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RESEARCH ARTICLE

Effects of DHA-Enriched Fish Oil Supplements on Dopamine Receptor Gene Expression in the Cerebral Cortex and Hippocampus Related to the Male Rat's Weight Gain

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Abstract

Background: Docosahexaenoic acid (DHA) can penetrate through the barrier between the blood and the brain through simple diffusion and obtains benefits for neurons. Supplementation of DHA-enriched fish oil for seven weeks in young male Wistar rat could alter dopamine (DA) receptor modulation, and it may be related to the increased weight gain. Aim: This present study aims to explore the alteration of the dopamine receptors (D1DR and D2DR) gene expression in the cerebral cortex and hippocampal area, as a top-down circuit control, related to the role of body weight gain as an indicator of food intake. Methods: Twenty samples of Rattus novergicus, aged 7-8 weeks, were randomly grouped into two groups as follows: 10 rats as the control group (CG) and 10 rats in the group that received treatment with fish oil - omega 3 capsules (FOG). CG and FOG were fed with standard laboratory food and received water ad libitum. The treated group (FOG) received 0.2 ml added supplementation of DHA-enrich fish oil (FO) capsules with the daily dose of 30 mg EPA and 45 mg of DHA for 7 weeks via gavage every morning. The body weight of each rat was weighed and recorded every 2 weeks. Results: The weight had increased significantly in the FOG during the first 4 weeks of treatment and continued to grow until the end of the 7 weeks of treatment ($p=0.036$). The relative D1DR gene expression in the cerebral cortex was significantly higher compared to the relative expression mRNA in the hippocampus region (D1DR 1.6 fold, $p < 0.05$). It was similar to D2DR FOG ($p<0.05$, 1.1 fold increase compare FOG in HC). Conclusion: Supplementation of DHA-enriched fish oil caused an alteration of the D1DR and D2DR gene expression, as neural circuitry regulating eating correlated with the altering weight gain in young rats with healthy feeding.

Keywords: *DHA, D1DR, D2DR, Weight gain.*

Introduction

Previous studies have found that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) enriched with fish oil omega 3 (FO) can influence the bio-physiological. In the meta-analysis of human and animal studies, the combination of fish oil supplementation with life modification intervention has benefited by effectively lowering weight gain in obese adults [1]. Reduction of body fat with supplementation of long-chain of n-3 family of Polyunsaturated Fatty Acid (PUFA) intake is possible through several mechanisms, such as increase feelings of satiety (appetite effect), increasing energy production and lowering storage of energy in the form of fat [2, 3].PUFA is mostly involved in the development of the brain in the neural membrane glycerophospholipid, related to its role in neuroprotective: neurogenesis, synaptogenesis, differentiation and stabilization of membrane fluidity [4]. Naturally, mammals cannot synthesize DHA, which is only consumed by dietary intake. In the brain, DHA transmits across the brain-

blood barrier by simple diffusion. Mice with a deficient of DHA in its brain have an alteration in synaptic transmission and plasticity. An accumulation of DHA in the human brain occurs during late gestation and early childhood. Comparatively, in rodents, this happens in the last three days of gestation through weaning period [5].

Previous studies have stated that a 0.2 ml daily dose of fish oil capsules containing 30mg EPA and 45mg DHA during an intervention period of 6 weeks can alter the lipid peroxidation in aged brain tissue [6]. The dietary supplementation of 40 mg/kg/day DHA for 30 days significantly increased DHA serum levels and DHA levels in the brain [7].

This previous study was used as a baseline time duration and dosage of DHA enriched fish oil in this study. In the DA system, there is a lack of n-3 PUFA in the dietary intake correlating with the decreased density of DA receptors in the brain [8]. The limbic area (nucleus accumbens and hippocampus), prefrontal cortical brain regions (orbitofrontal cortex and insula et al.) and neurotransmitter (dopamine and serotonin) play a role in rewarding the effects of food [9].

