membrane fluidity. On the other hand, stearate enrichment did not have any effect on uptake or degradation of low-density lipoprotein, nor did it affect membrane fluidity. This suggests that one mechanism by which unsaturated fatty acids reduce low-density lipoprotein is by their

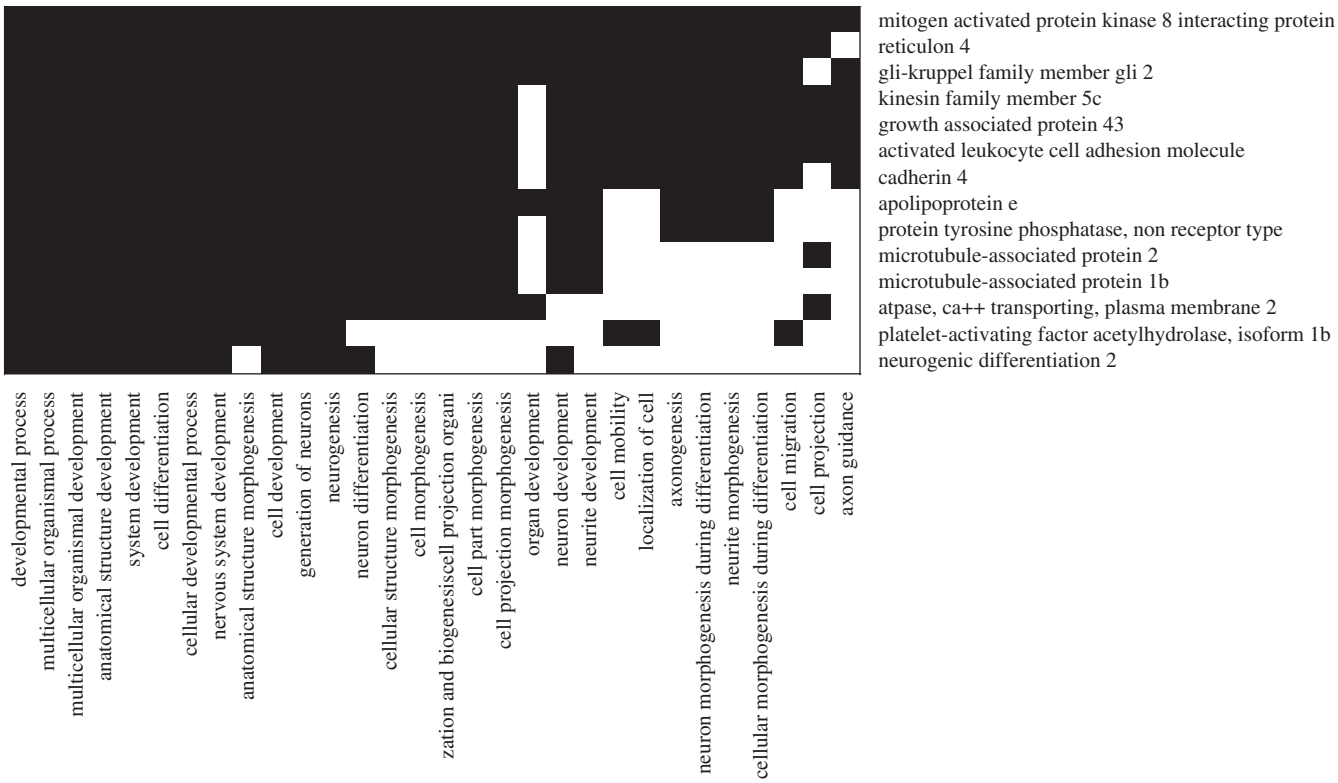

Fig. 2. Scheme showing gene ontology/pathway analysis of genes that showed altered expression in the *fat-1* transgenic mouse. Gene ontology analysis determines the biological significance of differentially expressed genes that can be used to determine the functional classification of the genes whose expression has been up-regulated/down-regulated. Black squares indicate that there is no relevance with the functional class indicated whereas green boxes are related to the functional classes indicated. The data shown here suggests that genes involved in neurogenesis and neuronal development are predominantly altered in the brain of *fat-1* mouse.

