Department of Pharmacology[1] and Department of Psychiatry[2], Medical University, Wroclaw, Poland

The anxiolytic activity of n-3 PUFAs enriched egg yolk phospholipids in rat behavioral studies

M. Rutkowska[1], W. Słupski[1], M. Trocha[1], M. Szandruk[1], J. Rymaszewska[2]

Received April 29, 2016, accepted June 3, 2016

Maria Rutkowska, Department of Pharmacology, Wroclaw Medical University, Mikulicza-Radeckiego 2, 50-345 Wroclaw, Poland
maria.rutkowska@umed.wroc.pl

Pharmazie 71: 655–659 (2016) *doi : 10.1691/ph.2016.6646*

Phospholipids play an important role in the biochemical and physiological processes of cells. An association between disturbed phospholipids metabolism in neuronal tissue and anxiety it was shown. The aim of this study was to examine the anxiolytic properties of phospholipids obtained from a new generation of eggs enriched in n-3 PUFA and its effect on locomotor activity in rat behavioral studies N-3 PUFA-enriched egg yolk phospholipids ("super lecithin") were added to the standard feed. Rats were fed by chow without (control group) or with (experimental group) addition of phospholipids. After six weeks of supplementation, the effect of phospholipids on locomotor activity in the open field test and anxiolytic properties in elevated plus maze and Vogel conflict test were examined. In the open field test the total distance traveled in the experimental group was similar to the control group. In the elevated plus maze test a six weeks phospholipids' administration significantly prolonged the time spent on the open arms by rats from experimental group compared to control group. The number of entries into the open arms was also increased but the difference was not statistically significant. The number of punished drinking water in the Vogel conflict test increased significantly in experimental *versus* control group. The obtained results suggest that the phospholipids isolated from n-3 PUFA enriched egg yolk have a specific anxiolytic effect, without general sedative influence.

1. Introduction

Phospholipids are the main constituent of neuronal membranes, and play a crucial role in the biochemistry and physiology of neurons. Based on their chemical structure, phospholipids may be divided into glycerophospholipids (e.g.: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol), and sphingophospholipids (Farooqui et al. 2000). Cerebral phospholipids contain exceptionally high amounts of polyunsaturated fatty acids (PUFAs), mainly arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:5n-6) of the n-6 and the n-3 series, respectively (Vancassel et. al 2001). Both, n-3 and n-6 PUFA are important for brain development and function, but n-3 PUFA in particular are often deficient in modern diets. DHA deficiency markedly affects neurotransmission (Chalon 2006), membrane-bound enzyme and ion channel activities (Yehuda et al. 2002), gene expression (Barcelo-Coblijn et al. 2003), intensity of inflammation and immunity (Calder et al. 2002), and synaptic plasticity (Bhatia et al. 2011). Human studies indicate that a lack of n-3 PUFA, or an imbalance between n-3 PUFA and n-6 PUFA is involved in the increasing prevalence of many diseases, including anxiety disorders (Green et al. 2006; Messamore and Namara 2016; Ross 2009). In addition, some clinical evidence suggests that the supplementation of n-3 PUFAs may ameliorate anxiety symptoms (Kiecolt-Glaser et al. 2011; Santos et al. 2013; Buydens-Branchey et al. 2008). Cerebral DHA level depends on dietary DHA content as well as on the liver synthesis from its nutritionally essential PUFA precursor, α-linolenic acid (α-LNA, 18:3n-3) – a shorter chain form (Rapoport et al. 2007). Cerebral astrocytes can also convert α-LNA to DHA, but the synthesis in the brain is limited. So, plasma DHA constitutes the main source of DHA for the brain (Bradbury 2011).

Fish is the richest dietary source of n-3 PUFA, mainly eicosapentaenoic acid (EPA) and DHA, playing an essential role in human health. However, fish consumption is below the recommended intake level in many countries (Hossain 2011). It is also notable that fish may be an important source of various toxic environmental contaminants, such as methyl mercury, polychlorinated biphenyls, dioxins, organochlorine pesticides and other environmental contaminants (Costa 2007). Therefore, it seems reasonable to introduce several n-3 PUFA-enriched food products that could be an alternative source of those healthy fatty acids.

