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# Fish oil promotes survival and protects against cognitive decline in severely undernourished mice by normalizing satiety signals☆,☆☆

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**Abstract**

Severe malnutrition resulting from anorexia nervosa or involuntary starvation leads to low weight, cognitive deficits and increased mortality rates. In the present study, we examined whether fish oil supplementation, compared with that of canola oil, would ameliorate the morbidity and mortality associated with these conditions by normalizing endocannabinoid and monoaminergic systems as well as other systems involved in satiety and cognitive function within the hypothalamus and hippocampus. Female Sabra mice restricted to 40% of their daily food intake exhibited decreased body weight, were sickly in appearance, displayed cognitive deficits and had increased mortality rates. Strikingly, fish oil supplementation that contains high omega-3 fatty acids levels decreased mortality and morbidity, and normalized the expression of genes and neurotransmitters in the hippocampus and hypothalamus. Fish oil supplementation, but not canola oil, increased survival rates, improved general appearance and prevented cognitive decline, despite the facts that both diets contained an equivalent number of calories and that there were no differences in weight between mice maintained on the two diets in 100% but decrease in the 40%. In the hypothalamus, the beneficial effects of fish oil supplementation were related to normalization of the endocannabinoid 2-arachidonylglycerol, serotonin (5-HT) ($P<.056$), dopamine, neuropeptide Y (NPY) and $Ca^{2+}$/calmodulin (CaM)-dependent protein kinase (Camkk2). In the hippocampus, fish oil supplementation normalized 5-HT, Camkk2, silent mating type information regulation 1 and brain-derived neurotrophic factor. In conclusion, dietary supplements of fish oil, as source of omega-3 fatty acids, may alleviate cognitive impairments associated with severe diet restriction and prolong survival independently of weight gain by normalizing neurochemical systems.


*Keywords:* Fish oil; Animal models; Cognition; Malnutrition; Gene expression; Endocannabinoids

## 1. Introduction

Anorexia nervosa (AN) is a life-threatening, psychiatric eating disorder characterized by insufficient caloric intake, low weight, distorted body image, cognitive deficits and other related problems that appear mostly among adolescent women from higher socioeconomic status [1]. This disorder usually begins as apparently benign diet attempts that deteriorate to a state of self-starvation with high risks of morbidity and mortality. There are presently no efficacious treatments for AN, a disease that has claimed the lives of about 20% of the patients over the last 20 years.

A variety of animal models have been developed to investigate AN, including diet restriction (DR), activity wheel and separation stress. Clearly, no single animal model is a true replica of the human disease, but the various assays are useful for focusing on different aspects of this condition. AN models include the following aspects: adolescent onset of the disease, predominance in females, decreased food intake and body weight, increased activity and abnormal neuroendocrine function. In the present study, we employed the DR model because it results in several phenotypes presented by AN patients, including profound weight loss, cognitive deficits, alterations in hormones and neurotransmitters, and increased mortality and morbidity [2,10].

Omega-3 polyunsaturated fatty acids (PUFAs) are hydrocarbon chains containing double bonds and constituting animal and vegetable oils. These precursors for the phospholipid membranes are essential not only for the functional development of the brain but also for the overall health and well-being of the organism. Docosahexanoic acid (DHA), found abundantly in fish oil, is important for the development of the nervous system in mammals [3,4]. Administration of food supplements rich in DHA to women during lactation or in the last trimester of pregnancy augmented the IQ of their offspring at the age of 4 [5]. There was also a correlation in aging between eating food supplements rich in omega-3 PUFAs and cognitive function [6].

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We previously investigated the roles of adenosine monophosphate-dependent kinase (AMPK), a "fuel gauge" for cellular metabolism [7] that regulates feeding behavior [8], and those of leptin, an adipokinin hormone, that plays a role in food intake and energy balance through hypothalamic circuits [9], in cognition and survival under severe caloric restriction [10]. Leptin restored AMPK phosphorylation level and cognitive function under caloric restriction to 40%, elevated antiapoptotic and reduced proapoptotic markers and promoted longevity [10]. omega-3 PUFAs were found to elevate plasma leptin in insulin-resistant rats [11]. Therefore, omega-3 PUFAs might have effects equivalent to those of leptin, that is, cognitive enhancement and promotion of survival and longevity. Since omega-3 PUFAs are natural substances found in humans and other mammals, regulatory hurdles are likely to be less onerous for their development than those for new drugs. Thus, in the present study, we examined the impact of fish oil supplementation in the diets of mice that were highly undernourished on their ability to thrive and on cognitive function. Thus, the overall objective of this study was to compare fish oil to canola oil supplementation on body weight, cognitive function and survival. Here, we report that fish oil supplementation improved cognitive function and prolonged survival rates in severely food restricted mice. In order to elucidate the underlying mechanisms of action, we assessed hippocampal and/or hypothalamic levels of endogenous cannabinoids (i.e., 2-AG and anandamide), cannabinoid receptor 1 (CB1) function and monoamine levels [serotonin (5-HT), dopamine (DA), and norepinephrine], as well as the expression of genes implicated in feeding, cognitive function, and survival, including $Ca^{2+}$/calmodulin (CaM)-dependent protein kinase (Camkk2), silent mating type information regulation 1 (SIRT1), brain-derived neurotrophic factor (BDNF) and NPY in these brain regions.

## 2. Materials and methods

### 2.1. Animals, diet and experimental design

In all experiments, the principles of laboratory animal care were followed and the protocols were authorized by the institutional animal care facility board at the Hebrew University of Jerusalem (ethics committee research no. MD 87.04-2). Female Sabra mice (Harlan, Jerusalem, Israel), 30 g weight, were used.

Sabra mice are a general purpose outbred strain that were bred at Hebrew University for a wide range of toxicological and behavioral studies. Females were used in these studies because of the high incidence of anorexia in women. The free body weights of mature female Sabra mice are 30–35 g.

Two different supplements were added to FAT FREE DIET (Cat. No. 901682, MP, Ohio). The first supplement was 5% canola oil (Oil Industries, Haifa, Israel) containing 59% of monounsaturated fatty acids, 26% of omega-6 fatty acids, 9% of omega-3 fatty acids, 6% of saturated fatty acids, 23 mg/100 ml of vitamin E and 603 mg/100 ml of phytosterol. The second diet consisted of 4% fish oil and 1% canola oil that was mixed with the basic food composition. The components of fish oil, as supplied by the manufacturer (American Naturavit Inc.; Distributor: Gramse Pharmaceuticals LTD, Yokneam, Israel), were as follows:

Fish oil 3182 mg/5 ml containing 1050 mg/5 ml (33% of fish oil) eicosapentanoic acid (EPA), 700 mg/5 ml (22% of fish oil) DHA and 200 U/5 ml vitamin E. Fish oil contains 27 mg/ml alpha-tocopherol and canola oil contains 0.23 mg/ml alpha, beta and gamma-tocopherol.

We compared the effects of supplementation of fish oil, which includes the derivatives of DHA and EPA, to canola oil in mice maintained on either a severely caloric-restricted diet or a normal diet.

### 2.2. Food restriction

Female Sabra mice (Harlan, Jerusalem), 30 g weight, were assigned to one of the following four groups:

Group 1: 100% diet with canola oil (weight maintenance) in which mice received 95 kcal/week per mouse with supplementation of 5% canola oil–3.6 g of FAT FREE DIET (Cat. No. 901682, MP, Ohio, composition as described [11]) per day per mouse, calculated for 30 g mouse weight. Group 2: 100% diet with fish oil in which mice received 95 kcal/day with supplementation of 4% fish oil and 1% canola oil that were mixed with the basic food (3.6 g). The mice on 100%+4% fish oil received 47.52 mg EPA and 31.68 mg DHA per day. Group 3: 40% diet with canola oil in which mice received 38 kcal/week per mouse in a 1.44-g fat-free diet per mouse per day with supplementation of 5% canola oil. Group 4: 40% diet with fish oil in which mice received 38 kcal/week per mouse in a 1.44-g fat-free food diet per mouse per day, supplemented with 1% of canola oil and 4% of fish oil which were mixed with the food powder. The mice on 40%+4% fish oil received 19 mg EPA and 12.67 mg DHA. Thus, the number of calories was exactly the same between the 40% restricted plus 5% canola oil and the 40% restricted plus 4% fish oil (and 1% canola oil) supplement mice, as each group received the same amount of FAT FREE DIET supplemented with 5% oil. The mice on 100% and 40% diet did not receive any EPA or DHA.