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Table 3
Differential gene expression data determined by DNA-microarrays *fat-1* vs. *WT* mouse hypocampus and QRT-PCR (italic, >2-fold repression; bold, >2-fold overexpression, bold italic between 1.5 and 2-fold repression).

Acc. number	Gene product	MA	SD	QPCR	SD	Confirm
NM_009128	Stearoyl-coenzyme A desaturase 2 (Scd2)	*−7.20*	1.43	***−1.84***	0.32	Yes
NM_008963	Prostaglandin D2 synthase (brain) (Ptgds)	*−5.33*	0.67	*−4.06*	0.52	Yes
NM_008791	Purkinje cell protein 4 (Pcp4)	*−4.25*	2.23	*−2.75*	0.31	Yes
NM_024441	Heat shock protein 2 (Hspb2)	*−2.90*	1.55	***−1.49***	0.09	Yes
NM_007470	Apolipoprotein D (Apod)	*−2.63*	0.75	*−6.28*	0.21	Yes
NM_025367	Sphingosine kinase 1 (Sphk1), transcript variant 2	*−2.29*	1.13	*−4.53*	0.53	Yes
NM_008512	Low-density lipoprotein receptor-related protein 1 (Lrp1)	*−2.21*	0.76	***−1.51***	0.32	Yes
NM_172266	Lysophosphatidylglycerol acyltransferase 1 (Lpgat1)	*−2.16*	0.85	***−1.58***	0.40	Yes
NM_177845	Phospholipase A2, group IVE (Pla2g4e)	*−5.52*	1.86	*−9.25*	0.44	Yes
NM_007878	Dopamine receptor 4 (Drd4	*−3.51*	0.51	*−4.03*	0.52	Yes
NM_016775	DnaJ (Hsp40) homolog, subfamily C, member 5 (Dnajc5)	*−2.02*	0.76	***−1.72***	0.06	Yes
NM_009508	Solute carrier family 32 (GABA ves. transporter), (Slc32a1)	*−2.00*	0.05	*−2.36*	0.08	Yes
NM_153193	Hydroxysteroid dehydrogenase-2, delta<5>-3-beta (Hsd3b2)	**8.44**	1.39	**2.69**	0.08	Yes
NM_172579	Signal-induced proliferation-associated 1 like 1 (Sipa1l1)	**3.08**	0.29	**3.32**	0.07	Yes
NM_020486	Basal cell adhesion molecule (Bcam)	*−2.73*	0.86	***−1.59***	0.11	Yes
NM_009917	Chemokine (C-C motif) receptor 5 (Ccr5)	*−2.38*	0.48	***−1.85***	0.11	Yes
NM_026769	Dopamine receptor D1 interacting protein (Drd1ip)	**2.68**	1.60	**2.28**	0.52	Yes
NM_011068	Peroxisomal biogenesis factor 11a (Pex11a)	*−3.55*	1.37	**1.42**	0.52	No
NM_008302	Heat shock protein 1, beta (Hspcb)	*−2.55*	1.90	**1.38**	0.44	No
NM_008071	Gamma-aminobutyric acid (GABA-A) receptor, b3 (Gabrb3)	*−2.12*	1.90	1.11	0.11	No
NM_007600	Calpain 1 (Capn1)	**3.53**	0.53	1.35	0.08	No
NM_207208	Chloride channel calcium-activated 6 (Clca6)	**4.65**	1.45	*−2.11*	0.07	No
NM_009371	Transforming growth factor, beta receptor II (Tgfbr2)	**2.52**	0.38	*−2.60*	0.11	No
NM_008254	3-hydroxy-3-methylglutaryl-coenzyme A lyase (Hmgcl)	**2.90**	1.02	*−1.37*	0.15	No

MA = microarray; SD = standard deviation.

physical effects on cell membranes as it relates to metabolism of the lipoprotein [22].