The hippocampus is also involved in the regulation of eating behaviours as mnemonic processes for memories and for encoding time, location in space and short term memories [10].The lateral entorhinal cortex (LEC) sends fibre to the hippocampus to the septal area that regulates feeding behaviour.

Projection fibre from the LEC to the hippocampal region activates the DA receptor (D2DR) cells which suggest association to the reduction of food intake in the rat [11]. Another study with rats found that activation of D1DR in the hippocampal region is associated with social learning and social interaction and might not involve food intake in male and female rats [12].

However, correlation studies of the DA gen receptor expression and the DHA intake are still limited. In this study, we use the supplementation of DHA in young rats to identify the pattern of D1DR and D2DR in the cerebral cortex and hippocampal region related with the bodyweight cue as an indicator of food intake.

Methods

Animals

Twenty male rats, ageing 7 to 8 weeks old, from the breed *Rattus novergicus* were bought from the Biofarma© Laboratories (73 to 105 grams in body weight). There was an acclimatization period in the facility for a total of 7 days before procedural laboratory experimental. They were grouped with 5 rats per cage. The light and dark cycle were maintained on a 12:12-h. The rats were also cared for in a low-stress environment (22°C, 50% humidity, no noise).

Food and water were provided ad libitum. The body weight was weighed in the 1st week, 4th week and 7th week during the study. The samples were divided into 2 groups as follows: 10 rats for the Control Group (CG) and 10 rats for treatment with fish oil omega 3 capsules (FOG). The CG was fed the normal standard laboratory food and received water ad libitum.

Those FOG was also supplied with the regular standard laboratory food, water ad libitum and received 0.2 ml of DHA-enriched FO capsules with the daily dose of 30 mg EPA and 45 mg DHA (capsules contains 600 mg EPA and 400 mg DHA) for 7 weeks via gavage every morning. The rats' body weights were weighed every 2 weeks. The procedure for the treatment of the animals was conducted according to the guide for the use and care of laboratory animals. It was approved by the Research Ethics Committee of Universitas Padjadjaran with the approval number: 1223/UN6.KEP/EC/2019.

Tissue Preparation and PCR

Brain Isolation

After 7 weeks of treatment, all rats were weighed and anaesthetized with isoflurane flow rate (concentration to 5% or higher). During the anaesthesia, the rats were euthanized by cervical based translocation. The brains were removed removal under 4 minutes. The sagittal section of the brain was conducted, and the right and left hippocampus were preserved in different tubes. The brain and hippocampal tissue were stored at -80º and were used to detect the gene expression of D1DR and D2DR.

RNA Extraction and Semi-quantitative PCR

A semi-quantitative standard RT-PCR was conducted to deduce the measurement of the gene of D1DR and D2DR in the brain.

By utilizing a 200 µl TRIzol Reagent (Qiagen), RNA was obtained from the brain.

CDNA was fused from 500 ng of total RNA in regards to the reverse transcription. This was done as per the Transcript or First Strand cDNA Synthesis kit (Takara Bio) by using the oligo dT and random primers in the following way; an addition of 0.5 lM sense and antisense primers, 200 lM dNTPs, 2.5 lL of the reverse-transcribed cDNA and 0.125 lL Taq polymerase (Roche) was made to the final volume of 25 lL. The prime number of PCR cycles was deduced to outline the PCR amplification's linear range.