Using this feeding protocol, we were able to evaluate the effect of fish oil supplementation, as canola oil contains all types of fatty acids. The study was terminated at 12 days to keep mortality rates at a minimum. The subjects were weighed on Days 1, 5, 8 and 12. Survival was recorded throughout the entire experiment.

### 2.3. Eight-arm spatial maze for cognitive assessment

Cognitive function studies were performed on Day 8 of the experiment. The mice were placed in an eight-arm maze, which is a scaled-down version of that developed for rats [12,13]. Mice were water deprived overnight and a reward of 50 μl of water was presented at the end of each arm. Mice were randomized into the treatment groups and put in a maze between 9:00 a.m. and 1:00 p.m. each day for 5 days. Observations were recorded until entries were made into all eight arms or until 24 entries were completed. Data were evaluated utilizing the formula of area under the curve (AUC) $(d2+d3+d4+d5)-4^{*}(d1)$. Hence, the lower the score, the better the cognitive function.

### 2.4. Measurement of monoamines

Mice were sacrificed by decapitation on the 12th day. Hippocampi and hypothalami were immediately dissected out and kept at −70°C for all measurements. Assay for NE, dopamine and 5-HT were performed by high-performance liquid chromatography/electrochemical detector using the same procedure reported previously [14].

### 2.5. Agonist-stimulated [35S]GTPγS binding

Hippocampal and hypothalamic tissue taken from animals on the specific diets were placed in 5 ml of cold membrane buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4) and homogenized. The samples were then centrifuged at 50,000×*g* at 5°C for 10 min. The supernatant was removed, and samples were resuspended in 5 ml of assay buffer A (50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4). Protein concentration was evaluated by the Bradford method (Bradford, 1976). Before assay, membranes (4–8 μg of protein) were preincubated for 20 min at 30°C with adenosine deaminase (3 mU/ml) in assay Buffer A. Concentration–effect curves were generated by incubating the appropriate amount of membrane protein (8 μg) in assay buffer B (assay Buffer A plus 1.25 g/l BSA) with 0.03 to 10 μM CP-55,940 in the presence of 30 μM GDP and 0.1 nM [35S]GTPγS in 0.5-ml total volume for 2 h at 30°C. Basal binding was measured in the absence of agonist, and nonspecific binding was measured in the presence of 20 μM unlabeled guanosine 5′-3-*O*-(thio) triphosphate. The reaction was terminated by vacuum filtration through Whatman GF/B glass fiber filters, followed by three to four washes with 4°C Tris buffer (50 mM Tris–HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency after 9-h extraction in ScintiSafe Econo 1 scintillation fluid.

### 2.6. [3H]SR141716A binding

Membranes were prepared as described above. Saturation analysis was performed by incubating 16 μg membrane protein with 0.2–3 nM [$^3$H]SR141716A in assay buffer (Buffer A+0.05 % BSA) in the presence or absence of 5 μM unlabeled SR141716A (to determine nonspecific and total binding, respectively) for 90 min at 30°C. The reaction was terminated by vacuum filtration through Whatman GF/B glass fiber filter that was pre-soaked in Tris buffer containing 5 g/l BSA (Tris–BSA), followed by four washes with 4°C Tris–BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency after extraction (1 h mechanical shaking) in ScintiSafe Econo 1 scintillation fluid.

### 2.7. Quantitative reverse transcription–polymerase chain reaction analysis

Mice were sacrificed by decapitation on the 12th day and total hippocampal and hypothalamic RNAs were extracted using Tri reagent according to the manufacturer's instructions and were reverse transcribed. RNA samples with no reverse transcription (RT) were amplified in the polymerase chain reaction (PCR) in order to rule out the possibility of amplifying genomic DNA contamination which was present in the RNA extracted from the tissue.

Quantitative RT-PCR was carried out with Power SYBR Green PCR Master Mix (Applied Biosystems, UK), in 7900HT instrument (Applied Biosystems). Volume reaction was 15 μl and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control. Threshold cycle (Ct) was determined by SDS software for each one of the samples tested, and the average Ct was calculated for each triplicate. ΔCt of each target gene was calculated by subtracting the average Ct for GAPDH of a given

sample from the average Ct for the target gene of the same sample. Average ΔCt of a certain target gene in the control group was subtracted from ΔCt of the same gene in samples from the treated groups (40% diet+Canola oil, 40% diet+Fish oil) to yield ΔΔCt of this gene in the sample. The quantity of a specific target gene in a certain sample relative to the control group was calculated as $2^{-\ \Delta\Delta^{Ct}}$ utilizing ΔΔCt determined for that sample. All primers were synthesized by Syntezza (Jerusalem, Israel). Gene specific primers are detailed in Table 1.

### 2.8. Quantifications of endocannabinoids

Mice were decapitated and brains were removed, snap frozen in dry ice and shipped to Virginia Commonwealth University under dry ice where they were stored at −80°C until the time of processing. On the day of processing, tissues were weighed and homogenized with 1.4 ml chloroform/methanol (2:1 v/v containing 0.0348 g PMFS/ml) after the addition of internal standards to each sample (2 pmol AEA-d8 and 1 nmol 2-AG-d8; Cayman Chemical, Ann Arbor, MI, USA). Homogenates were then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed and then centrifuged for 10 min at 4000 rpm (4°C). The aqueous phase plus debris were collected and extracted two more times with 0.8 ml chloroform. The organic phases from the three extractions were pooled and the organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml chloroform and mixed with 1 ml ice cold acetone. The mixtures were then centrifuged for 5 min at 3000 rpm and 4°C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml methanol and placed in autosample vials for analysis.

LC/MS was used to quantify AEA and 2-AG. The mobile phase consisted of (10:90) water/methanol with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 4.6* 15 cm, 3 μm (Supelco, USA). The mass spectrometer was run in electrospray ionization in positive mode. Ions were analyzed in multiple reaction monitoring mode and the following transitions were monitored: (348>62) and (348>91) for AEA, (356>62) for AEA-d8, (379>287) and (279>269) for 2-AG, and (387>96) for 2-AG-d8. A calibration curve was constructed for each assay based on linear regression using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.03 to 40 pmol for AEA and from 0.05 to 64 nmol for 2-AG.

### 2.9. Statistical analyses

Data are presented as mean±S.E.M. The following one-tail alternate hypotheses were tested: dietary restriction (40% diet+canola oil compared to 100% diet+canola oil) decreases 2-AG, AEA, NE, DA, 5-HT, BDNF and SIRT and enhances NPY and CAMKK2, whereas fish oil (40% diet+fish oil compared to 40% diet+canola oil in dietary restricted animals) has the opposite effect; fish oil in weight maintenance animals (100% diet+fish oil) is not expected to cause a significant change. Two-tail hypotheses were used to test weight and cognition. Exact Mann–Whitney *U* tests were employed throughout and the Holms modification of the Bonferroni correction for multiple comparisons [15] applied as appropriate :each parameter other than weight was considered a multiplicity of 2 , one for each use of the 40% diet+canola oil data, and weight a multiplicity of 6, the additional factor of 3 arising from the three days (5, 8, 12) on which the measurements were repeated. The survival rate was analyzed using Fisher's Exact Test for 2×2 tables corrected for multiple comparisons. In all cases, a *P* value less than .05 was deemed significant.

[35S]GTPγS binding data are reported as mean (±S.E.M.) of at least six experiments, each performed in triplicate. Nonspecific binding was subtracted from each sample. Net stimulated [35S]GTPγS binding was defined as agonist-stimulated minus basal [35S]GTPγS binding, and percent stimulation was defined as (net-stimulated/basal [35S]GTPγS binding)×100%. Nonlinear literative regression analyses of agonist concentration–effect and saturation binding curves were performed with Prizm 5.0 (Graphpad software, La Jolla, CA, USA). Statistical significance was determined by ANOVA followed by post hoc analysis with Dunnett's test ($\alpha$=.05).