Of the two types of PUFAs, n-3 has been reported to be more beneficial compared to n-6. This is in part attributed to the fact n-6 PUFAs form precursor to pro-inflammatory eicosanoids whereas those derived from n-3 are less pro-inflammatory and hence, have more favorable effects in the body [1–5]. In the light of this, *fat-1* transgenic mouse model is ideal to study the effects of tissue n-6/n-3 ratio in the body that would enable to clearly delineate the beneficial and harmful effects of various PUFAs. We hypothesized that increase in tissue concentrations of n-3 fatty acids may alter specific gene expression especially in the brain related to different cellular functions [6–8].

We have also analyzed the proximal promoters and distant regulatory elements (REs) such as enhancers, repressors, and silencers of the affected genes in the *fat-1* transgenic mouse. The top 10 hits of the DNA-binding factors are listed in Fig. 1 of which hepatocyte nuclear factor 4-alpha (HNF4) showed significant alteration in its expression (Fig. 1C) in the *fat-1* transgenic mouse. HNF4 binds to DNA sites required for the transcription of alpha 1-antitrypsin, apolipoprotein CIII, transthyretin genes, and HNF1-alpha. HNF4 concentration is high in liver, much lower in islets of Langerhans, endocrine pancreatic tumors, and cultured insulinoma cells, and is scarcely detectable in adult exocrine pancreas [23] and is known to play a critical role in regulating the expression of many genes essential for normal functioning of liver, gut, kidney, and pancreatic islets. A nonsense mutation (Q268X) in exon 7 of the HNF4-alpha gene is responsible for an autosomal dominant, early-onset form of non-insulin-dependent diabetes mellitus (maturity-onset diabetes of the young; gene named MODY1) [24]. MODY1 mutant protein showed that it has lost its transcriptional transactivation activity, fails to dimerize and bind DNA, implying that the MODY1 phenotype is because of a loss of HNF4alpha function. Further studies revealed that several genes encoding components of the glucose-dependent insulin secretion pathway such as glucose transporter 2, and the glycolytic enzymes aldolase B and glyceraldehyde-3-phosphate dehydrogenase, and liver pyruvate kinase expression is dependent upon HNF4-alpha. In addition, the expression of the fatty acid-binding proteins and cellular retinol binding protein also are down-regulated in the absence of HNF4-alpha, suggesting that HNF4-alpha is critical for regulating glucose transport and glycolysis and in doing so is crucial for maintaining glucose homeostasis [24]. In this context, it is interesting to note that we previously showed that both n-3 and n-6 fatty acids prevent chemical-induced diabetes mellitus in experimental animals [25,26]. This beneficial action of PUFAs could be attributed to their ability to alter the expression of HNF4-alpha since it (HNF4-alpha) plays a critical role in glucose homeostasis. This is supported by the observation that PUFAs suppress the glucose-6-phosphatase gene transcription by inhibiting the binding of HNF4-alpha to its cognate sites in the presence of polyunsaturated fatty acyl-CoA thioesters [27]. Furthermore, fatty acids are endogenous ligands for HNF4-alpha [28,29] that establishes a framework for the interaction between PUFAs and HNF4-alpha activity. In addition, HNF4-alpha is known to be down-regulated in renal cell carcinoma and has been shown to inhibit cell proliferation in the embryonic kidney cell line HEK293. HNF4-alpha regulates at least 14 genes that contribute to its inhibitory effect on cell proliferation, including well-known cancer genes, such as CDKN1A (p21), TGFA, MME (NEP), ADAMTS1, SEPP1, THEM2, BPHL, DSC2, ANK3, ALDH6A1, EPHX2, NELL2, EFHD1, and PROS1 [30]. Thus, HNF4-alpha is a multifaceted cell-specific transcription factor. Transcription factor CEBPa (for CCAAT/enhancer binding protein-α; encoded by the gene *CEBPA*) that is crucial for the differentiation of granulocytes also binds to the promoter and modulate the expression of the gene encoding leptin, a protein that plays an important role in body weight homeostasis [31].

Paired box protein Pax-8 is a cell differentiation-specific transcription factor and may play a regulatory role in mammalian development [32]. Two signal transducer and activator of transcription factors (STAT3 and STAT5B) are also found abundantly in the genes with altered expression. STAT3 binds to the interleukin-6 (IL-6)-responsive elements identified in the promoters of various acute-phase protein genes [33].