Eggs are a potential source of n-3 PUFA because they can be easily enriched with n-3 PUFA by dietary modifications of laying hens. Three n-3 PUFA-enriched eggs provide approximately the same amount of n-3 PUFA as one fish meal (Lewis et al. 2000). Eggs are also an excellent dietary source of choline (251 mg/100 g, mostly in the form of phosphatidylcholine), an essential nutrient for humans (Zeisel et al. 2003). The compound is necessary for synthesis of phospholipids in cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signaling, and lipid-cholesterol transport and metabolism. Choline can also be synthesized de novo by sequential methylation of phosphatidylethanolamine to phosphatidylcholine. However, de novo synthesis of choline alone is not sufficient to meet human requirements (Zeisel and Blusztajn 1994). Despite the significant role played by choline in the central nervous system (CNS), most studies focus on its role in the cognitive processes, as a precursor of acetylcholine. On the other hand, the effect of the compound on emotions is poorly understood. However, results of some large population-based studies suggest that choline deficiency may favor the development of anxiety disorders (Bjelland et al. 2009). The beneficial effect of choline on emotional function is confirmed in animals studies. Choline supplementation during pregnancy or adolescence was associated with prevention or reduction of anxiety-like behaviors in rats (McCall et al. 2015).

2. Investigations and results

The purpose of our study was to evaluate the effect of n-3 PUFA-enriched egg yolk phospholipids ("super lecithin", SL), on anxiety behavior in rat. SL obtained from modified eggs is a rich source of n-3 PUFA, particularly of DHA, and have a very good n-6/n-3 PUFA ratio of about 1.5:1.

2.1. *Effects of SL supplementation on the locomotor activity in the open field test (OF)*

As seen in Fig. 1 there was no significant difference in the distance traveled between the supplemented rats and control rats ($p > 0.05$), suggesting that SL supplementation had no effect on the locomotor activity of animals.

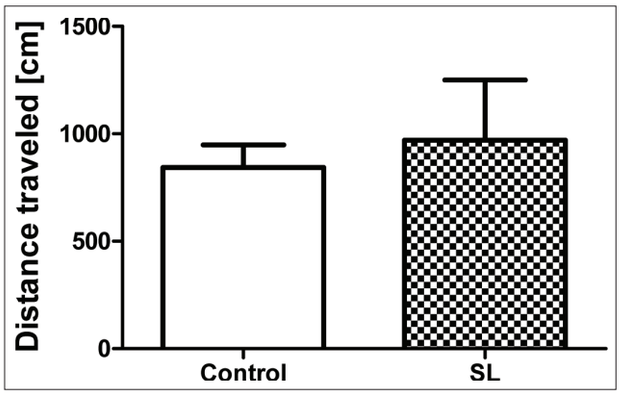

Fig. 1: Effect of "super lecithin" (SL) on rat's locomotor activity in the open field test. Data are expressed as mean ± SD (n = 10).

2.2. *Effects of SL supplementation on anxiety-like behavior in the elevated plus maze test (EPM)*

In the EPM we found that time spent on the open arms of the maze by SL supplemented rats was significantly longer compared to control group (128.83 *vs* 55.61, $p < 0.05$) and, conversely, less time was spent in the closed arms (145.43) when compared to controls (233.43); ($p < 0.02$). The number of entries into the open arms of the maze was also increased among the group of animals treated with phospholipids relative to the control group (4.6 *vs* 3.1), however the difference was not statistically significant (Fig. 2).

2.3. *Effects of SL supplementation on anxiety-like behavior in the Vogel conflict test (VCT)*

SL added to the standard chow significantly increased the number of punished licks in comparison to the control group (8.6 *vs* 4.6; $p < 0.001$) (Fig. 3), indicating an anxiolytic-like effect.

3. Discussion

This study examined the effect of SL supplementation on the anxiolytic-like behavior and exploratory activity of rats. The anxiolytic effect was evaluated using two standard animal models of anxiety: the EPM and the VCT, both widely used in research of new anxiolytic agents (Calabrese 2008).

A 6-week SL supplementation significantly prolonged the time spent in open arms of the EPM and increased the number of punished licks in the VCT. These results indicate the anxiolytic effect of SL (Millan 2003). SL had no effect on rats' motor activity in the OF, suggesting that the supplement has no sedative adverse effect.

