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14. ABSTRACT
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Contributions of polyunsaturated fatty acids (PUFA) on cerebral neurobiology: an integrated omics approach with epigenomic focus

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Abstract

The epigenetic landscape is vulnerable to diets. Here, we investigated the influence of different polyunsaturated fatty acids (PUFA) dietary supplements on rodents' nervous system development and functions and potential consequences to neurodegenerative disorders. Our previous nutrigenomics study showed significant impact of high n-3 PUFA-enriched diet (ERD) on synaptogenesis and various neuromodulators. The present study introduced a second equicaloric diet with n-6 PUFA balanced by n-3 PUFA (BLD). The typical lab diet with high n-6 PUFA was the baseline. Transcriptomic and epigenetic investigations, namely microRNA (miRNA) and DNA methylation assays, were carried out on the hemibrains of the C57BL/6j mice fed on any of these three diets from their neonatal age to midlife. Integrating the multiomics data, we focused on the genes encoding both hypermethylated CpG islands and suppressed transcripts. In addition, miRNA: mRNA pairs were screened to identify those overexpressed miRNAs that reduced transcriptional expressions. The majority of miRNAs overexpressed by BLD are associated with Alzheimer's and schizophrenia. BLD implicated long-term potentiation, memory, cognition and learning, primarily via hypermethylation of those genes that enrich the calcium-releasing neurotransmitters. ERD caused hypermethylation of those genes that enrich cytoskeletal development networks and promote the formation of neuronal precursors. We drew the present observations in light of our limited knowledge regarding the epigenetic influences on biofunctions. A more comprehensive study is essential to understand the broad influences of dietary supplements and to suggest optimal dietary solutions for neurological disorders.

1. Introduction

Dietary compositions can induce the epigenomic changes and, consequently, can perturb the long-term physiological and psychological health. Lasting implications of the prenatal diet on the offspring's health have been demonstrated *in vivo* [1–3]. Human epidemiological studies have found that the epigenetic signatures in individuals conceived during the Dutch Hunger Winter of 1944–45 persisted throughout their lives [4,5].

DNA methylation is a major epigenetic mechanism that can potentially control gene expression, genetic imprinting and genomic stability *via* mitotic and meiotic mechanisms [6]. DNA methylation requires choline, other methyl donors and sufficient amounts of energy [7,8]. Hence, foods enriched with or deprived of such supplements enable control of the methylation process [9,10]. Fish oils derived from salt water fish are choline enriched [11] and, therefore, potentially facilitate DNA methylation. Polyunsaturated fatty acids (PUFA)-enriched maternal diets were found to induce higher methylation in the offspring's gene body, which was further

linked to homeostasis in cellular and humoral immunity, and to the growth and development of children [12,13]. The details of the mechanisms of these processes and the consequences are still poorly understood. In fact, the role of methylation in regulating gene silencing has been recently critically challenged [6,14–16].

Noncoding microRNA (miRNA) mediates another class of epigenetic mechanisms. Evidence suggesting polymorphic characteristics of miRNA-mRNA interactions [17] endorses miRNAs as distinct post-translational modifiers. The miRNAs can influence a wide range of functions *via* suppressing the stability of targeted mRNA and augmenting its degradation. The causal association of miRNA and DNA methylation toward epigenetic manifestation is a subject of growing interest [8,18]. As with DNA methylation, the diet plays a key role in miRNA expression [19]. Of particular interest are the reports suggesting the role of omega-3 PUFA (n-3 PUFA) in modulating a pool of miRNAs that control the oncogenic and apoptotic networks [20–22].

The benefits of n-3 PUFA have been evaluated in the context of a wide variety of health issues [23]. The escalated risks of pathological and psychological disease have been connected to the greatly reduced proportion of n-3 PUFA in modern western diets in concert with the growing proportion of omega-6 PUFA (n-6 PUFA) [24–26]. However, time and again, clinical studies have failed to confirm the beneficial consequences of n-3 PUFA [27,28]. In this context, we demonstrated

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the contrasting implications of the n-3 PUFA-enriched diet (ERD) as compared to the standard diet *in vivo* [29]. We reported that n-3 PUFA-enriched diet resulted in attenuation of oxidative stress and reduction of amyloidal burden coupled with suppressed inflammatory and apoptosis networks. This suggests that n-3 PUFA-enriched diets have possible beneficial impacts on Alzheimer's disease, Parkinson's disease and affective disorder. ERD-mediated hyperactive dopaminergic, adrenergic, cholinergic and GABAergic networks have differential implications; these neuromodulators can ameliorate depression but can also trigger bipolar disorder, schizophrenia and suicidal inclination. Since the high concentration of n-3 PUFA may adversely impact certain psychological disorders, it becomes imperative to find an optimal concentration of n-3 PUFA to derive its full benefits.

To meet this challenge, we introduced an additional diet type containing a balanced ratio of n-3 and n-6 PUFA (BLD), where absolute amounts of both PUFAs were much higher than in the standard lab diet (STD) (Table 1A). All three diets used in this project have a similar solid texture and physical appearance, in addition to being equicaloric as constituted by equivalent amounts of proteins, carbohydrates, vitamins and minerals.

The purpose of the present study is to identify the epigenomic shifts caused by the diets supplemented with varying amounts of n-3 and n-6 PUFAs that were consumed by mice from their neonatal age to middle age. This age group was identified as particularly and increasingly susceptible to major health issues, including psychological disorders [30,31]. In interpreting the omics readouts of brains from these mice, we focused our investigation on the hypermethylated CpG islands (CpGI) mapped to the genes with suppressed transcripts. We also included the overexpressed miRNAs targeting the genes showing transcriptomic suppression. Finally, we identified the patterns connecting the suppressed transcripts, hypermethylated CpGIs and overexpressed miRNA profile. These genes and miRNAs were annotated to the networks associated with nervous system development and functions and psychological and neurological disorders. This pilot experiment showed the differential effects of diets captured at the epigenetic level. The study further underscored the need for more comprehensive nutrigenomics studies to understand the long-term impacts of diets.

2. Materials and methods

2.1. Diet composition

The details of ERD and STD diets are discussed in our previous communication [29]. Briefly, 16% by weight of PUFA was used in ERD and BLD diets (Harlan Laboratories, Inc., MD, USA) so that the appearance and structure of the food pellets would be consistent with that of STD (Certified Rodent Diet 5002*, Purina LabDiet, MA, USA). The resulting food pellets preempted individual prejudice for the smell and texture of any of the particular diets. Furthermore, their palatable structures helped maintain the tidiness of the cages. Hence, all of the habitants followed the same schedule of handling and caging. Given practice minimized any potential risk of cerebral plasticity attributed to the mouse handling with variable frequencies [32].

Within the limits of total PUFA percentage, we varied their sources in order to change the n-3 and n-6 PUFA ratios from the maximum possible (6:1 in ERD) to a balanced measure (1:1 in BLD). We composed the BLD with the following composition: 4% fish oil, 4% flaxseed oil and 8% safflower oil. While fish oil (n-3: n-6 FA=8:1) and safflower oil (n-3: n-6 FA = nil: 78) primarily contributed n-3 and n-6 PUFA, respectively, flaxseed oil (n-3: n-6 PUFA=3.7:1) contributed both types of PUFAs to meet the balance. Table 1B demonstrates that these three diets were similarly

supplemented by proteins, carbohydrates and crude fibers; vitamins and minerals were also supplemented proportionately, including vitamin E. Moreover, the gross caloric contributions were comparable. To maintain the food quality across the study duration and to minimize the oxidative damage to n-3 PUFA, freshly prepared food was purchased every 2 months and stored at 4ºC in sealed bags, and the food chambers were replenished daily with fresh supplies.

2.2. Animal model and nucleic acid extraction from hemibrains

Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (NRC 2011) in facilities that are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. The protocol was approved by the IACUC committee of Walter Reed Army Institute of Research, Silver Spring, MD, USA.

C57BL/6j male mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) at the age of 3 weeks, singly housed, randomly and equally sorted into three groups and immediately introduced to one of the three diets, namely ERD, BLD and STD. Henceforth, each diet group contained six animals. Mice had free access to their respective diets and standard lab-supplied liquids for the next 5 months or until they reached 25 weeks of age. At that time, the animals were euthanized by cervical dislocation without using any anesthesia. This method was AVMA Panel on euthanasia — approved and implemented to minimize the potential confounding effects of anesthetic agents on the brain molecular landscape [33]. The left hemibrains were collected for multiomics analysis. For nucleic acid extraction, ice cold hemibrain in Trizol (Invitrogen Inc., MA, USA) was homogenized, and total RNA and DNA were phase separated. The miRNAeasy mini kit from QIAGEN (MD, USA) was used to isolate total RNA that includes miRNA (RNA of 20–40 bases long) and mRNA following the vendor's protocol. DNA was isolated from the interphase and phenol-chloroform phase by ethanol precipitation and washed multiple times with sodium citrate/ethanol solution (0.1-M sodium citrate in 10% ethanol, pH 8.5). The resulting DNA pellet was air-dried and resuspended in 8-mM NaOH. We used Tape Station (Agilent Technologies, Inc., CA, USA) to check the qualities of nucleic acids. The samples showing RIN and DIN values >8.0, respectively were selected for further analysis.