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Table 4
Proteins that are either up-regulated or down-regulated in the brain of *fat-1* transgenic mouse.

	Fold change
Down-regulated proteins	
Caspase 4	−1.74
Connexin 32	−1.71
HSP70	−1.69
Cytokeratin 19	−1.64
Cytokeratin 8.12	−1.63
TRF1	−1.59
cdk6	−1.56
Desmin	−1.v4
Cytokeratin 13	−1.48
Cytokeratin 7	−1.46
Calmodulin	−1.46
pan Cytokeratin	−1.45
Nedd8	−1.43
aCatenin	−1.43
Up-regulated proteins	
Phospolipase A2 group V	1.40
FAK Phospo (pY577)	1.45
Nicastrin	1.46
b-NOS	1.46
CRK-L	1.47
S-100	1.47
SGK	1.48
Caveolin1	1.49
Bcl-xl	1.50
ARTS	1.50
i-NOS	1.51
CAM Kinase IV	1.52
PTEN	1.52
PAR4	1.52
Neurofilament 200	1.54
Gamma Tubulin	1.56
MAP Kinase(Erk1)	1.56
Phospolipase c gamma 1	1.56
MAPK-activated protein kinase-2	1.56
EGF receptor	1.58
MAP Kinase-activated phospotyrosine	1.58
ERK5	1.58
Protein phosphatase 1	1.60
p35	1.60
PAK phospo (Ps212)	1.60
PKC alfa	1.62
PKD	1.62
Glutamate receptor NMDAR 2a	1.63
S-100 beta	1.63
Protein phosphatase 1	1.64
FAK Phospo (pS772)	1.65
DOPA Decarboxylase	1.66
PKC gamma	1.66
NFKB	1.67
JNK-activated diphospo	1.69
Tau Phospho (pS199/202)	1.79
Synuclein monoclonal	1.79
Estrogen receptor	1.81
GRB-2	1.95
MAP Kinase-activated phospothreonine	1.98

It is evident from the results of the present study shown in Figs. 1 and 2 and Tables 1–4 that there is not only a significant change in the concentrations of various n- fatty acids in the *fat-1* transgenic mouse compared to wild-type but also a specific alteration in the expression of certain genes that seem to play critical role in various diseases. For instance, in the present study, we noted a significant alteration in the expression of stearoyl-Coenzyme A desaturase 2 (Scd2) that has been documented to play a role in Alzheimer's disease (AD). Uryu et al. [34] reported that stearoyl-coenzyme A desaturase-1 (SCD-1), lipogenetic enzyme, was specifically up-regulated in macrophages exposed to amyloid-A and interferon-γ suggesting of a link between AD and SCD-1. This is further substantiated by the report of Unger et al. [35] who noted that when gene expression patterns between the frontal cortical (Fc) and hippocampal (Hc) transcriptomes of wild-type, humanized presenilin-1 (PS1 [wt hPS1]) and Alzheimer's disease-linked DeltaE9 hPS1 mutant mice was studied, a subset of genes showed disturbed regional Fc-Hc gene expression ratios in the transgenic mice carrying the DeltaE9 hPS1 mutation especially for stearoyl-Coenzyme A desaturase-2 (Scd2) and prostaglandin D2 synthase (PGDS) that have been implicated in the pathology of AD. In this context, it is interesting to note that both n-3 and n-6 PUFAs regulate proteolytic maturation of sterol regulatory element binding proteins (SREBPs) that accounts for their ability to suppress hepatic lipogenic gene expression. PUFAs regulate the stearoyl-coenzyme A desaturase gene, a key enzyme in the cellular synthesis of monounsaturated fatty acids from saturated fatty acids, indicating that PUFA suppress gene transcription by a mechanism independent of SREBP maturation [36]. Stearoyl-coenzyme A desaturase (SCD) gene expression is up-regulated by diets high in saturated fatty acids whereas PUFAs suppress its expression. High-density glass microarray analysis of approximately 7800 genes confirmed that the expression of several key genes involved in cholesterol metabolism, fatty acid beta-oxidation and lipogenesis were affected by diet rich in DHA. Of all the genes studied stearoyl-coenzyme A (Delta-9) desaturase, a key enzyme involved in the regulation of triglyceride biosynthesis and secretion, was the most suppressed gene on treatment with DHA. In addition, DHA also decreased the expression of farnesoid X receptor and thus, n-3 PUFAs could lower plasma triglyceride and cholesterol levels [37]. In this context, it is noteworthy that n-3 PUFAs are useful both in the prevention and treatment of AD [38–40]. In *fat-1* transgenic mouse, the expression of caspase-4 is down-regulated suggesting that n-3 fatty acids could suppress apoptosis of neurons and thus, are useful in AD. Furthermore, prostaglandin D synthase activity is known to be increased in patients with Alzheimer's disease whereas n-3 PUFAs suppress its activity [41,42], which lends additional support to their beneficial action in AD. In addition, gene ontology/pathway analysis on the microarray output list (see Fig. 2) revealed that there is a significant alteration in the expression of genes in the *fat-1* transgenic mouse that are involved in neurogenesis and neuronal development that further implicates the role of n-3 PUFAs in AD and other neurological conditions. A similar analysis was undertaken for the protein array data (see Table 4) that indicated that caspase 4, HSP70, and dystrophin proteins are down-regulated whereas phospholipase A2, NOS, PTEN, and MAPK-activated protein kinase-2 proteins are up-regulated which indicates that proteins that are essential for neuronal survival and function are enhanced in the brain of *fat-1* transgenic mouse. This indicates that increase in w-3 PUFAs enhance the survival and function of neurons in the brain.