Table 1: Primers used for semi-quantitative PCR Analysis

Gene Symbol	Primer sequence (5´ to 3´) Upper strand: sense Lower strand: antisense	Product Size (bp)	Annealing	Cycle
D1DR	5'- TATCTCCAGCCCTTTCCAGTATGA-3' 5'- ATTCCACCAGCCTCTTCCTTCTTC-3'	858	62	37
D2DR	5'- GTGGATCCATTGGGGCAGTG- 3' 5'- GTGCTTGACGCGGATGGTGA- 3'	794	57	34

Table 1 illustrates the annealing stage and the cycles repeated for mRNA D1DR and D2DR. As an internal control method, each sample of the PCR results obtained was normalized through the ß-actin mRNA level as an internal control. The experiments were repeated thrice to ensure that consistent results were obtained. Throughout the repetitions, the conditions and parameters of the tests remained unchanged. Image J software version 1.4.3 was utilised to analyze the imaged. By using the comparison kinetic amplification of ß-actin which acted as endogenous control, a significant amount of RNA from D1DR and D2DR was determined. Data analysis using IBM SPSS v.25 with independent t-test and one-way ANOVA. The sample size was determined based on our previous study conducted in our laboratory involving similar methods and intervention. Statistical significance is shown as $p < 0.050$

Results

The Gain of Body Weight Related to the Duration Supplementation of Dha Enriched Fish Oil (Fo) was Given

Table 2 showed an increasing body weight with the variation of the duration of supplementation of FO: initial periods 0 to 4 weeks, 4 to 7 weeks and initial periods 0 to 7 weeks. The weight had increased significantly in the FOG during the first 4 weeks of treatment and grew until the end of the FO supplementation at the 7th week. With the t-test, the difference average weight gain between the groups was significant at 0 to 4 weeks ($p=0.0072$, $p<0.05$) and 0 to 7 weeks ($p=0.036$, $p<0.05$). The weight gain was associated with the FO supplementation and tended to increase after 7 weeks of treatment. However, the FOG increase in weight gain was not higher than in CG (Figure 1).

Table 2: Male Wistar body weight at baseline, 4 weeks, and 7 weeks of treatment

	Control group (CG)			Fish oil omega 3 group (FOG)		
	Initial periods	**4 weeks**	**7 weeks**	**Initial periods**	**4 weeks**	**7 weeks**
Mean BW± SD	75.67±2.3	175.33±9.5	260.33±27.32	108.4±8.65	181.6±9.55	251±25.50
Δ Initial periods vs 4 weeks ($p = 0.0072$)*	99.67 ± 9.71			73.2 ± 8.76		
Δ 4 weeks vs 7 weeks ($p= 0.45$)	85 ± 31.05			69.4 ± 23.44		
Δ Initial periods vs 7 weeks ($p= 0.036$)*	184.67 ± 25.1			142.6 ± 19.3		

Note:
Δ : increasing weight gain of male *Rattus novergicus*; BW: body weight; SD: standard deviation
*p<0.05 indicates significant changes in levels over time within groups at the initial period 0 to 4 weeks and 0 to 7 weeks respectively. No considerable variation change in BW was noticed between 4 to 7 weeks.

The initial bodyweight of the rats in this study had a large variability in the range of body weight. Based on the paper previously referred to, for rats aged 7 to 8 weeks, the body weight ranged from a minimum of 58 grams to a maximum of 108 grams [13].This study used the rat that had the minimum body weight as a base and CG (73 to 76 grams) and the rat with maximum bodyweight as the DHA group/FOG (97 to 108 grams). It is assumed that based on the previous study, DHA is associated with decreasing body weight in the obese condition [12].

Dopamine1 Receptor (D1DR) Gene Expression in the Cerebral Cortex and the Hippocampal Region of the Male Wistar Rats

We examined the dopamine receptor gene expression using a semi-quantitative PCR; specifically the PCR bands of dopamine receptor 1 (D1DR) and dopamine receptor 2 (D2DR) gene. The study used ß-actin for normalization (Figure 2). The result shown in Figure 3 illustrates that the relative expression mRNA D1DR in the cerebral cortex is significantly higher compared to the relative expression mRNA in the hippocampus region (D1DR 1.6 fold, p=0.03, p<0.05, independent t-test). Although the expression mRNA in in CG in the cerebral cortex is not significantly different to the FOG, there is still significantly different between CG and FOG in the hippocampal region (p=0.036, p<0.05, the D1DR mRNA expression is decreased 0.7 fold compared to CG as seen through an independent t-test).