Our results support some previous observations that phospholipids are an useful source of n-PUFA and have a favorable effect on various types of behavior, including anxiety-related one. Carrie et al. (2000) reported that eggs or brain phospholipids supplementation restored

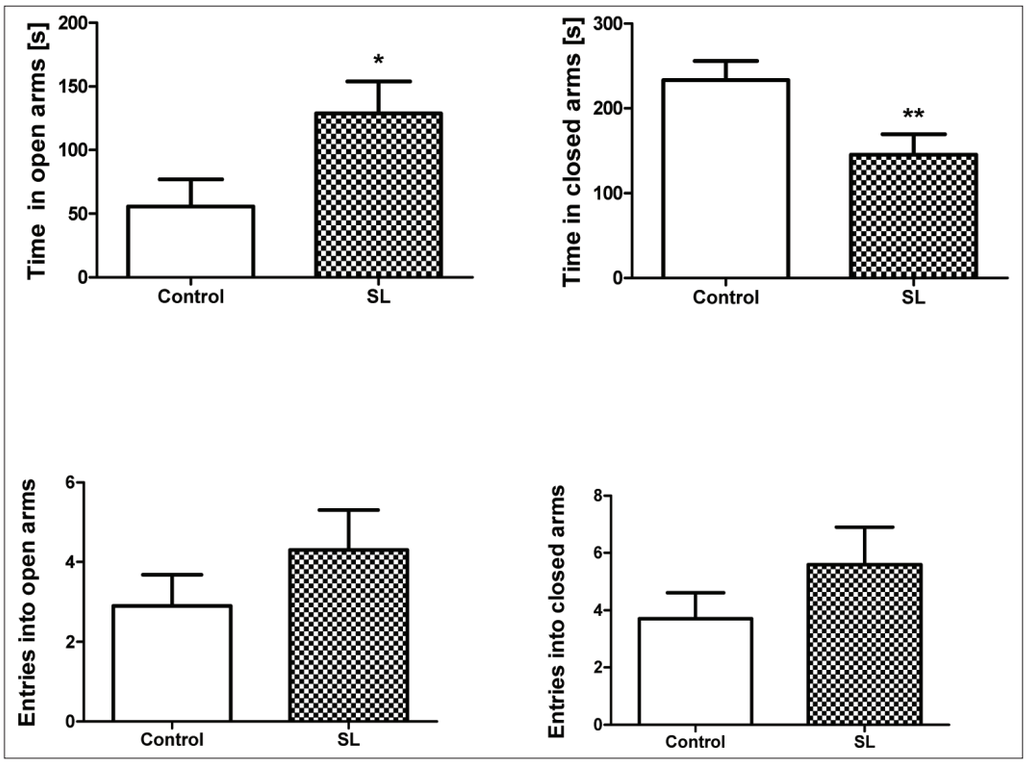

Fig. 2: Anxiolytic activity of "super lecithin" (SL) in the elevated plus maze in rats. Data are expressed as mean ± SD (n = 10). Independent samples t-test: *- $p < 0.05$, ** - $p < 0.02$ compared to the control.

normal fatty acid composition in the brain and significantly reduced the level of anxiety induced by n-3 PUFA deficiency in mice. Similar findings have been made in rats which exhibited increased level of anxiety upon chronic n-3 PUFA dietary deficiency and decreased anxiety upon DHA supplementation (Ferraz et al. 2011). A positive effect of n-3 PUFA on anxiety was also demonstrated in the study on adult mouse lemurs, a non-human primate (Languille et al. 2012; Vinot et al. 2011). However, some animal studies demonstrated that

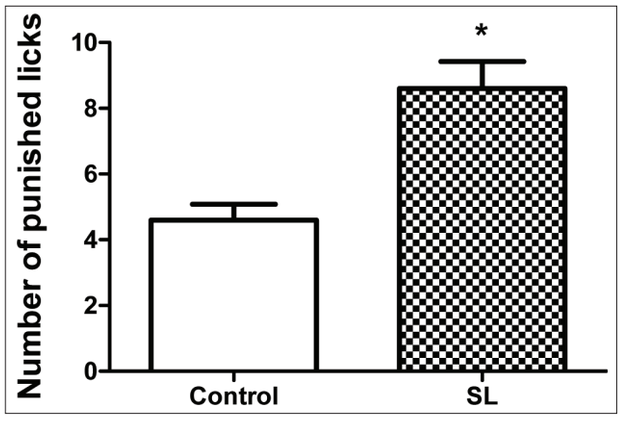

Fig. 3: Anxiolytic activity of "super lecithin" (SL) in the Vogel conflict test. Data are expressed as mean ± SD (n = 10). Independent samples t-test: * - $p < 0.001$ compared to the control

n-3 PUFA-enriched diet had no effect on anxiety-related behavior (Chalon et al. 1998; Farkas et al. 2002).

Similar discrepancies were observed in human studies. N-3 PUFA dietary supplementation has been found to reduce anxiety in medical students (Kiecolt-Glaser et al. 2011) and in substance abusers (Buydens-Branchey et al. 2006) as well as anxiety during pregnancy (Vaz Jdos et al. 2013). On the other hand, however, clinical trials demonstrated that supplementation of n-3 PUFA had no effect on anxiety symptoms in nondepressed, older individuals (van de Rest et al. 2008). Similarly, n-3 PUFA supplementation was ineffective in relieving symptoms of obsessive compulsive disorder (Fux et al. 2004).