2.3. Transcriptomic assay and analysis

The dual dye microarray was carried out using the SurePrint G3 mouse GE 8×60k microarray kit (Agilent Technologies, Inc., CA, USA) following the vendor's protocol and our methods previously reported [29]. Cy-5 labeled 200 ng of purified RNA was cohybridized with the reference RNA (Agilent Technologies, Inc., CA, USA) labeled with Cy-3 dyes. Overnight hybridization at 55 °C was followed by a series of washes. The slides were scanned using an Agilent DNA microarray scanner, and the features were extracted using the default setting of the Feature Extraction software (Feature Extraction software v.10.7, Agilent Technologies, Inc., CA, USA).

GeneSpring v.10.1 (Agilent Technologies, Inc., CA, USA) software was used to conduct the preliminary data filtration and statistical analysis. Each chip was subjected to intrachip normalization using the locally weighted scatter-plot smoothing method (LOWESS). Pair-wise moderated t tests at a cutoff of $P<.05$ and a ± 1.5-fold change were used to identify differentially expressed probes from among 62,976 probes anchored to the array that represented the whole mouse genome.

2.4. Epigenomic assay and analysis

The epigenomic analysis of DNA methylation profiles was performed using methylated DNA immunoprecipitation (MeDIP) followed by Agilent's Mouse 105 K CpG island microarray (Design ID: 015279). The array contains 88,737 probes in or within 95 bp of the CpG islands (CpGI) covering 15,342 CpGIs embedded in 16,664 unique genes.

MeDIP was initiated by preparing an antibody/magnetic bead mixture according to the manufacturer's protocol. Genomic DNA was sonicated to 200–1000-bp size using

Table 1A
Distribution profile of major dietary components of ERD, BLD and STD

	ERD		BLD		STD	
	% by weight	% kcal	% by weight	% kcal	% by weight	% kcal
Protein	18.3	17.2	18.3	17.2	23.9	24.11
Carbohydrate	51.8	48.8	51.8	48.8	53.8	62.67
Fat	16	33.9	16	33.9	5.7	13.22
Kcal/g	4.2		4.2		4.07	

Table 1B
Distribution profile of n-3 and n-6 PUFA in ERD, BLD and STD

		ERD	BLD	STD
		% of fat	% by weight	% by weight
n-3 PUFA	EPA	2.08	0.72	0.2
	DHA	1.6	0.6	
	ALA	0.29	3.12	
n-6 PUFA	LA	0.29	4.82	1.22
	AA	0.37	0.128	<0.01
n-3 PUFA: n-6 PUFA		7:1	1:1	1:6
EPA:DHA		1.5:1	1.2:1	–
EPA:AA		6:1	6:1	>20

the Sonics Vibra-Cell™ VCX130 Sonicator under standardized conditions. Five micrograms of DNA were added to the antibody-bead complex and incubated overnight at 4 °C. The resulting immunoprecipitated DNA was labeled using Cyanine 5-dCTP, and 250 ng of the corresponding sonicated genomic DNA was labeled using Cyanine 3-dCTP. Purification of labeled products, array hybridization and scanning were performed according to the standard protocol by following the default settings. Data from scanned images (TIFF) were extracted using Feature Extraction software Version 9.0 (Agilent Technologies, Inc., CA, USA).

Genomic Workbench (v 7.0.4) applied the Z-score-based algorithm to identify the methylation events, and after comparing the moving average of log ratio and Z-score data with genomic region tracks, it reported the methylation results. The differentially methylated probes were identified using a moderated t test with a cutoff of $P<.05$. The CpGIs showing 1.3 times more methylation were identified as the hypermethylated probes.

The microarray data were submitted to the Gene Expression Omnibus (GEO). This can be searched using the Platform ID: GSE83543 and GSE83544.

2.5. microRNA sequencing and analysis

One microgram of the RNA isolated from individual hemibrains was used to construct sequencing libraries with the Illumina (Illumina, CA, USA) TruSeq Small RNA Sample Prep Kit, following the manufacture's guideline. Briefly, 3′ and 5′ adapters were sequentially ligated to small RNA molecules, and the ligated products were reverse transcribed, amplified and subsequently size-selected screened by gel purification and by microfluidics using the Agilent Bioanalyzer High Sensitivity DNA chip. The indexed libraries were pooled in equimolar amounts, clustered and loaded into different lanes of a HiScanSQ Illumina instrument to generate 50 base-pair reads. Image analysis and base calling were performed using Illumina pipeline Version 1.5.15.1 and Illumina CASAVA sequencing analysis software Version 1.7.32.0.

The raw sequence reads were processed using a robust miRNA-seq quality control pipeline. First, the samples were evaluated as per quality control assurance using matrices that included acceptable duplication, k-mer or GC content generated using FASTQC. The samples that showed more than 30% disagreement with rest of the samples were excluded henceforth. The low quality reads (>20 quality score threshold) were filtered out, and adaptor sequences were pruned. The ensuing products were 48%–75% mapped against the mature miRBase miRNA database Version 21 for *Mus musculus* (mm9), a pool of RNA species were curated and the effective library sizes were normalized using the trimmed mean of M-values (TMM) normalization method provided by edgeR (Bioconductor.org). In a second phase of filtration, the samples with <1% abundance were also discarded. Meanwhile, we validated the miRBase output by mapping the same filtered sequence reads against the UCSC reference genome for *Mus musculus* (mm9 build) (University of California Santa Cruz; http://genome.ucsc.edu/). The short read aligner Bowtie (v 1.0.0) allowed one pair of mismatch. Differentially expressed miRNAs were mined by pair-wise comparison using moderated t test with the cutoff at $P<.05$.

To determine the mRNA targets of the selected miRNA modulators, we used the Ingenuity Pathway Analysis (IPA) platform (Ingenuity® Systems, www.ingenuity.com). The microRNA Target Filter predicted the mRNA targets by mining four databases, namely TargetScan, TarBase, miRecords and the Ingenuity® Knowledge Base. The list was screened further to identify negatively correlated (r<−0.5) miRNA:mRNA pairs as described elsewhere [34].

2.6. Probe mapping and functional annotations

We mapped the probes anchored to the Agilent CpG island array to locate their positions in the gene body and beyond using the genome browser available at the University of California Santa Cruz (UCSC; http://genome.ucsc.edu/). The probes were characterized as the promoter, divergent promoter, downstream and inside regions based on their distances from the transcription start sites (TSS). Henceforth, we define all the promoters and divergent promoters together as the *promoter*. "Inside" CpGIs are located within 10-kb upstream of TSS; arguably, these probe regions can drive the gene expression mechanism to variable degrees [6,14–16]. The probes located downstream of TSS were not considered for any transcription–methylation-based integration analysis.

The prevalence of the differentially methylated genes in the brain regions were estimated using the mouse brain atlas (mouse.brain-map.org). We used GeneSpring v.10.1 (Agilent Technologies, Inc., CA, USA) to perform two-dimensional hierarchal clustering using the Pearson correlation algorithm, and IPA (www.ingenuity.com) was used to mine the pathways and networks relevant to the present study. Networks meeting $P<.05$ by hypergeometric test were presented for further consideration.

3. Results

Six animals were recruited per diet group, namely *ERD*, *BLD* and *STD*. The animals were fed on the particular diet types for the last 22 weeks of their 25-week life span. The high-fat diet, as expected, increased the body weights: the average bodyweight of ERD mice was 36.5 g at age of 25 weeks [29]. At the same age, BLD mice were 39.6 g on

average, which was significantly higher ($P<.001$, Welch's t test) than the STD mice, whose average weight was 31 g. At 25 weeks of age, the mice were euthanized to collect hemibrains as the source of RNA, DNA and miRNA for the respective analyses.

3.1. Characterization of the differentially methylated probes

Principal component analysis (PCA) of 88,463 differentially methylated CpGIs revealed a considerable separation of methylation status among three diet types (Fig. 1A). Together, the top three principal components explain 57% of the cumulative variance.

Comparing ERD and STD, we identified 13,573 differentially methylated CpGIs annotating 8001 genes at the moderated t test cutoff of $P<.05$. There were 5010 hypermethylated CpGIs representing 3742 genes showing ≤1.3 times more methylation in ERD than STD (Fig. 2A). Likewise, we identified 8798 CpGIs representing 5924 genes differentially methylated between BLD and STD that included 5040 hypermethylated CpGIs annotated to 3720 genes (Fig. 2A).

3.2. Characterization of the differentially expressed transcripts

PCA of 62,976 transcripts displayed a considerable variability of their expressions induced by the three diet types. Together, the three top-ranked principal components explained 42% of cumulative variance (Fig. 1B).