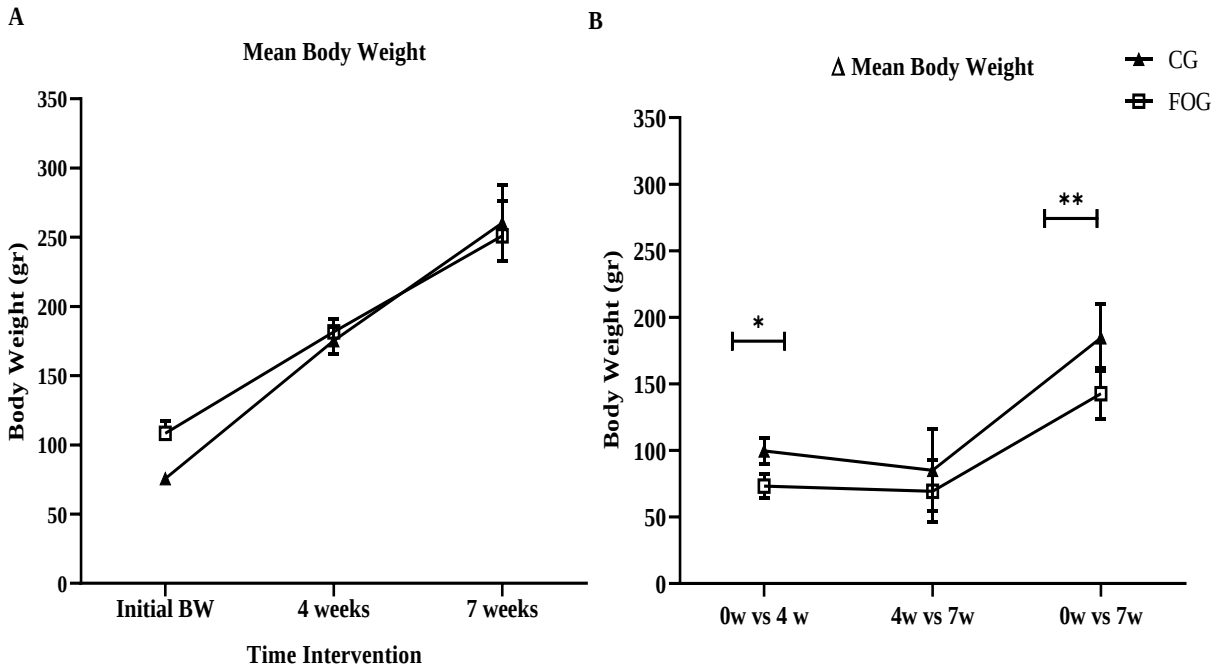

Figure 1: Trends of the FO supplementation increasing body weight between the 2 groups (A). The mean body weight gain for rats in FOG tends to increase after 4 weeks of the treatment, but the weight gain was not extremely high compared to CG (B). *Note*: *significant at *p*=0.0072; **significant at *p*=0.036