Some neurophysiological mechanisms have been proposed for the apparent relationship between PUFA and anxiety. According to one theory n-3 PUFA supplementation causes reduction of activation of the hypothalamic-pituitary-adrenal axis, promoting an anxiolytic effect (Pérez et al. 2013). That hypothesis is supported by studies by Barbarodo et al. (2013), demonstrating that fish oil supplementation caused a reduction of stress symptoms in abstinent alcoholics, and the effect is correlated with a reduced level of cortisol. The anxiolytic effect of n-3 PUFA is also associated with inhibition of pro-inflammatory cytokines synthesis. These cytokines promote secretion of corticotrophin-releasing hormone (CRH), a primary gateway to hormonal stress responses (Raison et al. 2006). Moreover, CRH stimulates the amygdala, a key brain region for fear and anxiety (Hibbeln 2004). In support of this, n-3 PUFA dietary supplementation has been found to reduce anxiety in medical students as well as lower synthesis of pro-inflammatory cytokines (Kiecolt-Glaser et al. 2011).

Long-term n-3 PUFA supplementation increases the level of brain-derived neurotrophic factor (BDNF) (Bousquet et al. 2009; Wu et al. 2004), regulating synaptic plasticity and increasing neuron survival (Poo 2001; Cohen-Cory et al. 2010). It is believed that BDNF may play a role in the pathophysiology of anxiety and in response to anxiolytic therapy (Kobayashi et al. 2005). In rats, decreasing the BDNF signal in the central amygdala and the medial amygdala caused development of anxiety (Pandey et al. 2006, 2008). Some clinical trials reported decreased BDNF levels in patients with anxiety-related disorders (Suliman et al. 2013). Moreover, patients with panic disorder and lower initial serum BDNF levels are inferior responders to the cognitive-behavioral therapy compared to patients with a higher level of the factor (Kobayashi et al. 2005).

The anxiolytic effects may be also the result of interactions between n-3 PUFA and serotonin, norepinephrine or dopamine pathways. Animal studies have demonstrated that chronic omega-3 deficiency or enriched diet feeding may modify metabolism, release and uptake of monoamines as well as may induce changes in 5-HT_2 and D_2 receptors (Delion et al. 1996; Innis et al. 2001; Schipper et al. 2011; Zimmer et al. 1998). N-3 PUFA may also interact with the endocannabinoid system. Larrieu et al. (2012) demonstrated that reduced cerebral DHA level caused reduction of the cannabionoid signal in structures responsible for mood control, and eliminates the anxiolytic effect of cannabinoid agonist.

Based on a previous animal study demonstrating that choline positively modulates emotional function, it may be presumed that the anxiolytic effect of SL also results from choline level increase. McCall et al. (2015) reported that supplementing male rats with choline (from weaning into early adulthood) reduced anxiety-like behaviors in the open field and the predator odor test. These behavioral effects were accompanied by an increase in hippocampal neurogenesis, suggesting a biological basis of choline anxiolytic-like effect.

As a precursor of acetylcholine, choline may intensify the cholinergic transmission (Magil et al. 1981). According to some clinical and experimental studies, the increased transmission causes reduction of the anxiety level. It was demonstrated in animal models that stimulation of the hippocampal cholinergic system induced anxiolytic effects (File et al. 2000b). In contrast, muscarinic receptor blockade through the M1-specific muscarinic antagonist pirenzepine, caused an increase of anxiety-like behavior in rats (File et al. 1998). Similarly, the muscarinic antagonist scopolamine has been reported to have anxiogenic effects in health volunteers (Curran et al. 1991) and in patients with geriatric depression (Newhouse 1988). The nicotinic cholinergic mechanisms also participate in the regulation of anxiety. However, nicotine appears to have both anxiolytic and anxiogenic effects (File et al. 2000a; Ouagazzal et al. 1999).

Concluding, probably due to a synergistic effect of two active elements: n-3 PUFA and choline, SL has an anxiolytic effect. Therefore, it seems that SL may be a potential candidate for nutritional adjuvant treatment of anxiety disorders.