Comparing ERD and STD, we identified 5213 significantly expressed transcripts representing 3100 unique genes. This cluster included 2150 transcripts (1104 genes) overexpressed and 3064 transcripts (2025 genes) suppressed by ERD. Likewise, 4153 probes corresponding to 2236 unique genes were found differentially expressed between BLD and STD, which included 2501 (1245 genes) and 1653 (1009 genes) transcripts overexpressed and suppressed, respectively (Fig. 2A).

3.3. Integration of the epigenetic and transcriptomic readouts

The transcriptomic data were integrated with DNA methylation readouts, and we subsequently focused on the hypermethylated CpGI-bound suppressed transcripts. Thereby, we found 308 genes in ERD-fed mice encoding hypermethylated CpGIs-bound suppressed transcripts. This cluster is defined as Probes of Interest 1 (PoI-1). Nearly 33% of these CpGIs were promoter bound, and the remainders were within the inside region (Fig. 2A). In comparison with STD, BLD perturbed 131 genes encoding hypermethylated CpGIs and suppressed transcripts (PoI-2); 36% of these CpGIs were promoter bound, and the rest were within the inside region (Fig. 2A).

Likewise, we found 206 genes encoding hypermethylated CpGIs and transcriptional suppression caused by ERD in comparison to BLD (PoI-3), which included 40% promoter-bound CpGIs (Fig. S1). In comparison to ERD, BLD hypermethylated 28 genes and suppressed their transcriptional expressions. Eleven of these hypermethylated CpGIs are promoter bound (Fig. S1).

3.4. Characterization of differentially expressed miRNAs and miRNA: mRNA pairs

PCA of 1156 miRNAs that crossed the quality control threshold displayed a considerable separation among three diet types. Together, three top-ranked principal components from each diet explained 80% of cumulative variance (Fig. 1C).

In comparing ERD with STD, a moderated t test ($P<.05$) identified differentially expressed 49 miRNAs targeting 8964 mRNAs. Within this pool of miRNA:mRNA pairs, there were 18 overexpressed miRNAs that significantly suppressed (r<−0.5; $P≤.05$) 580 mRNA targets [34]. Eleven of these miRNAs targeted 10 or more mRNAs (the top five are shown in Table 2). In this cluster of 580 suppressed mRNAs, a subgroup of 109 mRNAs overlapped with PoI-1 (Fig. 2A).
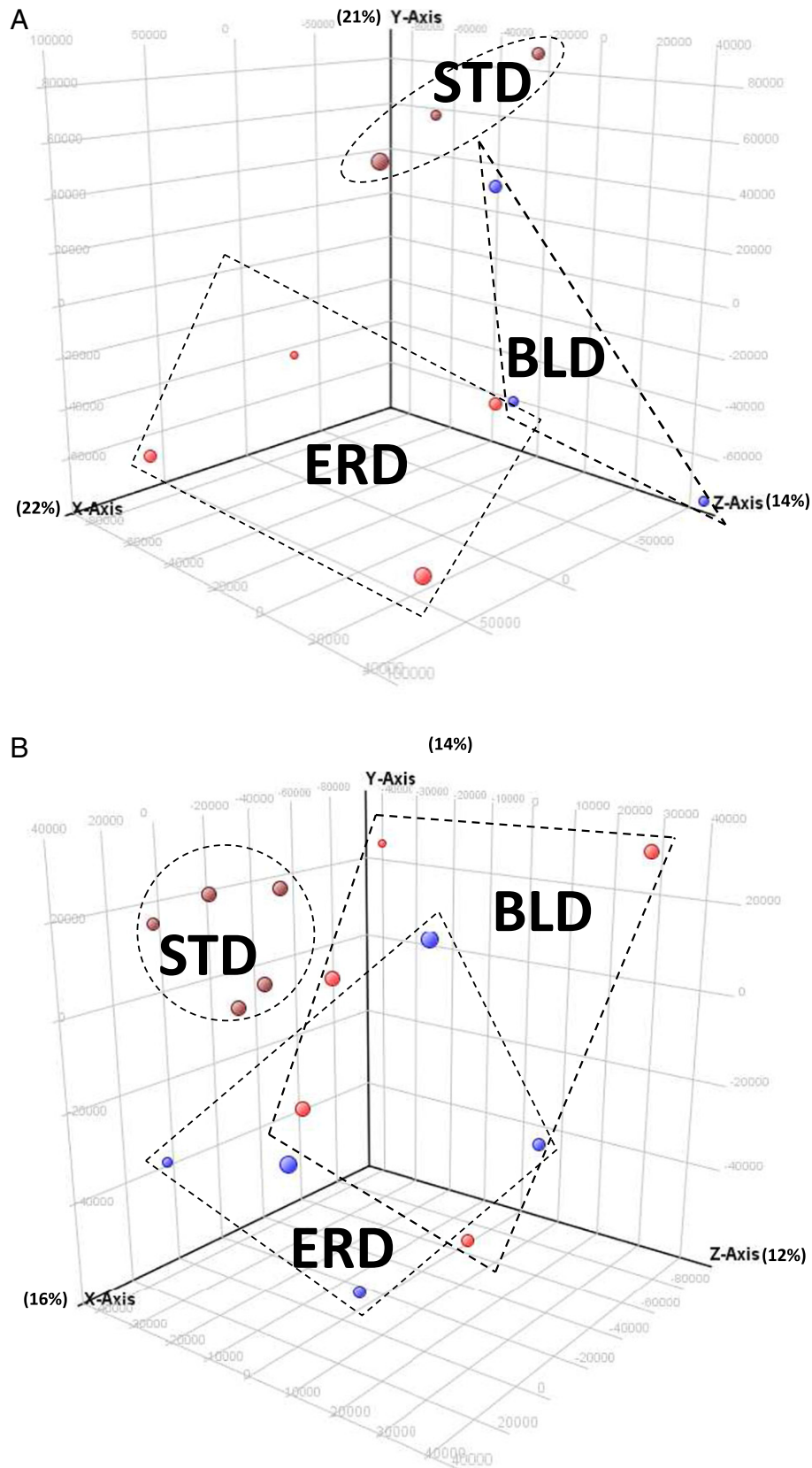
Fig. 1. (A) Principal component analysis (PCA) of the differentially methylated probes (CpG islands) altered by the three diet types. The samples showing DIN values >8.0 were selected for this assay; each colored point represents one animal. (B) Principal component analysis (PCA) of differentially expressed transcripts altered by the three diet types. The samples showing RIN values >8.0 were selected for this assay; each colored point represents one animal. (C) Principal component analysis (PCA) of differentially expressed miRNAs altered by the three diet types. miRNA samples selected for the assay were screened in two steps: First, the samples were evaluated as per quality control assurance using following matrices including acceptable duplication, k-mer or GC content generated using FASTQC. The samples showing more than 30% disagreement with rest of the samples were excluded henceforth. In a second phase of filtration, the samples with <1% abundance were discarded. Each colored point represents one animal.
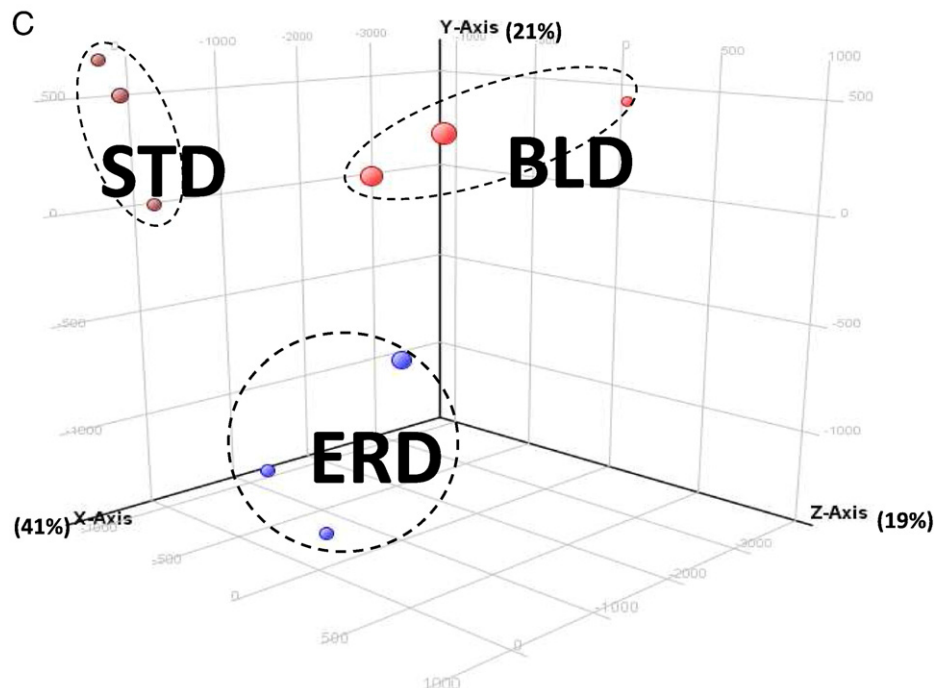
Fig. 1. (*continued*).