Table 1
PCR primers used in this study, including accession numbers, sequences and location in the transcript

| Gene | Accession number (NCBI) | Primer sequence | Primer location in the transcript |
|---|---|---|---|
| GAPDH | N M 008084 | F:5′CTCTGCTCCTCCCTGTTCCA-3′<br>R:5′CTGGCACTGCACAAGAAGATG-3′ | 172–191<br>222–202 |
| Camkk2 | N M 145358 | F:5′-AAAGGGCTCCTATGGTGTTGTC-3′<br>R:5′-GTATTGTCATTTTCATTGTAGGCCA-3′ | 566–587<br>616–592 |
| NPY | N M 023456 | F:5′-CTCCGCTCTGCGACACTACA-3′<br>R:5′-AATCAGTGTCTCAGGGCTGGA-3′ | 220–239<br>295–276 |
| SIRT-1 | N M 019812 | F:5′AACGTCACGCCAGCTCTA-3′<br>R:5′ATAGGTCCATATACTTTTGTTCAGCAAC-3′ | 539–558<br>609–582 |
| BDNF | N M 007540 | F:5′CACTGAGTCTCCAGGACAGCAA-3′<br>R:5′CTCTTCTCACCTGGTGGAACATT-3′ | 609–630<br>659–637 |

## 3. Results

### 3.1. Body weight

Mice maintained on a 40% restricted diet displayed a significant decrease in body weight compared to the 100% diet on Days 5, 8 and 12 (Fig. 1) (*P*<.05a) vs. control regardless of oil supplementation. Four percent fish oil supplementation enhanced significantly the weight decrease in diet-restricted mice on Days 5, 8 and 12 (*P*<.05b vs. DR to 40%); *n*=10 mice per group. However, no change was observed in the control group following fish oil supplementation. (The letters 'a' and 'b' following P values throughout the article signify statistical comparison between groups.)

### 3.2. Mice appearance

Mice maintained on the 100% diet presented a healthy appearance and appeared well groomed, regardless of oil supplementation. However, the 40% diet supplemented with canola oil displayed matted, ungroomed and piloerect fur, and were sickly in appearance. Strikingly, fish oil supplementation to mice on the 40% diet appeared to be protective in terms of general appearance, as these mice appeared more similar to the mice maintained on the 100% diet than those supplemented with canola oil on the 40% diet, despite their profound weight loss. Representative mice from each group are presented in Fig. 2.

### 3.3. Cognitive function

Mice maintained on the 40% diet supplemented with canola oil displayed significantly impaired performance in radial arm maze acquisition as compared to the control group (Fig. 3; *P*<.05a) as reflected by the higher AUC values. In the 40% DR group, fish oil supplementation improved significantly the performance compared to the canola oil supplementation (*P*<.05b).

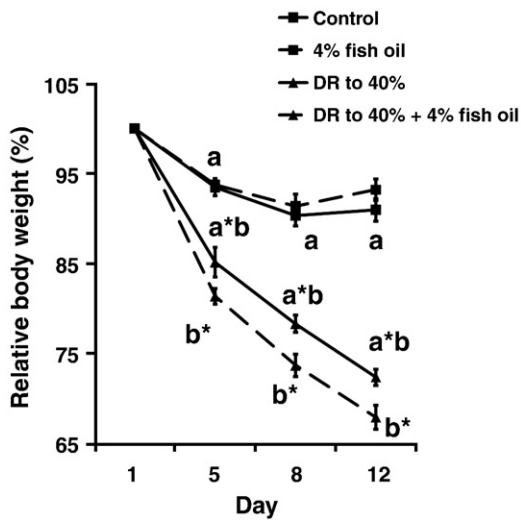


Fig. 1. Female Sabra mice were assigned to one of the following four groups: Group 1, 100% diet with canola oil (weight maintenance); Group 2, 100% diet with fish oil; Group 3, 40% diet with canola oil; Group 4, 40% diet with fish oil. The study was terminated at 12 days. The subjects were weighed on Days 1, 5, 8, and 12. Mice weight decreased following DR to 40% and was further decreased by fish oil supplementation on the 8th day. Values are means±S.E.M. *n*=10 mice per group. Means with a common letter differ. *P<.05 corrected for multiple comparisons.

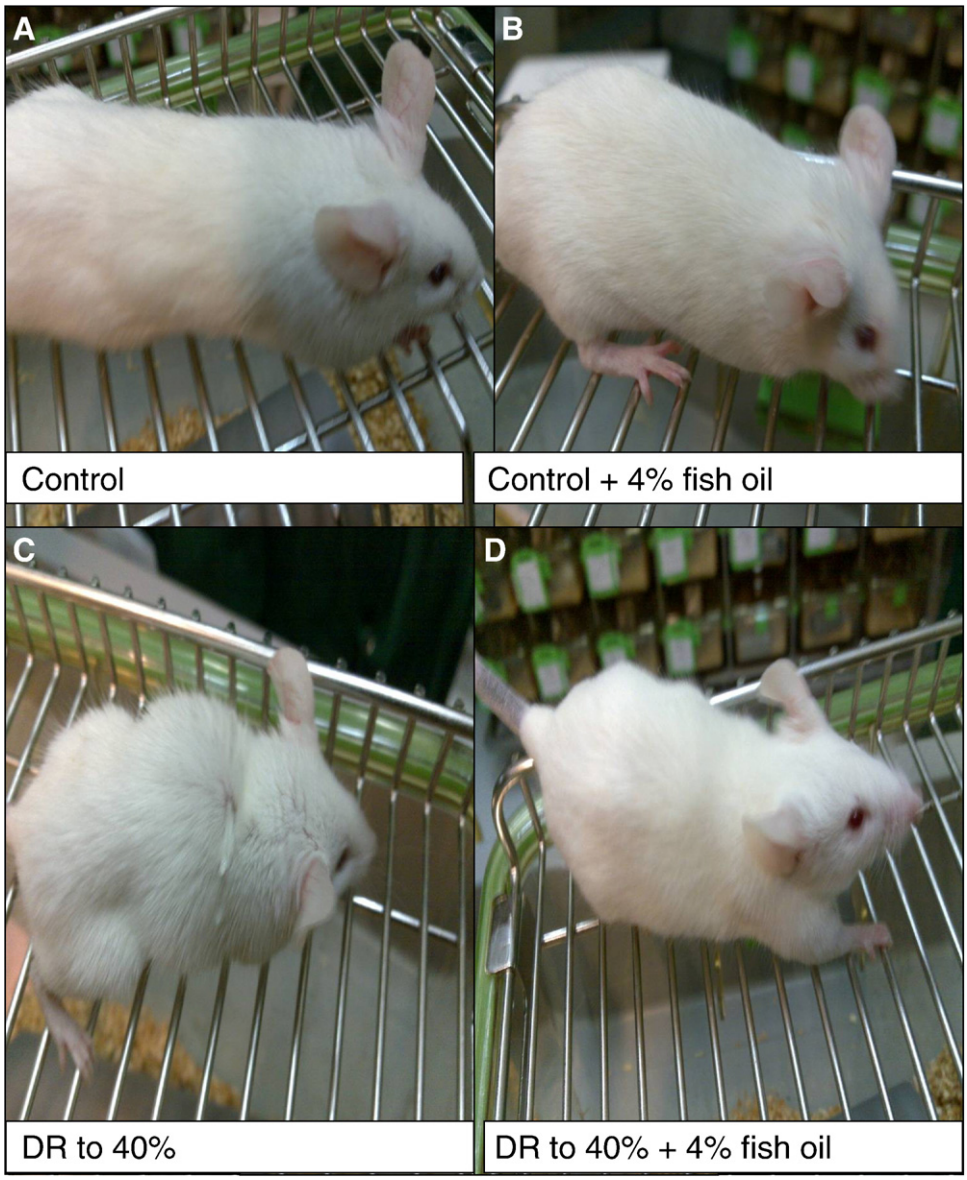


Fig. 2. Mice appearance following control, DR and fish oil supplementation (representative mice from each group are presented). Mice maintained on the 100% diet presented a healthy appearance and appeared well groomed, regardless of oil supplementation (A, B). However, the 40% diet supplemented with canola oil displayed matted, ungroomed and piloerect fur, and were sickly in appearance (C). Strikingly, fish oil supplementation to mice on the 40% diet appeared was protective in terms of general appearance (D), as these mice appeared more similar to the mice maintained on the 100% diet than those supplemented with canola oil on the 40% diet, despite their profound weight loss.