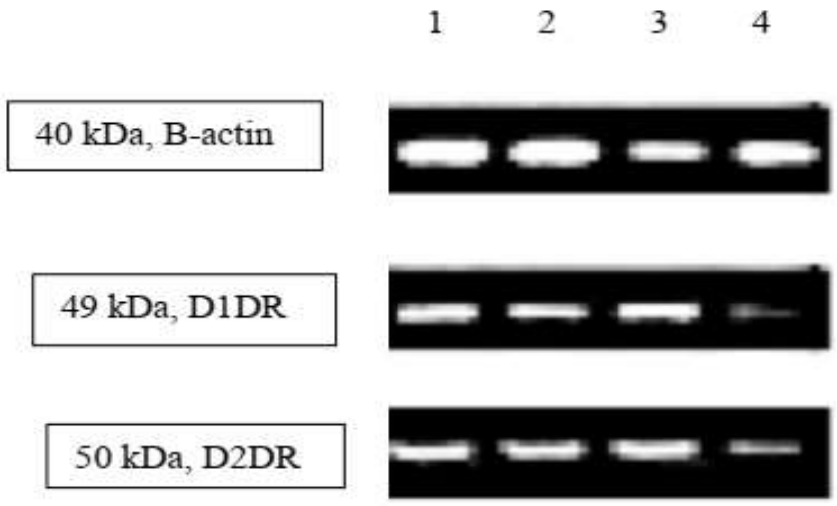

Figure 2: The different density of mRNA in the cerebral cortex and the hippocampal region after FO supplementation. *Note*: Group 1: control group (CG) in cerebral cortex; Group 2: fish oil-omega 3 group (FOG) in cerebral cortex; Group 3: control group (GC) in hippocampal; Group 4: fish oil-omega 3 group (FOG) in hippocampal

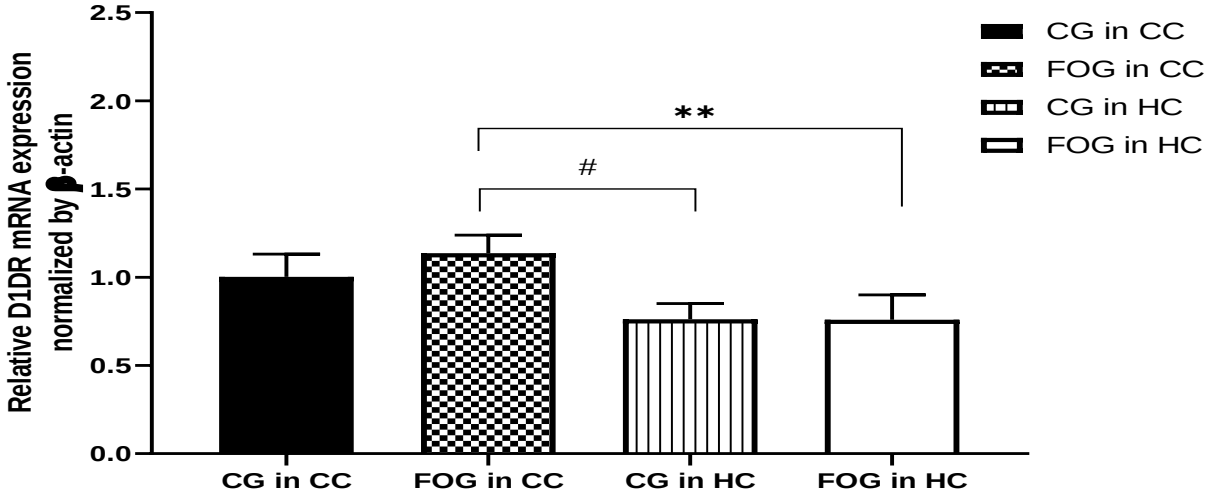

Figure 3: Seven weeks of FO supplementation in DIDR group. *Note*: #the density of the mRNA D1DR is significant highly (p=0.036) in the FOG's cerebral cortex compared to the FOG's hippocampal region; **the density of the mRNA D1DR in the FOG's cerebral cortex was significantly higher than FOG in the hippocampal region (p=0.037; independent t-test)