Comparison of BLD with STD using a moderated *t* test ($P<.05$) identified 105 differentially expressed miRNAs targeting 11,404 mRNAs. Within this pool of miRNA:mRNA pairs, there were 39 overexpressed miRNAs that significantly suppressed ($r<-0.5$; $P≤.05$) 796 mRNAs. Twenty-nine of these miRNAs targeted 10 or more mRNAs (the top five are shown in Table 2). In this cluster of 796 suppressed mRNAs, a subgroup of 106 mRNAs overlapped with PoI-2 (Fig. 2A).

3.5. Functional annotations

The present communication is primarily focused on the nervous system development and function and neurological and psychological disorders. Functional annotations were performed in two phases. The first phase included those miRNAs which were overexpressed by either ERD or BLD. Most of the miRNAs are associated with organismal injury and abnormalities. Table 2 lists the top five miRNAs ranked by the number of mRNA targets suppressed by individual miRNAs. Fig. 2B shows the miRNAs of PoI-1 and PoI-2 associated with the most enriched functional annotations and disorders. Many studies, including our previous observations [29], linked a fish oil-enriched diet to Alzheimer's disease [25,27,35] and schizophrenia [36,37]. Fig. 3 shows those miRNAs which are epigenetically associated with either of these two neurodegenerative disorders. The majority of these miRNAs are elevated by BLD in comparison to ERD.

The second phase of the functional analysis was focused on the genes listed in PoI-1 through PoI-4. Following the same protocol, we curated the genes associated with nervous system development and function (Table S1A), neurological diseases (Table S1B) and psychological disorders (Table S1C). The patterns displayed how PoI-1 and PoI-2 are associated with memory and relevant functions (Fig. 4A) and brain formation and relevant mechanisms (Fig. 4B). Particular attention was given to the nervous system development and functions, which was further sorted based on their association with (a) cellular functions; (b) cerebral health; and (c) cerebral functions (Fig. 5).
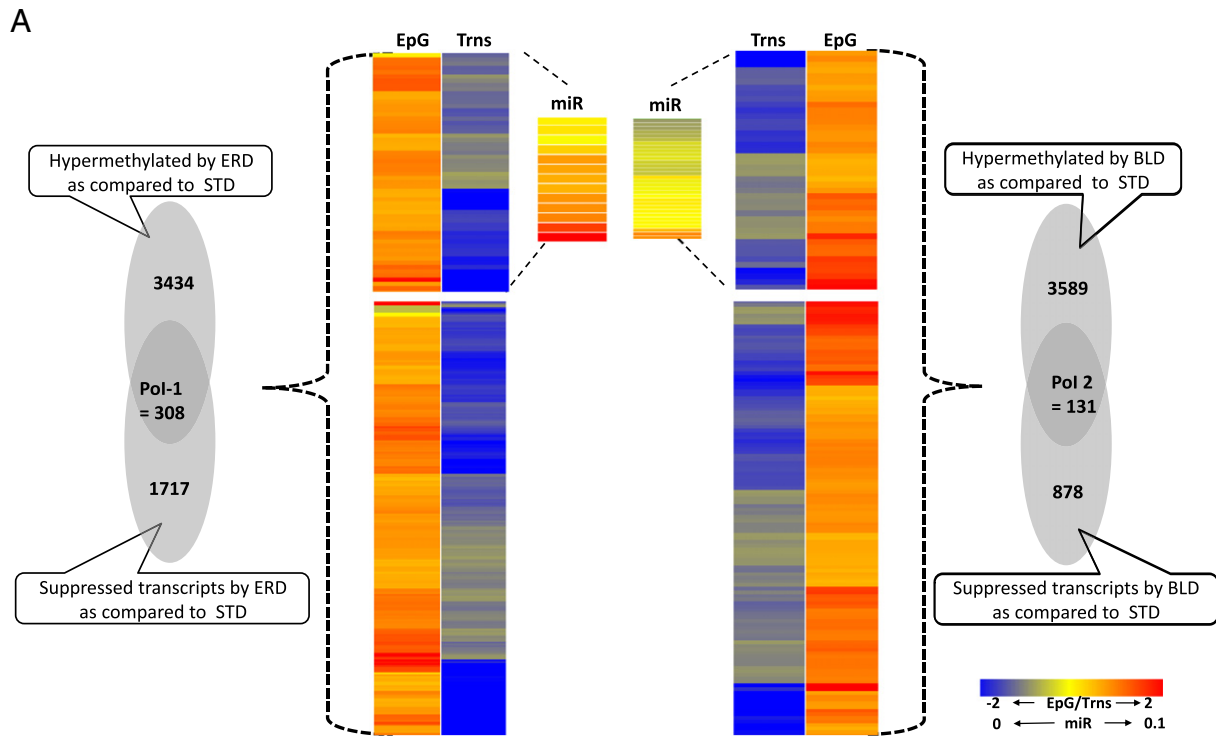
We mapped the genes annotated from PoI-1 through PoI-4 to the brain regions typically associated with stress negotiation. However, very few genes were mapped to the brain regions of interest, and therefore, no additional functional analysis was conducted. PoI-1 to PoI-4 were further mapped to two neural cell types, namely *astrocytes* and *neurons*. This cell-specific database was curated from developing and mature mouse forebrain [38], and we used genes that showed more than twofold changes. From PoI-1, there were 41 and 48 genes overexpressed in neurons and astrocytes, respectively. Similarly, there were 17 and 27 genes in PoI-2 overexpressed in neurons and astrocytes, respectively. Mapping the genes to oligodendrocytes returned a small sample set with negligible statistical power. The same conclusion held true for PoI-3 and PoI-4.

4. Discussion

Our previous study about the transcriptional profile of rodent hemibrain showed a significant impact of a fish oil-enriched diet on nervous system development and neurological diseases [29]. By stimulating the calcium-induced growth cascade and downstream PI3K-AKT-PKC networks, ERD augmented neuritogenesis, reinforced synapse and potentially promoted LTP. Furthermore, ERD reduced the amyloidal burden, attenuated oxidative stress and assisted in somatostatin activation, thereby displaying potential benefits for attenuating Alzheimer's disease, Parkinson's disease and affective disorder. These results motivated us to comprehend the epigenetic consequences of the variable enrichments of n-3 and n-6 PUFA in daily diets.

The present study was designed to understand the epigenetic vulnerability of the brain to diets with maximum enrichment of PUFAs, where n-3 PUFA was either equivalently balanced by n-6 PUFA or not. Hence, both diets contained a maximum amount (*i.e.*, 16% by weight) of PUFA that allowed the food pellets to retain the same solid texture. The basic diet structure and the philosophy behind such diet construction are discussed elsewhere [29]. Almost the entire portion of fat in ERD was constituted by n-3 PUFA (n-3: n-6 PUFA=6:1) following many such precedents [39–41]. Equicaloric BLD, on the other hand, was composed of equal portions of n-3 and n-6 PUFA (n-3: n-6 PUFA=1:1).

Fig. 2. (A) Hierarchical clustering of the significantly altered genetic and epigenetic molecules induced by ERD and BLD, both in comparison to STD. The columns with "Trns" header cluster corresponding genes with suppressed transcripts. The columns with "EpG" header cluster the hypermethylated genes. The columns with "miR" header cluster the overexpressed miRNAs targeting a subgroup of genes with silenced transcripts. The left and right Venn diagrams identify the probes of interest (PoI) where genes include both hypermethylated CpG islands and suppressed transcripts. The color scale depicting the range of fold change is in the lower right hand corner. (B) Characterization of probes of interests-1 and -2 (PoI-1 and PoI-2): Corresponding overexpressed miRNAs targeting the same gene list and the functional annotations are reported.

To meet the 1:1 ratio between n-3 and n-6 PUFA, we had to use different oil types in BLD. Besides PUFA content, the rest of the diet constituents were optimally comparable between ERD and BLD. Nevertheless, it is beyond the scope of the present study to understand the residual effect of the oils other than fish oil on rodent health. STD is also equicaloric to ERD and BLD but has almost a reverse of PUFA proportion as compared to ERD (n-3: n-6 PUFA=1:6) [39–41]. Furthermore, both ERD and BLD contained a nearly equivalent distribution of major n-3 and n-6 PUFAs (EPA:DHA=1.5–1.2:1 and EPA:AA=6:1), which was designed according to some suggested health benefits [42–45].

The assay used six male mice per three diet groups, namely ERD, BLD and STD. Diets were consumed by the mice from 3 weeks of age till 25 weeks of age, which is approximately equivalent to human middle life (50 years) [46]. This middle life or near retirement age is a critical junction of human life as many physiological and psychological complications set in during this time [30,31]. Hemibrains collected from the mice fed any of these three diets were probed. The brain is a complex system composed of a number of regions committed to play definite and often feedback-driven roles. Therefore, brain region-specific analyses are always of great interest, but this is beyond the scope of the present study.