### 3.4. Survival rates

After 12 days, all mice in the 100% diet groups survived, regardless of oil supplementation ($n$=40 mice/group). However, only 22 of 40 (i.e., 55%) of the 40% DR mice that received canola oil supplementation survived (Fig. 4; Fisher's exact test with Bonferroni–Holm modification: $P$<.01 vs. control). Fish oil supplementation led to a dramatic improvement in survival rates of the 40% DR mice to 82.5% (i.e., 33 of 40 mice; $P$<.01 vs. DR to 40%). Because of the high mortality rates in the 40% diet, canola oil supplemental group, the experiment was ended at 12 days.

### 3.5. Hypothalamus

#### 3.5.1. Endocannabinoid system

Because of the important roles that the endogenous cannabinoid system plays in regulating feeding behavior and learning, we examined endocannabinoid levels as well as CB1 receptor function in both hypothalamus and hippocampus of the mice in each of the four conditions. As shown in Fig. 5A, 40% diet with canola oil supplement resulted in a significant decrease in hypothalamic 2-AG compared to the 100% diet groups ($P$<.05a). Fish oil supplementation normalized 2-AG levels in this brain region of mice receiving 40% diet ($P$<.05b). DR significantly decreased anandamide levels (Fig. 5B) ($P$<.05a), while fish oil supplementation did not affect anandamide levels. We next examined whether DR and oil supplementation would affect CB1 receptor binding and function. Curiously, significantly more CB1 receptor binding (Fig. 5C) and GTPγS binding activity (Fig. 5D) were found in the 100% diet group given canola oil compared to each of the other three groups, which did not differ from one another. The $K_D$ values did not differ among the four groups; however, a significant difference in $B_{max}$ values ($P$<.05a) was found between the control and the other three groups in the various concentrations (Fig. 5C) (Table 2).

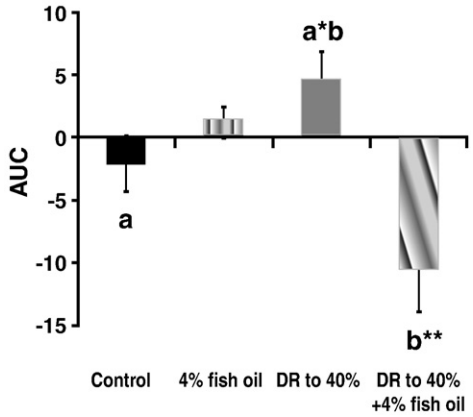


Fig. 3. Cognitive function in the eight-arm maze test. Cognitive function was impaired following DR to 40% and was improved by fish oil supplementation. Values are means± S.E.M. $n$=34 for control, 20 for 4% fish oil, 32 for DR to 40% and 25 for DR to 40%+4% fish oil. Means with a common letter differ. $P$<.05 corrected for multiple comparisons.

### 3.5.2. CB1 receptor function

ANOVA followed by Dunnett's post hoc (P α<.05) showed that the $E_{max}$ values for 100% DR+fish oil, 40% DR+canola oil and 40% DR+fish oil were significantly lower than that 100% DR+canola oil suggesting a reduction in CB1 receptor function; groups 100% DR+fish oil, 40% DR+canola oil and 40% DR+fish oil were not significantly different to each other (Fig. 5D).

The decrease in CB1 receptor activity and function following fish oil supplementation in 100% DR has metabolic value for weight maintenance and health (Table 3).

### 3.5.3. Monoamine concentrations

The 40% diet group supplemented with canola oil possessed significantly decreased levels of 5-HT in the hypothalamus compared to the 100% diet group supplemented with canola oil. Fish oil supplementation increased almost significantly ($P$<.056) 5-HT levels in the hypothalamus in the 40% diet group (Fig. 6A).

Dopamine concentration decreased in the hypothalamus in the 40% diet group given canola oil extract (Fig. 6B) ($P$<.05a). Fish oil supplementation restored dopamine concentration in the 40% diet group ($P$<.05b).

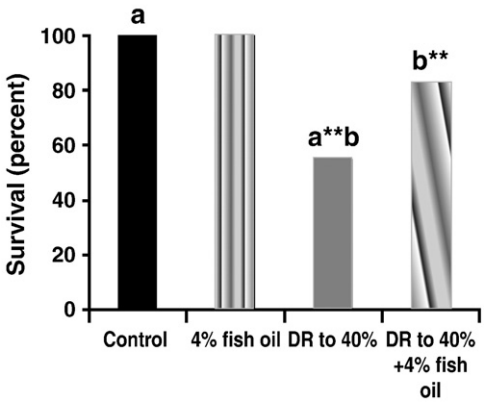


Fig. 4. Survival rates after 2 weeks of experiment. 100% of the mice not subjected to DR survived while only 55% of the DR to 40% mice and 82.5% of the DR mice fed with fish oil survived. $n$=40 mice per group at the beginning of the experiment. $n$=40 for control and 4% fish oil, 22 for DR to 40% and 33 for DR to 40%+4% fish oil at the end of the experiment. Means with a common letter differ. $P$<.05, $P$<.01.

### 3.5.4. Gene expression

The expression of mRNA for the NPY gene increased in the 40% diet group (Fig. 6C; $P$<.05b vs. control) and 4% fish oil supplementation restored it ($P$<.05c). The expression of the Camkk2 gene increased in the 40% DR group (Fig. 6D; $P$<.05a vs. control) and fish oil restored it ($P$<.05b vs. DR to 40%).

## 3.6. Hippocampus

### 3.6.1. Endocannabinoid system

In contrast to the results in the hypothalamus, no significant difference was detected in hippocampal 2-AG levels caused by DR; however, oil supplementation increased 2-AG levels ($P$<.05a) (Fig. 7A). However, neither DR nor fish oil supplementation change anandamide level (Fig. 7B). However, as found above, significantly more CB1 receptor binding and GTPγS binding activity was found in the 100% diet group given canola oil compared to each of the other three groups, which did not differ from one another (Fig. 7C, D). ANOVA followed by Dunnett's post hoc (P α<.05) showed that the $B_{max}$ values for 100% DR+fish oil, 40% DR+canola oil and 40% DR+fish oil were significantly lower than that diet of 100% DR+canola oil, suggesting a reduction in CB1 receptor level; groups 100% DR+fish oil, 40% DR+canola oil and 40% DR+fish oil were not significantly different to each other (Fig. 7C).

ANOVA followed by Dunnett's post hoc (P α<.05) showed that the $E_{max}$ values for 100% DR+fish oil, 40% DR+canola oil and 40% DR+fish oil were significantly lower than that of 100% DR+canola oil, suggesting a reduction in CB1 receptor function; groups 100% DR+fish oil, 40% DR+canola oil and 40% DR+fish oil were not significantly different to each other (Fig. 7D).

### 3.6.2. Monoamine concentrations

The 40% diet group supplemented with canola oil possessed significantly decreased levels of 5-HT in the hippocampus compared to the 100% diet group supplemented with canola oil ($P$<.05a). Fish oil supplementation increased 5-HT levels in the hippocampus in the 40% diet group compared to the canola oil-supplemented 40% diet group (Fig. 8A; $P$<.05b).

In contrast, no significant changes were found in hippocampal DA (data not shown). Interestingly, the 40% diet group supplemented with fish oil possessed significantly higher concentrations of norepinephrine than the 40% groups (Fig. 8B; $P$<.05a), which is consistent with the enhanced performance of these mice in acquisition of the radial arm task.