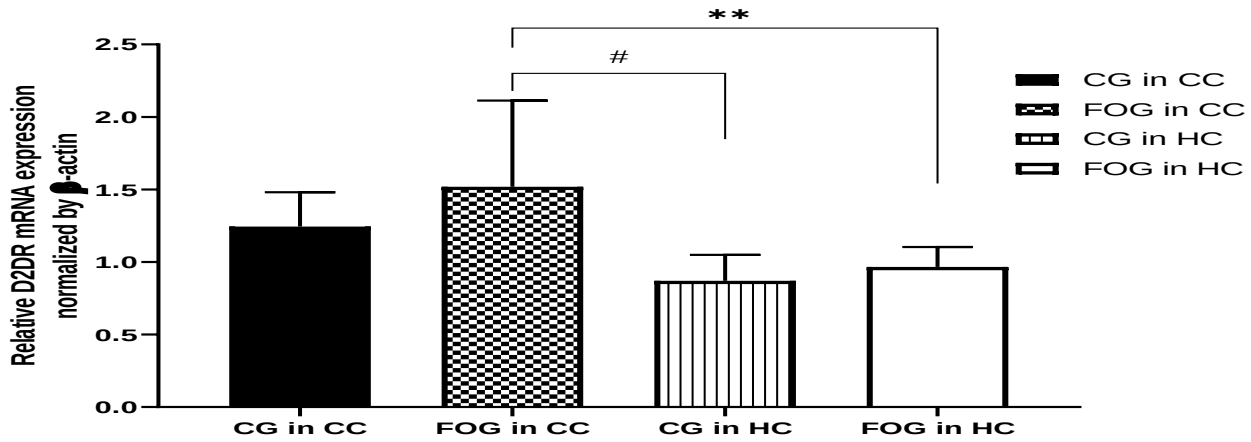

Figure 4: Seven weeks of FO supplementation in D2DR group. *Note*: #the density of the mRNA D2DR in the FOG cerebral cortex is significantly higher than CG in the hippocampal region (p=0.008); **the density of the mRNA D2DR is significantly higher in the FOG's cerebral cortex compared to the FOG's hippocampal region (p=0.020; ANOVA test)

Dopamine 2 Receptor (D2DR) MRNA Expression in the Cerebral Cortex and hippocampal Region of Male Wistar Rats

We also examined the D2DR gene expression in the rats' cerebral cortex and hippocampal region using a semi-quantitative PCR. D2DR bands gene normalized using the ß-actin (Figure 4). In the cerebral cortex, the relative expression of mRNA D2DR for FOG significantly higher compared to the hippocampal region ($p=0.02$, $p<0.05$, 1.1 fold increase compared to FOG in HC through independent t-test).

Discussion

There are three major results in this study. First, there were different trends of increasing weight gain between the CG and DHA enriched fish oil group (Figure 1). Second, the results also show that the density of D1DR and D2DR in the cerebral cortex is different than the hippocampal region (Figure 3). Third, there is a correlation between the weight gain and density of the DA receptor (Figure 4). The two major classes of polyunsaturated fatty acids (PUFA) are omega-3 and omega-6 fatty acids. PUFA has two or more double bonds between carbons within the fatty acid chain.

Alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is part of omega-3 PUFA. EPA and DHA are also natural ligands of PPAR-γ, nuclear receptor, which distribute and are expressed in many tissues, including in brain tissue. The role of synaptic endings and transport of dopamine system is associated with appetite-

regulating molecules and closely with the PUFA intake in the DHA study [2]. The DHA (cis C22:6 fatty acid) serum was increased with DA uptake through the blood-brain barrier, and it was assumed that DHA potentially acts as a carrier-mediated transport of dopamine to the brain [14].

In this study, 0.2 ml of DHA enriched fish oil (30mg EPA and 45mg DHA) in 7 weeks can increase D1DR and D2DR in group treatment; either in the cerebral cortex or hippocampal region when compared to CG (Figure 3 and 4). Since the previous study stated that DHA could move across the blood-brain barrier, mapping brain vasculature plays an important role. Development of vascular in the cerebral cortex is dominant in the hypothalamus and periventricular zone.