The general concept describing DNA methylation as a "silencing" epigenetic mark has been challenged by the advent of high throughput technologies that screen the whole genome [6,14–16]. Although the full characterization of the dynamic associations between transcription and DNA methylation are yet to be comprehended, the genomic context, such as the transcriptional start sites (TSS with or without

Table 2
The top five significantly enriched miRNAs expressed by the pair-wise comparison of three diet types

miRNAs overexpressed by	miRNA (Alias)	Number of genes significantly suppressed	Major functions
miR (ERD > STD)	mmu-miR-29c-3p (miR-29b-3p)	122	Alzheimer's disease; Schizophrenia
	mmu-miR-200a-3p (miR-141-3p)	101	Differentiation of neural precursor cells
	mmu-miR-1192 (miR-495-3p)	96	Bipolar disorder
	mmu-miR-148b-3p (miR-148a-3p)	84	Muscular dystrophy; Alzheimer's disease
	mmu-miR-144-3p	32	Hematopoiesis
miR (ERD > BLD)	mmu-miR-124-3p	58	Maintenance of neural stem cells, LTP of CA1 neuron
	mmu-miR-200a-3p (miR-141-3p)	55	Differentiation of neural precursor cells
	mmu-miR-129-5p	43	Hippocampal synaptoneurosomes
	mmu-miR-375-3p	13	Differentiation of neurites
	mmu-miR-488-3p	11	Hypothalamus energy balance
miR (BLD > STD)	mmu-miR-384-5p (miR-30c-5p)	59	Alzheimer's disease; Schizophrenia
	mmu-miR-19b-3p	57	Alzheimer's disease
	mmu-miR-195-5p (miR-16-5p)	56	Alzheimer's disease; Schizophrenia
	mmu-miR-20b-5p (miR-17-5p)	54	Hematopoiesis; Alzheimer's disease; Schizophrenia
	mmu-miR-26b-5p (miR-26a-5p)	39	Cell cycle progression of cortical neurons; Alzheimer's disease; Schizophrenia
miR (BLD > ERD)	mmu-miR-24-3p	8	Schizophrenia
	mmu-miR-301a-3p (miR-130a-3p)	7	Regulates axon terminal protein (aquaporin 4) expression
	mmu-miR-367-3p (miR-92a-3p)	7	Alzheimer's disease; Schizophrenia; Progressive motor neuropathy
	mmu-miR-23b-3p (miR-23a-3p)	6	Brain maturation
	mmu-miR-19b-3p	5	Alzheimer's disease; Progressive motor neuropathy

CpG islands) or the CpG island's location (near *vs.* distal promoter regions) is emerging as the key mediator of their interplay. Hence, the present study was essentially focused on the probes which were (a) hypermethylated by either ERD or BLD and (b) located in either a promoter or an inside region upstream. All of our observations were presented on the pretext about our poor knowledge about the implications of methylated CpG islands at distal promoter regions and beyond.

Integrated analysis examined the role of miRNAs, another posttranslational modifier that suppresses the genomic expressions. In the context of emerging literature to understand the interplay between epigenetics and miRNA, the present study is possibly one of the first attempts to comprehend their combined impact on the nutritional biology. However, a lack of temporal analysis has limited us in soliciting which one of the three mechanisms, namely *transcriptomics*, *DNA methylation* and *miRNA*, are the causes or consequences of their biological interactions. Furthermore, it is beyond the scope of the present study to classify any methylation events as the inducer or inhibitor of the biofunctions annotated herein. Hence, we took extreme caution in interpretation of the functional trend in the context of epigenetic changes.

The present work is limited in not assessing the bioavailability of PUFA in rodent brains. However, many previous studies suggested a direct correlation of the diet types with the cerebral fatty acid compositions [47]. It is also possible that the potential side effects of fat-enriched diets, such as risk of obesity and insulin resistance, could have certain impacts on the functions of interest, but this is beyond the purpose of the present study.

4.1. PUFA contributes epigenetically in brain development and neurogenesis

Beneficial contributions of PUFA to the cerebral health and neuroprotection are well documented [48–51]. The present study pursued the epigenetic contexts of the functional activities in brain formation and development caused by n-3 PUFA-enriched diets with or without the supplement of n-6 PUFA. ERD epigenetically perturbed a large number of genes associated with neurons, which, in turn, are linked to brain formation and organization of cytoskeleton. Interestingly, ERD epigenetically perturbed a gene cluster comprising CD9, ACTR3, NLGN1, GDA, RPS6KA3 and MAPK8IP3 associated with

filopodia (Fig. 4B), the precursors for dendritic spines in neurons [52]. In addition, ERD hypermethylated genes, such as CAMKK2 and CAMSAP1, which encode the members of a calcium/calmodulin binding family that typically regulate filopodia growth and spine formation [53]. *Camkk2* directly regulates the kinase activity of calcium/calmodulin protein kinase [54], and *Camsap1* engages calcium-induced messengers of PRKCD-mediated cell signaling pathway [55].

Synaptic health critically depends on neurite development. Dietary effects on neuritogenesis could have long-lasting influence. Alcohol treatment, for instance, adversely influences the neurite outgrowth *in vitro* [56]. ERD and BLD hypermethylated the genes associated with neuritogenesis but potentially adapted two different mechanisms (Figs. 2B and S1B). Neurite formation in neuronal cells is typically driven by two independent pathways, both shown to be affected by ERD. This diet epigenetically controlled VAPA. Co-regulated with VAMP2, VAPA mediates the actin-driven neuritogenesis [57]. In parallel, ERD possibly influenced the integrin-dependent neuritogenesis network in neuronal cells *via* hypermethylated MAPK8 [57]. On the other hand, BLD potentially modulated neuritogenesis *via* a cholinergic pathway initiated in the astrocyte cells. Epigenetically modified by BLD, c-JUN is overexpressed in astrocytes and coexpresses with muscarinic proteins [58]. Stimulation of the muscarinic cholinergic receptor protein in astrocytes augments neuritogenesis *via* controlling the load of neuritogenic extracellular matrix proteins such as laminin and fibronectin [59].

4.2. PUFA plays key roles in long term regulation of memory and LTP

Neurotransmitters and receptor proteins, such as G-protein coupled receptors, play active roles in neuromodulation and consequently influence the memory architecture, learning and behavioral paradigms [60,61]. We previously suggested that ERD promoted long-term potentiation by optimally driving the calcium and dopamine messengers to phosphorylate the protein phosphatase 1 regulatory inhibitors [62]. A nearly similar cluster of biofunctions was epigenetically disturbed by ERD and BLD. For instance, BLD preferentially regulated the methylation status of a cluster of genes encoding proteins associated with potassium ion channels and the GABAergic pathway (KCNC1, KCNH3, GABRA2 and GRIN2A); they are associated with spatial and working memory [63] (Fig. 4A). The downstream
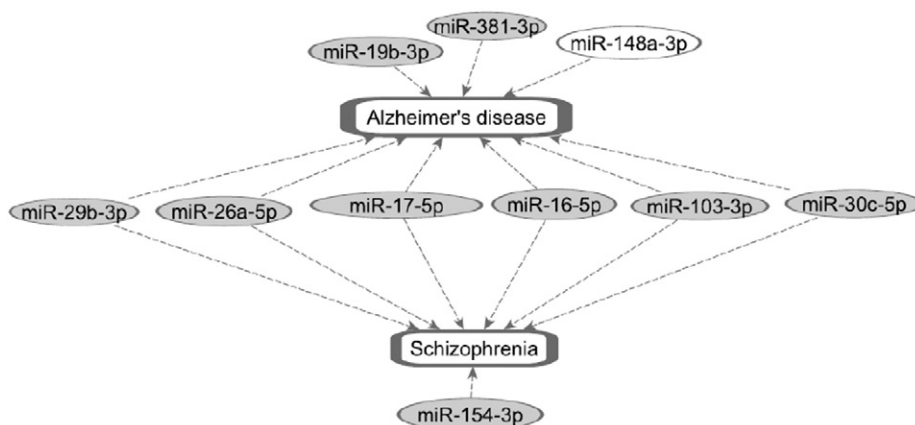
Fig. 3. Network linking the miRNA regulators of schizophrenia and Alzheimer's disease. The miRNAs overexpressed by BLD (shaded) and a miRNA overexpressed by ERD (clear) were evaluated in comparison to STD.

epigenetic influence of BLD was highlighted by the hypermethylated PPP1R1A, a member of a family of protein phosphatases that are major regulators of LTP [64]. The epigenetic influence of BLD on cell membrane architecture was highlighted as JPH3 and JPH4 were found to be hypermethylated. These genes encode junction proteins that modulate memory and LTP *via* calcium/calmodulin messengers [65].