### 3.6.3. Gene expression

The expression of mRNA for Camkk2 increased in the hippocampi of the 40% diet group supplemented with canola oil compared to the corresponding 100% diet group (Fig. 8C; $P$<.05a). The 4% fish oil supplementation in the 40% diet group fully normalized Camkk2 mRNA ($P$<.05b).

BDNF expression increased following fish oil supplementation to both the control group (Fig. 8D) and the DR group ($P$<.05a and $P$<.05b, respectively). Finally, SIRT-1 expression increased following fish oil supplementation, both in the control group (Fig. 8E; $P$<.05a) and the DR group ($P$<.05b).

## 4. Discussion

The major findings reported here were that fish oil supplementation improved appearance, enhanced survival and reversed cognitive dysfunction following severe DR in mice. The metabolic stress induced by caloric restriction was ameliorated by fish oil, as indicated by the normalization of genes and neurochemistry in the hypothalamus and

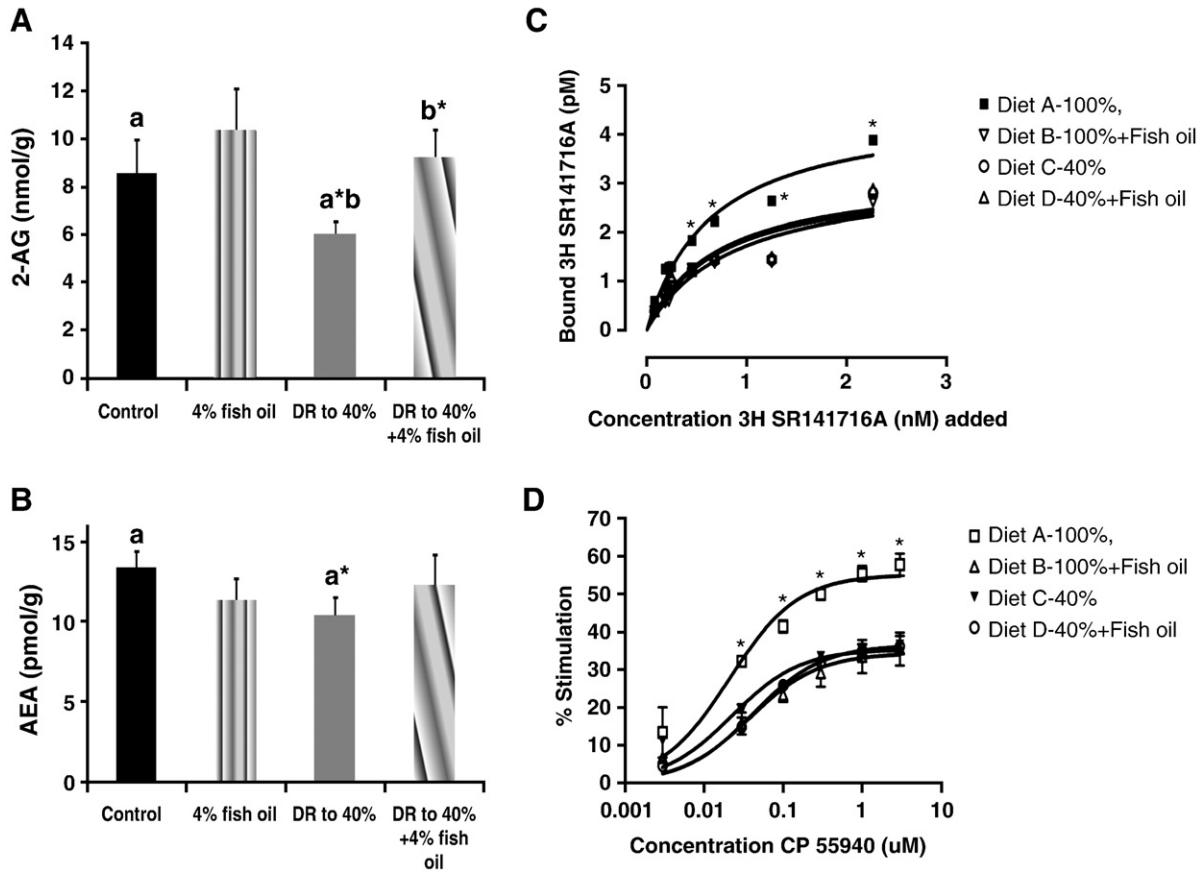


Fig. 5. Alterations in the endocannabinoid system in the hypothalamus. (A) 40% diet with canola oil supplement resulted in a significant decrease in hypothalamic 2-AG compared to the 100% diet groups ($P<.05$a). Fish oil supplementation normalized 2-AG levels in this brain region of mice receiving 40% diet ($P<.05$b). (b) DR decreased significantly ($P<.05$a), while oil supplementation did not affect anandamide levels. (C) More CB1 receptor binding and (D) GTPγS binding activity were found in the 100% diet group given canola oil compared to each of the other three groups, which did not differ from one another.

hippocampus. Fish oil supplement rescued NPY and Camkk2 gene expression as well as 2-AG, 5-HT ($P<.056$) and dopamine concentrations in hypothalami of 40% diet-restricted mice. Likewise, fish oil supplement normalized Camkk2 gene expression in the hippocampus and enhanced BDNF and SIRT-1 mRNA expression in the hippocampus. Thus, supplementation of fish oil "fooled" the body by producing satiety signals.

Fish oil supplementation improved appearance (Fig. 2), cognitive function (Fig. 3) and enhanced survival rate following severe DR (Fig. 4), without increasing body weight (Fig. 1). Importantly, both groups subjected to 40% DR received the same number of calories, but different percentages of the omega-3 fatty acids. The original working hypothesis of this study was that the behavioral effects associated with severe DR were caused by hypoactivity in endocannabinoid signaling, which would be normalized by fish oil supplementation. Consistent with this hypothesis was the observation that the significant reductions in hypothalamic 2-AG levels following 40% DR were normalized by fish oil. 40% DR did not affect hippocampal 2-AG levels; however, fish oil supplementation enhanced hippocampal 2-AG levels suggesting the possible involvement of the endocannabinoid system in these effects. Moreover, significant decrease in anandamide were found in the hypothalamus and no change in anandamide level following fish oil supplementation to 40% DR was noticed indicating that 2-AG is the primary endocannabinoid in the current model. Curiously, the 100% diet group that received canola oil supplementation showed increased CB1 receptor binding as well as increased CP55,940-induced stimulation of Gi/o protein activity in both brain regions compared to the other three groups, which did not differ among each other. Although the mechanisms that led to diet-induced changes in CB1 receptor expression and function remain to be elucidated, it is clear that CB1 receptor activity in these brain areas does not account for differences in the protective effects of fish oil in mice exposed to severe DR.

Searching for another mechanism to explain these beneficial effects of omega-3 fatty acids, we examined the expression of the genes encoding for NPY and Camkk2, which are involved in energy status, in the hypothalamus, the brain region responsible for energy regulation. We also measured the concentration of 5-HT and dopamine which are

Table 2
CB1 receptor binding in the hypothalamus and hippocampus

| | | 100% Diet+5% canola | 100% Diet+fish oil | 40% Diet+5% canola | 40% Diet+fish oil |
|---|---|---|---|---|---|
| Hypothalamus | $B_{max}$±S.E.M. | 4.59±0.449 | 3.236±0.625 | 3.1783±0.623 | 3.227±0.640 |
| | $K_D$±S.E.M. | 0.645±0.145 | 0.879±0.358 | 0.727±0.317 | 0.704±0.314 |
| Hippocampus | $B_{max}$±S.E.M. | 5.22±0.12 | 3.32±0.11 | 3.61±0.15 | 3.74±0.12 |
| | $K_D$±S.E.M. | 0.494±0.030 | 0.464±0.046 | 0.536±0.064 | 0.527±0.047 |

Table 3
Effect of diet on CP-55,940 stimulation of GTPγS binding in hypothalamus and hippocampus

| | | 100% Diet+5% canola | 100% Diet+fish oil | 40% Diet+5% canola | 40% Diet+fish oil |
|---|---|---|---|---|---|
| Hypothalamus | $E_{max}$±S.E.M. | 55.1±2.7 | 34.4±1.7 | 35.5±2.5 | 36.6±0.7 |
| Hippocampus | $E_{max}$±S.E.M. | 87.7±3.2 | 51.6±1.4 | 60.9±1.4 | 60.6±1.7 |

Values represent percent stimulation.

known to regulate feeding behavior in this brain region ([16,17] respectively). NPY is known to stimulate appetite [18]. Injection of NPY to animals leads to increased feeding [19] and production of fat cell precursors [20]. We have shown that prolonged, severe DR led to increased NPY expression [21], which was prevented by supplementation of 4% fish oil to the diet (Fig. 6C). These findings suggest that food-restricted animals were more "satiated" when their food was supplemented with fish oil than with canola oil. The high increase in NPY expression following severe food restriction implies an energetic deficiency existing in such a state and its prevention by fish oil supplementation, following decreased body weight of the treated related to untreated 40% DR groups (Fig. 1).