Prostaglandin that have pro-inflammatory actions are believed to play a major role in inflammatory conditions such as bronchial asthma, collagen vascular diseases (such as rheumatoid arthritis, lupus), sepsis, infections [43–46], AD, type 2 diabetes mellitus, hypertension, obesity, depression, and schizophrenia since plasma levels of IL-6, TNF-α, and high sensitive C-reactive protein (hs-CRP) are increased in them [47–50]. In these conditions prostaglandin metabolism is altered [51,52] whereas n-3 PUFAs are of significant benefit [43–46,53] in view of their anti-inflammatory action.

Activation of phospholipase A_2 (PLA_2) induces the release PUFAs from the cell membrane lipid pool for the formation of various pro-inflammatory eicosanoids. Hence, enhanced activity of PLA_2 could be considered as a marker of inflammation. In the present study, we noted that in *fat-1* transgenic mouse the

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expression of PLA_2 is decreased. It is known that PLA_2 activity is increased in AD, type 2 diabetes and hypertension, emphasizing the pro-inflammatory nature of these conditions [54–56]. Thus, the observed decreased expression of PLA_2 in *fat-1* transgenic mice supports the belief that n-3 PUFAs bring about their anti-inflammatory action, at least in part, by suppressing the activity of PLA_2.

Abnormalities in dopamine receptor have been noted in Alzheimer's disease, attention deficit hyperactivity disorder (ADHD) and other neurological conditions, type 2 diabetes and obesity [57–61] in which n-3 PUFAs are known to be of some benefit. This implies that n-3 PUFAs could have regulatory role in dopamine metabolism. This is supported by the observation that n-3 PUFAs do indeed modulate dopamine receptor expression, metabolism, and action [62–64].

In addition, in the present study, alterations in the expression of genes concerned with apolipoprotein D that has a role in AD [65], and sphingosine kinase that modulates cell cycle and apoptosis [66], hydroxysteroid dehydrogenase-2 that is involved in the pathobiology of obesity and insulin resistance [67] was also noted. Sphingosine kinase not only participates in apoptosis but also induces the production of pro-inflammatory prostaglandins by stabilizing cyclo-oxygenase-2 enzyme [66] suggesting that there is a close interaction between these two (sphingosine kinase and prostaglandins) pathways. Hydroxysteroid dehydrogenase plays a role in abdominal obesity and is negatively associated with insulin resistance [67]. It is interesting to note that Alzheimer's disease is considered as type 3 diabetes since significant insulin resistance is seen in the brain of these patients [68]. Furthermore, n-3 PUFAs interact with several genes and modulate their expression that could explain their beneficial actions in various conditions. These results emphasize the close interaction between various genes and their products with n-3 PUFA and how such an interaction is relevant to various diseases.