4.3. Disease footprints of ERD and BLD

Genes involved in movement disorders were epigenetically altered by both ERD and BLD, while BLD showed biased influence toward genes affecting Alzheimer's disease. A number of longitudinal cohort studies have highlighted the temporal association of PUFA intake and Alzheimer's advancement. In the elderly, the risk of Alzheimer's was 60% less among those who consumed fish verses those who rarely ate fish. A 6-year follow up study showed the potential role of n-3 PUFA in slowing down the age-related loss of cognitive power [66]. Similar observation was reported elsewhere [35].

Our previous study showed that ERD suppressed the transcriptomic expression of APBA1, a key gene encoding the amyloid beta (A4) precursor protein-binding family. Consequently, there was an increased chance of reducing the amyloidal toxicity, a typical precursor of Alzheimer's onset [67]. The present study mined miR-381-3p, miR-30c-5p and miR-103-3p, all overexpressed by BLD (Fig. 3), that directly target APBA1. We anticipate a potentially long-term preventative effect provided by diets supplemented with an optimal proportion of n-3 and n-6 PUFA on the development of Alzheimer's.

Among the miRNAs overexpressed by ERD, miR-16-5p and miR-30c-5p are the upstream regulators of complement component C4, and miR-103-3p and miR-16-5p are the upstream regulators of DISC1. A recently published work identified C4 as a potential biomarker of schizophrenia [68]. Transgenic manipulation of the DISC1 gene displayed schizophrenia-like phenotypic endpoints; the knockout mice displayed reduced dendritic complexity and impaired spatial working memory [69]. Additional research could identify how an optimally balanced PUFA diet contributes to ameliorating schizophrenia.

4.4. Conclusions

Emerging studies indicate the necessity of apportioning n-3 PUFA and n-6 PUFA to maximize the health benefits. The balanced ratio of n-3 and n-6 PUFA has already showed inhibitory outcome in studies of tumorigenesis and cardiovascular atrophies [70–72]. Brain development mechanisms were found to benefit from balanced supplementation of n-3 and n-6 PUFA [73]. In fact, high amounts of n-3 PUFA may have adverse impacts on early growth unless the diet was suitably enriched by n-6 PUFA [74]. Rodent memory and LTP were reinforced by diets constituted by n-3 and n-6 PUFA at a 4:1 ratio [75]. The debate about an optimal ratio of n-3 and n-6 PUFA was further fueled by multiple studies focusing on various permutations and combinations of n-3 and n-6 PUFA types such as the EPA:DHA ratio [76], the arachidonic acid (AA):EPA ratio [42,77] and the AA:alpha-linolenic acid (ALA) ratio [78]. In this context, the present study intends to throw light on how these dietary supplements impart lasting signatures on major neurological functions.

Overall, we found a good correlation between the epigenetic outcome of this study and our previous study [29] exploring the genetic plasticity caused by ERD. Perturbations of calcium ion-mediated networks with the downstream consequences were emerged as the major outcome coperturbed by epigenetic and genetic structure. In comparing ERD and BLD, one highly enriched with n-3 PUFA and the other with a balanced ratio of n-3 and n-6 PUFA, we identified some instances where their epigenetic contributions were different. For instance, BLD epigenetically perturbed a unique set of networks associated with cognition, startle response and LTP, which could be attributed to the preferential influences on the membrane junction proteins and calcium-releasing messengers. It is intriguing to note that BLD selectively augmented a miRNA cluster associated with Alzheimer's and schizophrenia. We also noted how ERD and BLD adapted two distinct cell type-based paths associated with neuritogenesis, which warrants a more systematic investigation.

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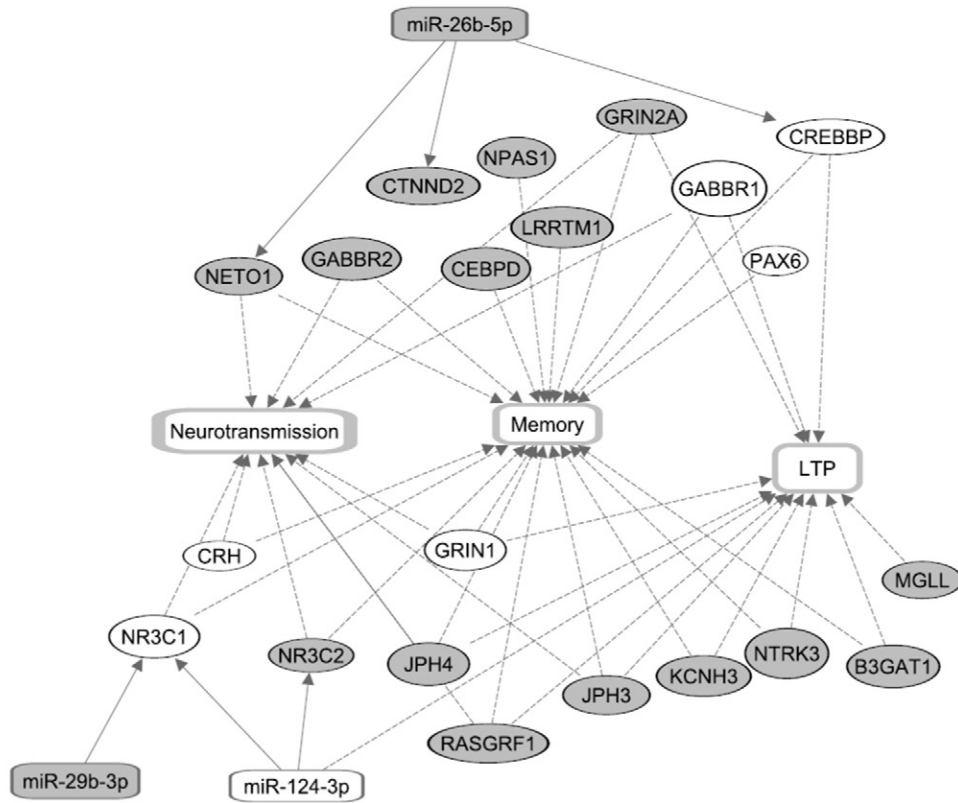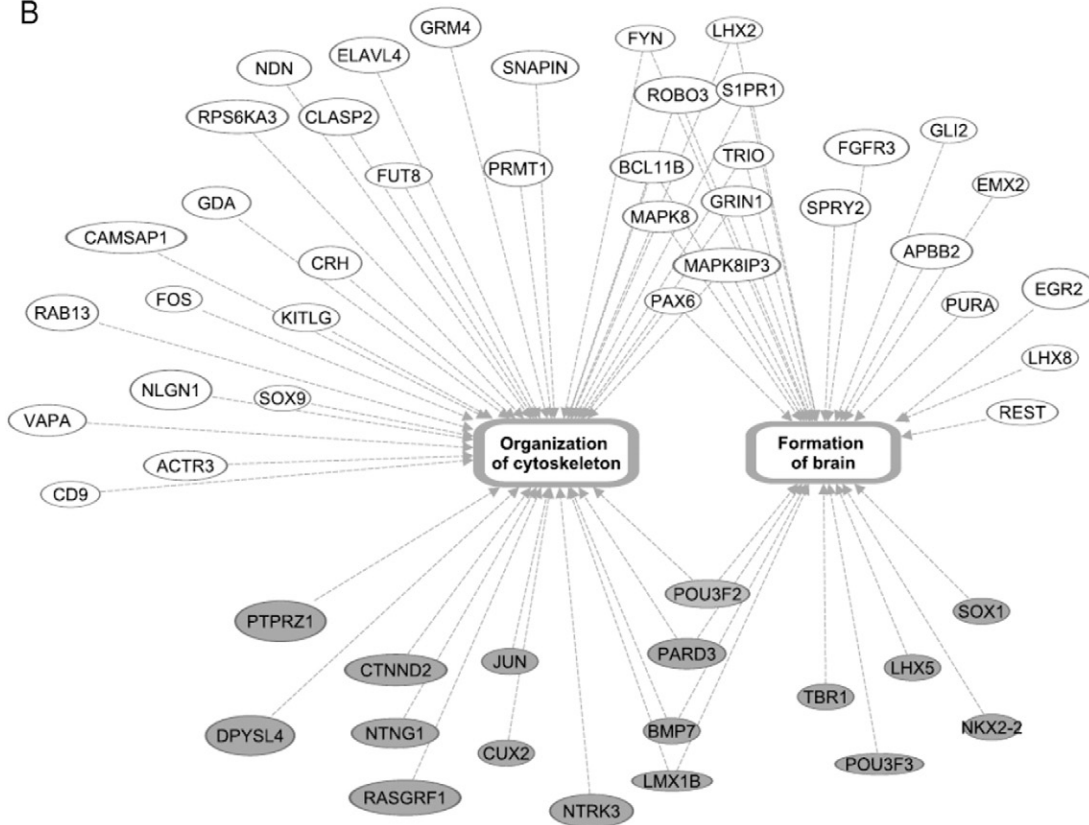
Fig. 4. (A) The gene networks associated with neurotransmission, memory and long-term potentiation (LTP): The genes include both hypermethylated CpG islands and suppressed transcripts and are shown with the overexpressed miRNAs induced by either ERD (clear) or BLD (shaded). (B) The gene networks associated with organization of cytoskeleton and formation of brain: The genes include both hypermethylated CpG islands and suppressed transcripts and are shown with the overexpressed miRNAs induced by either ERD (clear) or BLD (shaded).
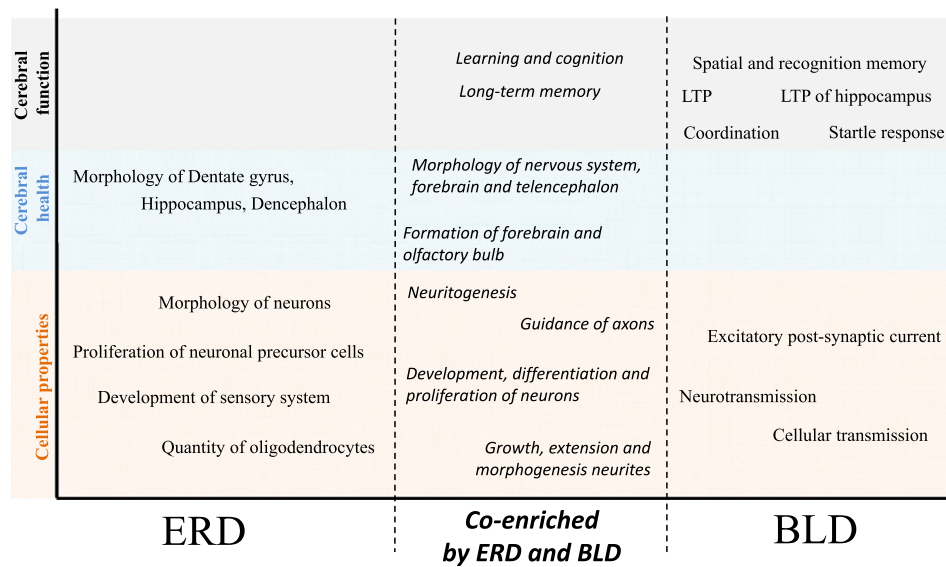
Fig. 5. Differential pathway enrichment by ERD and BLD associated with nervous system development and functions: Significantly enriched terms of nervous system development and functions are sorted into three groups, namely *cellular properties*, *cerebral health* and *cerebral functions*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2016.12.006.