We have previously reported that increased phosphorylation of AMPK enhances activation of this molecule [10], which is known to stimulate feeding [22] and weight gain [23]. In addition, we previously found that an increase in the phosphorylation and activation of this molecule enhances NPY expression [24]. AMPK responds to acute changes in the energetic regulation at the cellular level, and when activated, it turns off anabolic pathways in the body. It consumes adenosine triphosphate (ATP), such as in synthesis of fat and carbohydrates, and triggers catabolic pathways, which promote ATP production from fatty acid and glucose in order to maintain the body's energetic balance [25]. Many findings suggest that Camkk2 causes activation or phosphorylation of AMPK [26]. In experiments performed on mice lacking the gene for Camkk2, the hypothalamic activity of AMPK decreases, and as a result, NPY and Agouti related protein (AgRP) expression in NPY neurons decreases [27]. We found that as a result of 40% food restriction, Camkk2 expression increased, but fish oil prevented this effect (Fig. 6D). This finding is in line with the level of NPY expression. We postulate that this normalization results from the elevation of leptin, which was previously shown to

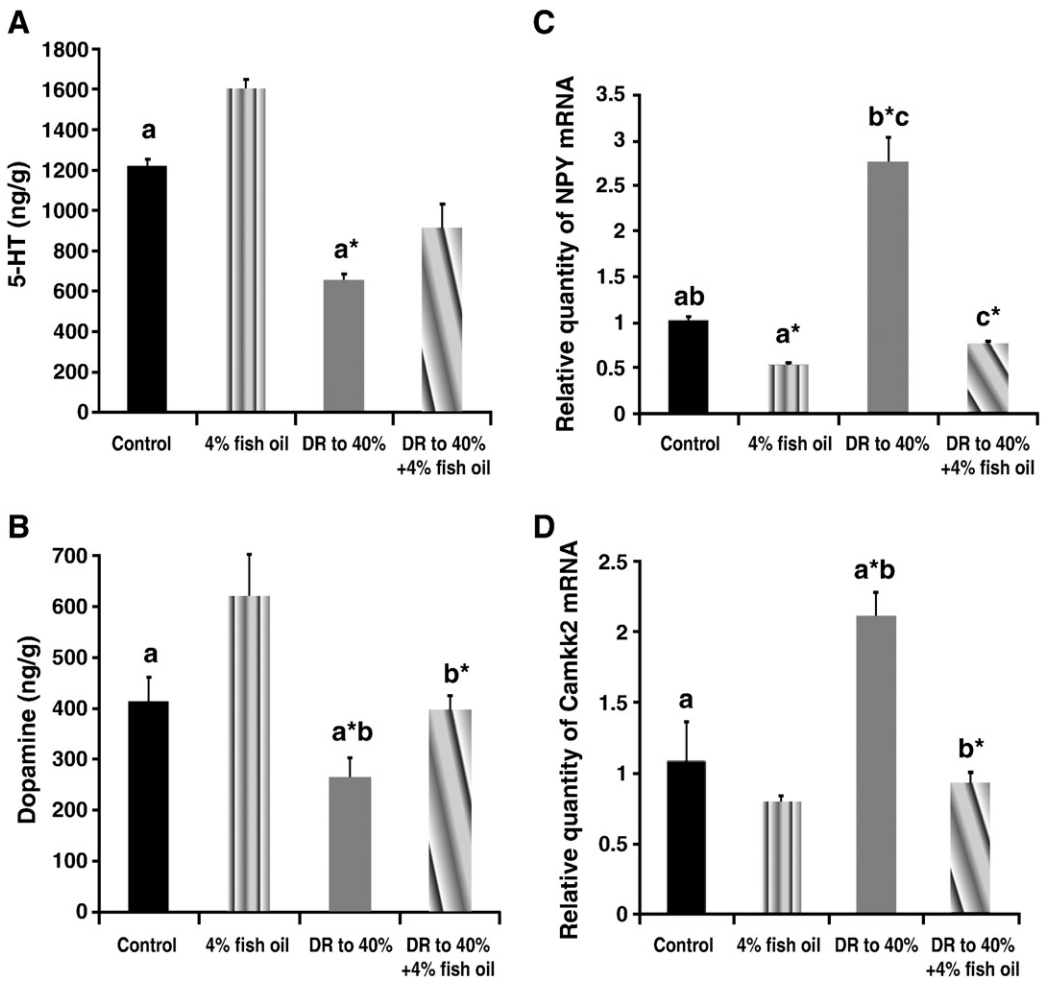


Fig. 6. Monoamine concentrations and gene expression in the hypothalamus. (A) The 40% diet group supplemented with canola oil possessed significantly decreased levels of 5-HT in the hypothalamus compared to the 100% diet group supplemented with canola oil ($P$<.05a). Fish oil supplementation increased 5-HT levels in the hypothalamus in 40% ($P$<.056) diet groups. (B) Dopamine concentration decreased in the hypothalamus in the 40% diet group given canola oil extract ($P$<.05a). Fish oil supplementation restored dopamine concentration in the 40% diet group ($P$<.05b). (C) NPY mRNA expression increased following DR to 40% ($P$<.05b) and was restored by fish oil supplementation ($P$<.05c). Camkk2 mRNA expression increased following DR to 40% ($P$<.05a) and was restored by fish oil supplementation ($P$<.05b). Values are means±S.E.M.