The hippocampus has less density of vascular than other areas in the cerebral cortex [15]. This might indicate that the density of DA in the cerebral cortex is higher than in the hippocampal region; either in CG or in group treatment. Several studies stated that chronic stress could alter the monoamine neurotransmitter system through activated cAMP-responsive element-binding protein (CREB) [16]. Supplementation of DHA enriched fish oil feeding via gavage during 7 weeks in young rats aged 6 to 7 weeks suggested that it comes to a critical point to increase dopamine density in this study.

However, receptor D1DR only increased in the cerebral cortex and not in the hippocampal tissue. It might be consistent with the previous study that D2DR is predominantly associated with feeding behaviours. The circuit of feeding behaviours sends reciprocal fibre from the lateral entorhinal cortex-hippocampal-septal region. In the study of rodents, by using as an assay manner of gen c-fos, the marker of neuronal activity in fasting and a fed condition stated that activity in the hippocampal cell neuron could be manipulated by influencing food intake [11, 17].

By using phosphor trap and an unbiased RNA profiling method, the study found that hippocampal neuron such as D2DR is activated by food stimulus [18].Optical stimulation on the lateral entorhinal cortex (LEC) projected onto the hippocampal region shows a significant decrease during the test with food consumption. It concluded that activation of D2DR in the hippocampal region was associated with the decreasing of food intake.[11] Consistent with this study, the weight gain trend in FOG did not increase high compared to CG; showing a correlation with the increasing D2DR in the DHA enrich fish oil group (Figure 3 and 4).

Neuron from LEC sending the projection fibre into the hippocampal region conveys sensory information from the gustatory, olfactory and visual cortex that is associated with a sense of food cues and can alter the food intake motivation [19].It is possible that the fish oil supplement induces “smell” that was not appropriate with the rat appetite, activated the LEC-Hippocampal region-septal area pathway and activated D2DR neuron in the hippocampal region as a regulator of food intake. The reward mechanism activated. The smell of fish oil could be reasons for negative response in terms of motivation to eat. Biological behaviour in rats was not tested in this study.

This might well be a subject for future research because the motivation to eat might be one of the important factors in researching eating behaviour. Land *et al.* in their previous study noted that the correlation of D1DR in the medial prefrontal cortex (mPFC) with optogenetic photostimulating of feeding. Activation of optogenetic photo-stimulating feeding caused an increasing neuron c-fos density as neural marker activity, and there was an increasing density of D1DR in mPFC. The studies also show the projection of fibre from mPFC to the basolateral amygdala, nucleus accumbens and dorsal striatum when the photo-stimulation of feeding is stimulated, which indicates control feeding behaviour.

Similarly, with our study, the density of D1DR increased in the cerebral cortex but not in the hippocampal region. It is assumed that the projection of D1DR neuron from mPFC in the cerebral cortex is dominant to the subcortical region such as the basolateral amygdala, nucleus accumbens and dorsal striatum less to the hippocampal region [20]. It might indicate why the density of mRNA of D1DR in the cerebral cortex significantly increased compared to the hippocampal region in the DHA enriched fish oil group. In this study, the exact mechanism by DHA can

increase D2DR and decrease D1DR in the hippocampus is unknown. The finding of neural circuitry regulating the eating needs by the DHA has been left for a future study.

Conclusion

Supplementation of DHA enriched fish oil stimulates the D1DR and D2DR genes expression, which plays a role in regulating the neural circuitry for eating. The DHA could alter the DA receptor gene expression, and it may be correlated with homeostasis regulation in body weight gain or loss.

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Author Contributions

All authors state that they meet the current ICMJE criteria for Authorship. Conceptualization, F.V., and H.G.; Methodology, F.V., A.R., A.P., and G.W.; Writing – Original Draft, F.V., L.L.F., and A.P.; Writing – Review & Editing, F.V., A.R., A.P., and G.W.; Supervision and Resources, F.V., A.R., A.P., R.L., and G.W.; Formal Analysis, F.V., L.L.F., A.P., R.L., H.G., and G.W.

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