References

[1] Grant WF, Gillingham MB, Batra AK, Fewkes NM, Comstock SM, et al. Maternal high fat diet is associated with decreased plasma n-3 fatty acids and fetal hepatic apoptosis in nonhuman primates. PLoS One 2011;6:e17261.

[2] Ma J, Prince AL, Bader D, Hu M, Ganu R, et al. High-fat maternal diet during pregnancy persistently alters the offspring microbiome in a primate model. Nat Commun 2014;5:3889.

[3] Thorn SR, Baquero KC, Newsom SA, El Kasmi KC, Bergman BC, et al. Early life exposure to maternal insulin resistance has persistent effects on hepatic NAFLD in juvenile nonhuman primates. Diabetes 2014;63:2702–13.

[4] Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet 2009;18:4046–53.

[5] Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A 2008;105:17046–9.

[6] Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. Nat Rev Genet 2008;9:465–76.

[7] Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. J Nutr 2002;132:2333S–5S.

[8] McKay JA, Mathers JC. Diet induced epigenetic changes and their implications for health. Acta Physiol (Oxford) 2011;202:103–18.

[9] Pogribny IP, Karpf AR, James SR, Melnyk S, Han T, et al. Epigenetic alterations in the brains of fisher 344 rats induced by long-term administration of folate/methyl-deficient diet. Brain Res 2008;1237:25–34.

[10] McKay JA, Waltham KJ, Williams EA, Mathers JC. Folate depletion during pregnancy and lactation reduces genomic DNA methylation in murine adult offspring. Genes Nutr 2011;6:189–96.

[11] Zeisel SH, Mar MH, Howe JC, Holden JM. Concentrations of choline-containing compounds and betaine in common foods. J Nutr 2003;133:1302–7.

[12] Lee HS, Barraza-Villarreal A, Biessy C, Duarte-Salles T, Sly PD, et al. Dietary supplementation with polyunsaturated fatty acid during pregnancy modulates DNA methylation at IGF2/H19 imprinted genes and growth of infants. Physiol Genomics 2014;46:851–7.

[13] Lee HS, Barraza-Villarreal A, Hernandez-Vargas H, Sly PD, Biessy C, et al. Modulation of DNA methylation states and infant immune system by dietary supplementation with omega-3 PUFA during pregnancy in an intervention study. Am J Clin Nutr 2013;98:480–7.

[14] Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 2012;13:484–92.

[15] Deaton AM, Bird A. CpG islands and the regulation of transcription. Genes Dev 2011;25:1010–22.

[16] Schubeler D. Function and information content of DNA methylation. Nature 2015;517:321–6.

[17] Ryan BM, Robles AI, Harris CC. Genetic variation in microRNA networks: the implications for cancer research. Nat Rev Cancer 2010;10:389–402.

[18] Chuang JC, Jones PA. Epigenetics and microRNAs. Pediatr Res 2007;61:24R–9R.

[19] Mathers JC, Strathdee G, Relton CL. Induction of epigenetic alterations by dietary and other environmental factors. Adv Genet 2010;71:3–39.

[20] Farago N, Feher LZ, Kitajka K, Das UN, Puskas LG. MicroRNA profile of polyunsaturated fatty acid treated glioma cells reveal apoptosis-specific expression changes. Lipids Health Dis 2011;10:173.

[21] Shah MS, Schwartz SL, Zhao C, Davidson LA, Zhou B, et al. Integrated microRNA and mRNA expression profiling in a rat colon carcinogenesis model: effect of a chemo-protective diet. Physiol Genomics 2011;43:640–54.

[22] Davidson LA, Wang N, Shah MS, Lupton JR, Ivanov I, et al. N-3 polyunsaturated fatty acids modulate carcinogen-directed non-coding microRNA signatures in rat colon. Carcinogenesis 2009;30:2077–84.

[23] Deckelbaum RJ, Torrejon C. The omega-3 fatty acid nutritional landscape: health benefits and sources. J Nutr 2012;142:587S–91S.

[24] de Lorgeril M, Salen P. New insights into the health effects of dietary saturated and omega-6 and omega-3 polyunsaturated fatty acids. BMC Med 2012;10:50.

[25] Hooijmans CR, Pasker-de Jong PC, de Vries RB, Ritskes-Hoitinga M. The effects of long-term omega-3 fatty acid supplementation on cognition and Alzheimer's pathology in animal models of Alzheimer's disease: a systematic review and meta-analysis. J Alzheimers Dis 2012;28:191–209.

[26] Bousquet M, Calon F, Cicchetti F. Impact of omega-3 fatty acids in Parkinson's disease. Ageing Res Rev 2011;10:453–63.

[27] Jicha GA, Markesbery WR. Omega-3 fatty acids: potential role in the management of early Alzheimer's disease. Clin Interv Aging 2010;5:45–61.

[28] da Silva TM, Munhoz RP, Alvarez C, Naliwaiko K, Kiss A, et al. Depression in Parkinson's disease: a double-blind, randomized, placebo-controlled pilot study of omega-3 fatty-acid supplementation. J Affect Disord 2008;111:351–9.

[29] Hammamieh R, Chakraborty N, Gautam A, Miller SA, Muhie S, et al. Transcriptomic analysis of the effects of a fish oil enriched diet on murine brains. PLoS One 2014;9:e90425.

[30] Leung MY, Pollack LM, Colditz GA, Chang SH. Life years lost and lifetime health care expenditures associated with diabetes in the U.S., National Health Interview Survey, 1997-2000. Diabetes Care 2015;38:460–8.

[31] Wraw C, Deary IJ, Der G, Gale CR. Intelligence in youth and mental health at age 50. Intelligence 2016;58:69–79.

[32] Rosenbaum MD, VandeWoude S, Johnson TE. Effects of cage-change frequency and bedding volume on mice and their microenvironment. J Am Assoc Lab Anim Sci 2009;48:763–73.

[33] Shi Q, Guo L, Patterson TA, Dial S, Li Q, et al. Gene expression profiling in the developing rat brain exposed to ketamine. Neuroscience 2010;166:852–63.

[34] Liu T, Papagiannakopoulos T, Puskar K, Qi S, Santiago F, et al. Detection of a microRNA signal in an in vivo expression set of mRNAs. PLoS One 2007;2: e804.

[35] Schaefer EJ, Bongard V, Beiser AS, Lamon-Fava S, Robins SJ, et al. Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and Alzheimer disease: the Framingham heart study. Arch Neurol 2006;63:1545–50.