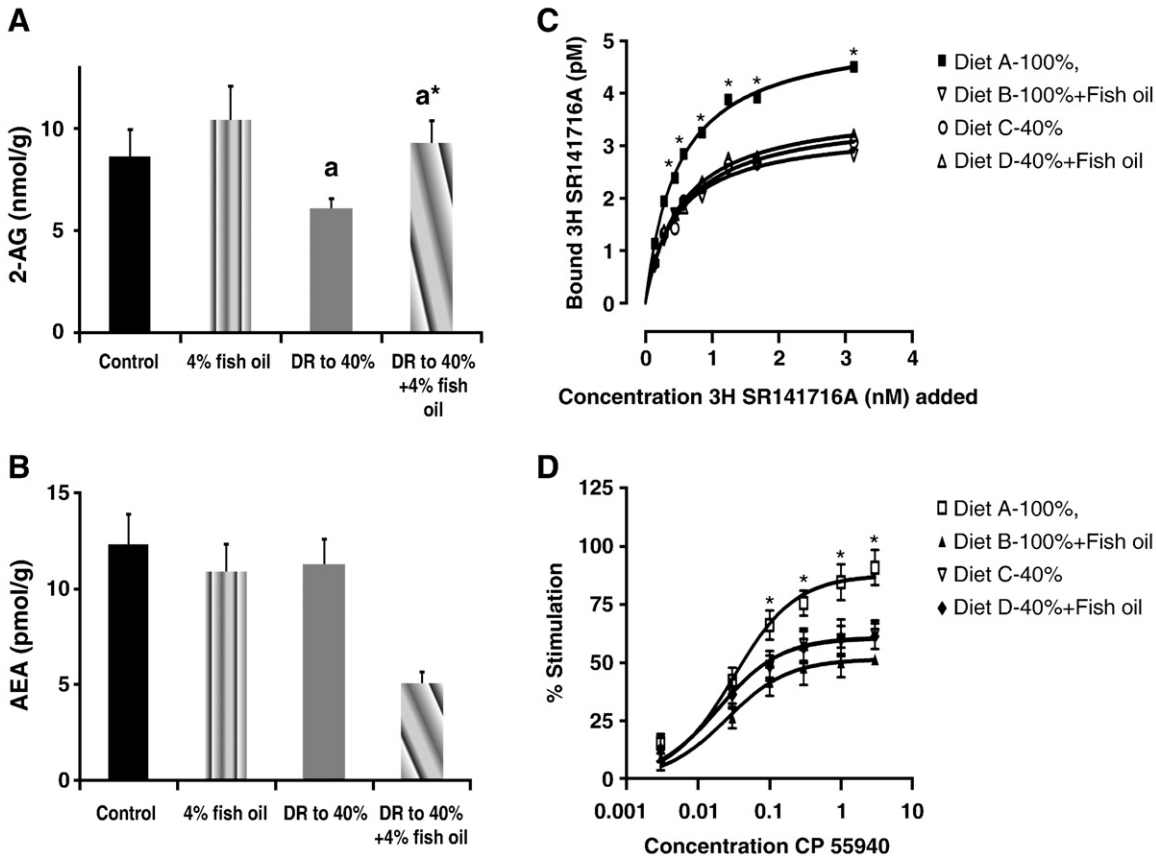


Fig. 7. Biochemical alterations in the endocannabinoid system in the hippocampus. (A) No differences were detected in hippocampal 2-AG levels following DR, while there was significant increase following oil supplementation. (B) Neither DR nor fish oil change anandamide levels. More CB1 receptor binding and GTPγS binding activity was found in the 100% diet group given canola oil compared to each of the other three groups, which did not differ from one another.

negatively regulate AMPK phosphorylation [10]. Indeed, others found that fish oil enhanced leptin levels in plasma [11].

In the present study, we measured the concentration of the monaminergic neurotransmitters 5-HT and dopamine in the hypothalamus. A decrease of 5-HT was observed as a result of 40% DR and was almost restored ($P$<.056) by fish oil supplementation in the severely food-restricted animals (Fig. 6A). Dopamine concentration in the hypothalamus tended to increase as a result of fish oil in the control group, decreased significantly in the 40% DR group compared to the control group and fish oil supplement restored them (Fig. 6B). The increase of dopamine and 5-HT concentrations as a result of fish oil indicates that fish oil induced satiety, as these parameters were reported before to be associated with satiety [28,29], without causing any increase in body weight. Thus, omega-3 supplementation "fools" the energy sensor of the body to signal a state of satiety despite severe undernourishment, thereby promoting survival.

Undoubtedly, the biological, sociological and psychological factors contributing to AN are highly complex and interrelated. Consequently, a single animal model cannot possibly capture its full scope. In this report, we used the DR model to focus on one aspect of this condition, the significant deficiency in the number of calories consumed over time. However, two major limitations of DR model must be acknowledged. First, the food restriction is involuntary, being manipulated by the experimenter and not the animal. Second, DR does not model the antecedents of this devastating disease. Despite these limitations, many of the changes in neuroendocrine function and other phenotypes found in AN patients can be mimicked by DR in mice, thus giving it some face validity as a model of certain aspects of the disease [2]. Indeed, restricted caloric intake models key phenotypes presented by an AN patient, including profound weight loss, cognitive deficits, alterations in hormones and neurotransmitters, and increased mortality and morbidity. Accordingly, we focused on the consequences of severe DR, one of the many manifestations of this disease, not its antecedents.

Long-term potentiation (LTP) is believed to play a large role in learning and memory [30]. A potential mechanism that accounts for the present results is that insufficient intra-membranous PUFA elicits adverse effects on cell membrane fluidity [31]. Omega-3 PUFA deficiency was reported to reduce the release of neurotransmitters vesicles, thus the signaling between the cells was impaired [32], and a decrease in membrane microviscosity was reported to be associated with a decrease in the density of muscarinic receptors which are involved in learning and memory [33]. To summarize this point, omega-3 PUFA deficiency may disrupt membrane fluidity, which may further weaken synaptic connections and thus impair learning and memory. Hence, the great importance of investigating this issue in AN in which many nutrients are deficient, including fatty acids. Due to these results and the cognitive deficits found in anorexia patients, we examined the effects of the addition of omega-3 PUFA-containing oils to diet in a murine model of anorexia. Cognitive function was found to decline following DR to 40% and was restored following fish oil supplementation (Fig. 3). In order to elucidate the underlying mechanism for this phenomenon, we have investigated catecholamine and gene expression in the mice hippocampi. As mentioned above, the hippocampus is the brain region responsible for cognitive function.