Synuclein-alpha protein was found to be differentially expressed and up-regulated in *fat-1* transgenic mouse when protein microarray study was performed. Synuclein-alpha forms insoluble cytoplasmic aggregates in Parkinson's disease (PD) and other neurodegenerative disorders. Synuclein-alpha has biochemical properties and a structural motif that renders it a novel member of the fatty acid-binding protein family and thus, may transport fatty acids between the aqueous and membrane phospholipid compartments of the neuronal cytoplasm [69]. Recombinant synuclein-alpha forms multimers *in vitro* upon exposure to vesicles containing physiological concentrations of arachidonic acid and docosahexaenoic acid and this process occurred much faster than in aqueous solution [70]. Furthermore, exposure of living mesencephalic neuronal (MES) neurons to PUFAs increased synuclein-alpha oligomer levels, whereas saturated fatty acids decreased them. PUFAs directly promoted oligomerization of recombinant synuclein-alpha, suggesting that synuclein-alpha interacts with PUFAs *in vivo* to promote the formation of highly soluble oligomers that precede the insoluble synuclein-alpha aggregates associated with neurodegeneration [71]. But, it is not clear as to how normally soluble synuclein-alpha protein assembles into insoluble aggregates associated with neuronal dysfunction. It was reported that highly soluble oligomers of synuclein-alpha could be detected in normal brain supernatants and their augmentation in Parkinson's disease and dementia with Lewy bodies' brains. Since PUFAs enhanced synuclein-alpha oligomerization in intact mesencephalic neuronal cells, further studies led to the observation that elevated PUFA levels occur Parkinson's disease and Lewy bodies' brain soluble fractions. Higher PUFA levels were also detected in the supernatants and high-speed membrane fractions of neuronal cells overexpressing wild-type or Parkinson's-causing mutant synuclein-alpha. The increased PUFA content in the membrane fraction was accompanied by increased membrane fluidity in the synuclein-alpha overexpressing neurons, whereas membrane fluidity and the levels of PUFAs were decreased in the brains of mice genetically deleted of synuclein-alpha. These results suggest that synuclein-alpha–PUFA interactions regulate neuronal PUFA levels as well as the oligomerization state of synuclein-alpha both under normal conditions and in Parkinson's disease [72]. In contrast, there is evidence to indicate that synuclein-alpha accumulation is associated with neuroprotection from oxidative stress [73]. Furthermore, synuclein-alpha reduced AA turnover, whereas synuclein-alpha ablation increased the incorporation of DHA and its turnover in brain phospholipids [74,75] suggesting that AA is not harmful whereas DHA could produce oligomerization of synuclein-alpha and thus, may have a role in Parkinson's disease. In the present study we observed that synuclein-alpha protein was up-regulated in *fat-1* transgenic mouse. These and other results [69–75] suggest that there is a dynamic equilibrium maintained between various PUFAs and synuclein-alpha and thus, both n-3 and n-6 fatty acids may play a vital role in the pathogenesis of Parkinson's disease and other neurodegenerative conditions.

In summary, based on the results of the present study in *fat-1* transgenic mouse and other studies, it is clear that n-3 PUFAs modulate the expression of several genes concerned with inflammation, apoptosis, cell cycle, neurotransmitters, and hormones and thus bring about their actions. In this context, the close interaction between n-3 PUFAs and their products and cytokines, pro-inflammatory eicosanoids, insulin, neurotransmitters, PLA_2, sphingosine kinase, caspases, farnesoid X receptor, PPARs, SREBPs, HMG-CoA reductase, transforming growth factor, cytokeratins, and nitric oxide synthase seems to be important (see Figs. 1 and 2 and Tables 1–4 for the genes that are modulated in *fat-1* transgenic mouse). Hence, future studies need to be performed that delineate the interaction between n-PUFAs and the molecules involved in the inflammatory, cytokine, and neurotransmitter pathways to get a handle on the pathophysiological role of these fatty acids in health and disease.

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