[36] Knochel C, Voss M, Gruter F, Alves GS, Matura S, et al. Omega 3 fatty acids: novel Neurotherapeutic targets for cognitive dysfunction in mood disorders and schizophrenia? Curr Neuropharmacol 2015;13:663–80.

[37] Zugno AI, Canever L, Mastella G, Heylmann AS, Oliveira MB, et al. Effects of omega-3 supplementation on interleukin and neurotrophin levels in an animal model of schizophrenia. An Acad Bras Cienc 2015;87:1475–86.

[38] Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, et al. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J Neurosci 2008;28:264–78.

[39] Yon MA, Mauger SL, Pickavance LC. Relationships between dietary macronutrients and adult neurogenesis in the regulation of energy metabolism. Br J Nutr 2013; 109:1573–89.

[40] Heerwagen MJ, Stewart MS, de la Houssaye BA, Janssen RC, Friedman JE. Transgenic increase in N-3/n-6 fatty acid ratio reduces maternal obesity-associated inflammation and limits adverse developmental programming in mice. PLoS One 2013;8:e67791.

[41] Lei X, Zhang W, Liu T, Xiao H, Liang W, et al. Perinatal supplementation with omega-3 polyunsaturated fatty acids improves sevoflurane-induced neurodegeneration and memory impairment in neonatal rats. PLoS One 2013;8:e70645.

[42] Adams PB, Lawson S, Sanigorski A, Sinclair AJ. Arachidonic acid to eicosapentaenoic acid ratio in blood correlates positively with clinical symptoms of depression. Lipids 1996;31:S157–61 [Suppl].

[43] Simopoulos AP. Evolutionary aspects of diet: the omega-6/omega-3 ratio and the brain. Mol Neurobiol 2011;44:203–15.

[44] Simopoulos AP. Importance of the ratio of omega-6/omega-3 essential fatty acids: evolutionary aspects. World Rev Nutr Diet 2003;92:1–22.

[45] Simopoulos AP. Importance of the omega-6/omega-3 balance in health and disease: evolutionary aspects of diet. World Rev Nutr Diet 2011;102:10–21.

[46] Dutta S, Sengupta P. Men and mice: relating their ages. Life Sci 2016;152:244–8.

[47] Yu H, Bi Y, Ma W, He L, Yuan L, et al. Long-term effects of high lipid and high energy diet on serum lipid, brain fatty acid composition, and memory and learning ability in mice. Int J Dev Neurosci 2010;28:271–6.

[48] Jiao J, Li Q, Chu J, Zeng W, Yang M, et al. Effect of n-3 PUFA supplementation on cognitive function throughout the life span from infancy to old age: a systematic review and meta-analysis of randomized controlled trials. Am J Clin Nutr 2014;100:1422–36.

[49] Schuchardt JP, Huss M, Stauss-Grabo M, Hahn A. Significance of long-chain polyunsaturated fatty acids (PUFAs) for the development and behaviour of children. Eur J Pediatr 2010;169:149–64.

[50] Bourre JM. Roles of unsaturated fatty acids (especially omega-3 fatty acids) in the brain at various ages and during ageing. J Nutr Health Aging 2004;8:163–74.

[51] Lauritzen I, Blondeau N, Heurteaux C, Widmann C, Romey G, et al. Polyunsaturated fatty acids are potent neuroprotectors. EMBO J 2000;19:1784–93.

[52] Mattila PK, Lappalainen P. Filopodia: molecular architecture and cellular functions. Nat Rev Mol Cell Biol 2008;9:446–54.

[53] Pereira L, Cheng H, Lao DH, Na L, van Oort RJ, et al. Epac2 mediates cardiac beta1-adrenergic-dependent sarcoplasmic reticulum Ca2+ leak and arrhythmia. Circulation 2013;127:913–22.

[54] Anderson KA, Means RL, Huang QH, Kemp BE, Goldstein EG, et al. Components of a calmodulin-dependent protein kinase cascade. Molecular cloning, functional characterization and cellular localization of Ca2+/calmodulin-dependent protein kinase kinase beta. J Biol Chem 1998;273:31880–9.

[55] Caino MC, von Burstin VA, Lopez-Haber C, Kazanietz MG. Differential regulation of gene expression by protein kinase C isozymes as determined by genome-wide expression analysis. J Biol Chem 2011;286:11254–64.

[56] Zhang X, Kusumo H, Sakharkar AJ, Pandey SC, Guizzetti M. Regulation of DNA methylation by ethanol induces tissue plasminogen activator expression in astrocytes. J Neurochem 2014;128:344–9.

[57] Gupton SL, Gertler FB. Integrin signaling switches the cytoskeletal and exocytic machinery that drives neuritogenesis. Dev Cell 2010;18:725–36.

[58] Herdegen T, Leah JD. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Brain Res Rev 1998;28:370–490.

[59] Guizzetti M, Moore NH, Giordano G, Costa LG. Modulation of neuritogenesis by astrocyte muscarinic receptors. J Biol Chem 2008;283:31884–97.

[60] Caroni P, Donato F, Muller D. Structural plasticity upon learning: regulation and functions. Nat Rev Neurosci 2012;13:478–90.

[61] Gogolla N, Galimberti I, Caroni P. Structural plasticity of axon terminals in the adult. Curr Opin Neurobiol 2007;17:516–24.

[62] Kotaleski JH, Blackwell KT. Modelling the molecular mechanisms of synaptic plasticity using systems biology approaches. Nat Rev Neurosci 2010;11:239–51.

[63] Auger ML, Floresco SB. Prefrontal cortical GABA modulation of spatial reference and working memory. Int J Neuropsychopharmacol 2015;18.

[64] Brown GP, Blitzer RD, Connor JH, Wong T, Shenolikar S, et al. Long-term potentiation induced by theta frequency stimulation is regulated by a protein phosphatase-1-operated gate. J Neurosci 2000;20:7880–7.

[65] Moriguchi S, Nishi M, Komazaki S, Sakagami H, Miyazaki T, et al. Functional uncoupling between Ca2+ release and afterhyperpolarization in mutant hippocampal neurons lacking junctophilins. Proc Natl Acad Sci U S A 2006;103: 10811–6.

[66] Morris MC, Evans DA, Tangney CC, Bienias JL, Wilson RS. Fish consumption and cognitive decline with age in a large community study. Arch Neurol 2005;62: 1849–53.

[67] Lorenzo A, Yuan M, Zhang Z, Paganetti PA, Sturchler-Pierrat C, et al. Amyloid beta interacts with the amyloid precursor protein: a potential toxic mechanism in Alzheimer's disease. Nat Neurosci 2000;3:460–4.

[68] Sekar A, Bialas AR, de Rivera H, Davis A, Hammond TR, et al. Schizophrenia risk from complex variation of complement component 4. Nature 2016;530: 177–83.

[69] Dawson N, Kurihara M, Thomson DM, Winchester CL, McVie A, et al. Altered functional brain network connectivity and glutamate system function in transgenic mice expressing truncated disrupted-in-schizophrenia 1. Transl Psychiatry 2015;5:e569.

[70] Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med (Maywood) 2008;233:674–88.

[71] Lu X, Ding X, Jing L. Effect of mechanism of action of different omega-6/omega-3 polyunsaturated fatty acids ratio on the growth of endometrial carcinoma mice. Cell Biochem Biophys 2015;71:1671–6.

[72] Hammamieh R, Chakraborty N, Miller SA, Waddy E, Barmada M, et al. Differential effects of omega-3 and omega-6 fatty acids on gene expression in breast cancer cells. Breast Cancer Res Treat 2007;101:7–16.

[73] Loef M, Walach H. The omega-6/omega-3 ratio and dementia or cognitive decline: a systematic review on human studies and biological evidence. J Nutr Gerontol Geriatr 2013;32:1–23.

[74] Scholtz SA, Colombo J, Carlson SE. Clinical overview of effects of dietary long-chain polyunsaturated fatty acids during the perinatal period. Nestle Nutr Inst Workshop Ser 2013;77:145–54.

[75] Yehuda S, Carasso RL. Modulation of learning, pain thresholds, and thermoregulation in the rat by preparations of free purified alpha-linolenic and linoleic acids: determination of the optimal omega 3-to-omega 6 ratio. Proc Natl Acad Sci U S A 1993;90:10345–9.

[76] Freeman MP, Hibbeln JR, Wisner KL, Brumbach BH, Watchman M, et al. Randomized dose-ranging pilot trial of omega-3 fatty acids for postpartum depression. Acta Psychiatr Scand 2006;113:31–5.

[77] Conklin SM, Manuck SB, Yao JK, Flory JD, Hibbeln JR, et al. High omega-6 and low omega-3 fatty acids are associated with depressive symptoms and neuroticism. Psychosom Med 2007;69:932–4.

[78] Lucas M, Mirzaei F, O'Reilly EJ, Pan A, Willett WC, et al. Dietary intake of n-3 and n-6 fatty acids and the risk of clinical depression in women: a 10-y prospective follow-up study. Am J Clin Nutr 2011;93:1337–43.