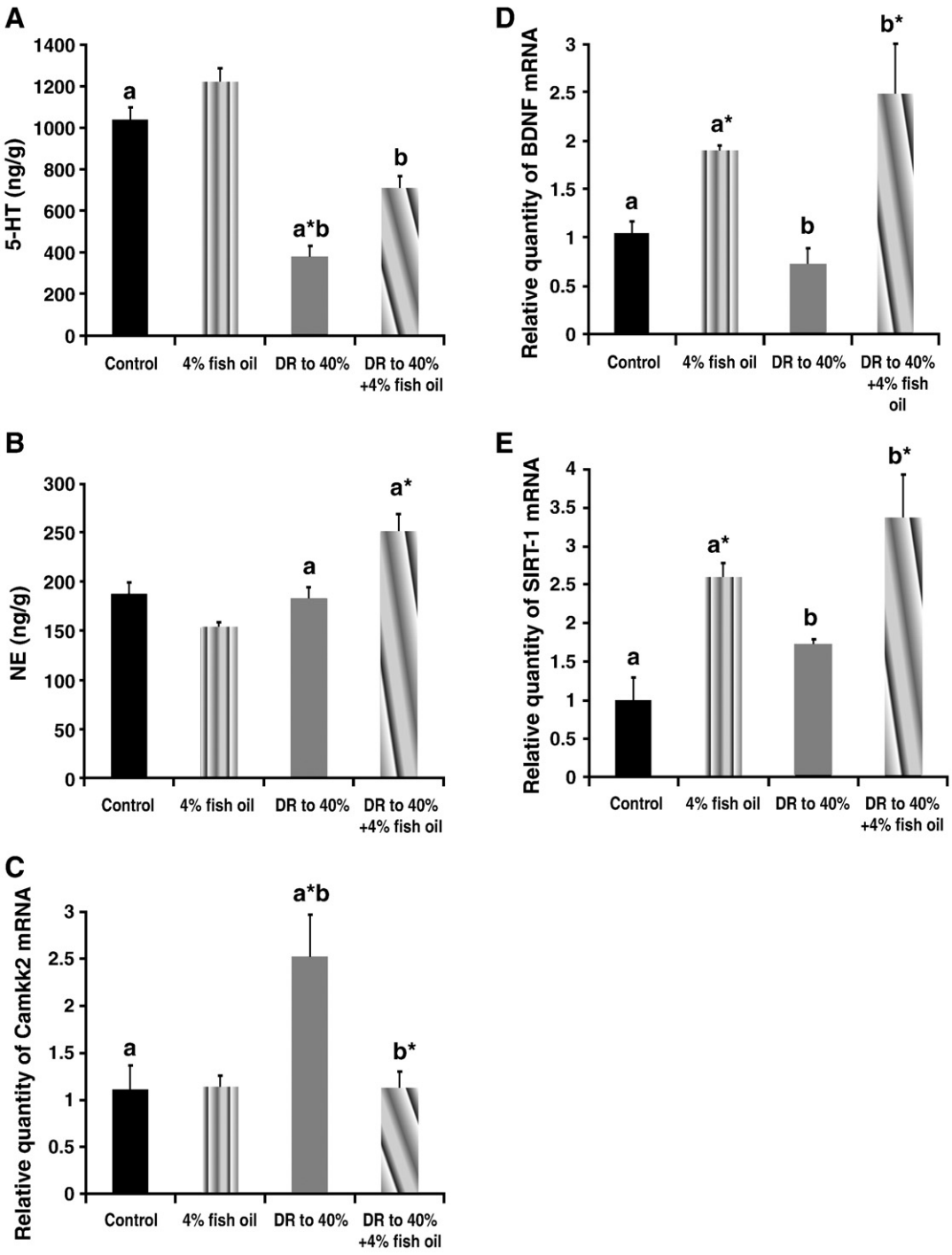


Fig. 8. Monoamine concentrations and gene expression in the hippocampus. (A)The 40% diet group supplemented with canola oil possessed significantly decreased levels of 5-HT in the hippocampus compared to the 100% diet group supplemented with canola oil ($P<.05$a). Fish oil supplementation increased 5-HT levels in the hippocampus in the 40% diet group compared to the canola oil-supplemented 40% diet group ($P<.05$b). (B) The 40% diet group supplemented with fish oil possessed significantly higher concentrations of norepinephrine than 40% groups ($P<.05$a), which is consistent with the enhanced performance of these mice in acquisition of the radial arm task. (C) The expression of mRNA for Camkk2 increased in the hippocampi of the 40% diet group supplemented with canola oil compared to the corresponding 100% diet group ($P<.05$a). The 4% fish oil supplementation in the 40% diet group fully normalized Camkk2 mRNA ($P<.05$b). (D) BDNF expression increased following fish oil supplementation to both the control group ($P<.05$a) and the DR group ($P<.05$b). (E) SIRT-1 expression increased following fish oil supplementation, both in the control group ($P<.05$a) and in the DR group ($P<.05$b).

Following the finding of cognitive decline after DR to 40% and its restoration by fish oil, we determined the expression of the gene for Camkk2, which is related to the regulation and activation of AMPK [26]. We showed previously that DR to 40% activates or phosphorylates AMPK, which was accompanied by cognitive decline, while leptin, an adipokinin hormone regulating body weight, normalized AMPK phosphorylation [10]. The equivalent to this finding in the present study is the increase in Camkk2 under DR to 40% and its decrease after fish oil supplementation (Fig. 8C), which may account for the cognitive improvement by fish oil (Fig. 3).

We also found a significant decrease in hippocampal 5-HT concentration as a result of caloric restriction to 40%. Fish oil elevated

hippocampal 5-HT levels in diet restricted to 40% (Fig. 8A). This increase in 5-HT following fish oil may indicate a reduction of the stress effect induced by the severe diet, and may also account for the cognitive improvement, as 5-HT is well known to play an important role in memory [33].

The major findings reported here are that fish oil supplementation enhanced survival and reversed cognitive dysfunction following severe DR. The metabolic stress induced by caloric restriction was ameliorated by fish oil supplementation, as indicated by the normalization of 2-AG, 5-HT and dopamine, as well as the genes for NPY and Camkk2 in the hypothalamus. Hence, it appears that fish oil may have beneficial effects in situations of severe food restriction by normalizing neurochemical systems and other factors within the hypothalamus. Dietary fish oil also normalized hippocampal norepinephrine and 5-HT concentrations, which are necessary for intact cognitive function, and restored the expression of Camkk2 in the hippocampus, which implies an elimination of the metabolic stress in this region, which is responsible for learning and memory. Thus, fish oil may have improved cognition by the above-mentioned mechanisms in the hippocampus [34].

According to Di Marzo et al. [35], the brain seems particularly resistant to changes in the fatty acid profile of dietary fat. Dietary manipulation of n-3 fatty acids in the brain is complicated by their relative constitutively high concentrations in the organ. Manipulation of brain levels of endocannabinoids with dietary fat is of great value in view of the physiological role of these compounds in the regulation of synaptic plasticity including eating, cognition, mood movement and neurogenesis. Few studies addressed the issue of whether dietary fatty acids can modulate brain endocannabinoid concentrations: Berger et al. [36] showed in piglets that milk formulations enriched in long-chain polyunsaturated fatty acids (LC-PUFA) were able after 1 month to significantly modify the levels of the corresponding NAEs in various brain regions and an increase in whole brain anandamide levels by dietary arachidonic. Both Berger et al. [36] and Watanabe et al. [37] have shown that n-3 PUFAs reduce anandamide and 2-AG levels in the brain of postnatal mice and hypothalamus of adult mice, respectively, while the opposite effect was obtained with n-3 LC-PUFA-deficient diet. In the first study, piglets were fed in large quantities for 18 days while in the second, either female mice were fed a diet for 23 weeks before mating with male mice and the male pups were fed the same diet for an additional 6 weeks or male mice were supplemented with 10% linoleate or 10% DHA-rich fish oil for 4 weeks. More recently, Di Marzo et al. [35] showed that n-3 PUFA reduce anandamide levels in the brain of Zucker rats. Artmann et al. [38] showed in rats that 2.6% of EPA and DHA administered for 1 week did not change the brain 2-AG and anandamide concentrations. Thus, in order to change fatty acid profile of dietary fat, a high percentage of dietary fat and for long duration is required.

Our studies show that 40% DR decreased 2-AG and anandamide levels in the hypothalamus ($P<.05$a); 4% fish oil normalized 2-AG levels while it did not change anandamide levels. In the hippocampus there was almost no change in 2-AG levels while there was significant increase following fish oil supplementation and no change in anandamide levels (Fig. 7B). The main difference between the studies is that in our studies the mice were on severe DR which decreased 2-AG levels in the hypothalamus. As the brain keeps homeostasis [39], incorporation of more n-3 PUFA might have caused the metabolism of n-6 PUFA to 2-AG. According to Mathieu et al. [40], n-3 PUFA-deficient rats exhibited an increase in brain arachidonic acid levels, which was significantly more pronounced following stress in brain of postnatal mice and hypothalamus of adult mice, respectively. More recently, Di Marzo et al. [35] showed that n-3 PUFAs reduce anandamide in the brains of Zucker rat. n-3 PUFAs and/or fish oil elevated leptin levels and produce effect of satiety. According to Di Marzo et al. [41], leptin is known to reduce anandamide and 2-AG levels in rodents as well as CB1 receptor expression. Di Marzo's experiment was done on ad libitum diet while following severe DR, there are decreases in both leptin [10] and 2-AG levels, which was normalized by fish oil which probably enhanced leptin levels [11]. However, following fish oil supplementation, there is a decrease in CB1 receptor expression, which is consistent with the role of leptin. In the hippocampus, fish oil may have caused an elevation of leptin signaling which reduced CB1 receptor expression.

Fish oil "fools" the energy sensors of the body to signal a state of satiety by enhancing the production of satiety factors: 5-HT and dopamine and possibly leptin, which decreases orexogenic and increases anorexogenic signals in the hypothalamus. 2-AG levels were normalized. Enhancing satiety factors in AN improve the physical appearance and performance of the 40% DR mice given fish oil supplement, in spite of the fact that these mice received the same amount of calories in their diet as 40% DR mice given canola oil supplement.

Recently, it was shown in food-deprived fish that CB1 receptor expression correlated with endocannabinoid tone [42]. This finding is the opposite of what was reported here (enhancement of 2-AG levels and reduction of NPY expression). This different pattern of effects could be attributed to species differences and to the severe DR in our case. The effect of AEA, administered via water, was evaluated after different exposure times at both physiological and molecular levels. The results obtained indicate that fish exposed to AEA via water present approximately 1000-fold higher levels of AEA in both the brain and liver, which was associated with a significant increase in food intake as well as with the elevation of CB1 and NPY mRNA levels in the brain. A peripheral effect of AEA was also observed, since a time-dependent increase in hepatic CB1 mRNA and protein levels was detected. These effects were attenuated by the administration, via water, of a selective CB1 receptor antagonist (AM251) [43]. These findings indicate that the endocannabinoid AEA, at doses that stimulate food intake in fish, concomitantly stimulates the expression of the orexigenic peptide NPY as well that of its own receptor, thereby potentially enhancing its effect on food consumption. We have found in the hypothalamus decreased levels of AEA following severe DR and increased level of NPY.

Our findings suggest that fish oil may possess efficacy in treating cognitive decline as well as normalizing neurochemical dysfunctions related to AN or other related problems, such as cancer, cachexia or starvation. In conclusion, these results provide a compelling argument for clinical studies investigating fish oil supplementation following severe DR.

## Acknowledgments

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