4. Experimental

4.1. Animals

Male Wistar rats, approximately 3 months old, obtained from the Experimental Medicine Centre of Medical University of Bialystok, were used. They were housed under standard laboratory conditions with a 12 h light/dark cycle (lights on at 7 a.m.) at a constant temperature (21± 2 °C) and given free access to food and water throughout the experiment. The experimental protocols were approved by the Ethical Committee on the Animal Research of the Institute of Immunology and Experimental Therapy Polish Academy of Sciences in Wroclaw in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

4.2. Diets

Egg yolk phospholipid fraction ("super lecithin") obtained from the Department of Animal Products Technology and Quality Management, Wroclaw University of Environmental and Life Sciences, from Lohmann Brown hens line was used. The pure phospholipid fraction, defined as the amount of substance insoluble in acetone was 73%. The content of phosphatidylcholine in this fraction was 81.73%, and phosphatidylethanolamine −18.27%. The test substance also contained ovo-lipids, proteins and small amounts of vitamins (especially E and group B vitamins). The fatty acid composition of a supplement was estimated by gas chromatography and shown in the Table. The test diet was prepared by supplementation a conventional laboratory diet (Labofeed H, Kcynia, Poland) with egg yolk phospholipids ("super lecithin",, SL; 5 g/100 g diet). The test diet was prepared every 2–3 days and stored at 4 °C. Rats were fed by chow without (control group, n=10) or with (experimental group, n=10) addition of SL (5%), respectively. After 6 weeks of supplementation animals were subjected to 3 behavioural tests : day 1 - open field test, day 3 elevated plus maze test, and days 4-6 - Vogel conflict tests.

4.3. Open field test

The open field apparatus consisted of a round arena (100 cm diameter), with 40 cm high walls. Each rat was placed individually into the center of apparatus and was left for 5 min. The distance traveled (cm) was recorded and analyzed with a video-tracking software (SMART v 1.2, Panlab, Spain). The luminosity at the center of the open field was 100 lux.

4.4. Elevated plus maze test

The EPM apparatus consisted of two open arms (25 × 12 cm) and two opposite closed arms (25 cm × 12 cm × 30 cm), situated like a plus sign with an open square (12 cm × 12 cm) in the center. The maze was elevated 50 cm from the floor. Rats were placed individually on the central square of the maze facing an open arm at the beginning of the test, and were allowed to explore the maze for 5 min. The number of entries into (with all four paws) and the time spent in both the open and enclosed arms were measured. The number of entries to the open arms and the total time spent in these arms of the maze were taken as an anxiety index. EPM behaviors were monitored and analyzed using the Panlab's video-tracking system described above.

Table : Fatty acid composition in phospholipids from egg yolk*

Fatty acids	
C14:00	0.40
C14:01	0.16
C16:00	26.36
C16:01	2.52
C17:00	0.24
C18:00	14.05
C18:01	29.66
C18:02	13.08
C18:03	3.12
C20:02	0.18
C20:03	0.12
C20:04	2.41
C20:05	0.58
C22:06	7.12
ω-3	10.82
ω-6	15.79
ω6/ω3	1.46
Total saturated	41.05
Total nonsaturated	58.95
Total monounsaturated	32.34
Total polyunsaturated	26.61

*- In g/100 total fatty acid

4.5. Vogel conflict test (VCT)

The VCT was performed in a Plexiglas box (50 cm x 26 cm x 15 cm) with a stainless grid floor. A metallic spout of a drinking bottle containing water was projected into the box. The simultaneous contact of the animal with the spout and the grid floor of the cage closed an electrical circuit controlled by a sensor (Panlab, Spain), producing seven pulses of water per second whenever the animal was in contact with both components. Each pulse was considered as a lick. After every 20 licks through the metallic spout of a drinking bottle the rat received a 0.3 mA shock for 0.5 s. Animals had limited access to water for 3 consecutive days before the test. 1st day: animals did not have access to water in the home cage except for 15 min in the test cage during training session to locate the drinking tube and become accustomed to drinking from it. 2nd day: animals had access to water only during training session (15 min) and for 60 min after it in the home cage. On the third day the test session was carried out (after 72 h water deprivation). The test period lasted for 3 min and the animals received a 0.3 mA, 500 ms shock every 20 licks. The number of licks and shocks delivered were recorded and analyzed with using Panlab's software – PackWin v 2.0.

4.6. Statistical analysis

The analysis of the data was performed using STATISTICA 9.0 (StatSoft, USA). All values presented are expressed as mean ± SD (standard deviation). To evaluate the differences between the control and the test group the independent samples t-test test was used. The differences were considered statistically significant at $p < 0.05$.

Acknowledgements: This study is the part of the project entitled *„Innovative technologies of biopreparations' production on the base of new generation of eggs"* co-financed by the European Union from the European Regional Development Fund under the Operational Program Innovative Economy, 2007-2013.

Conflicts of interest: None declared.

References

Barbadoro P, Annino I, Ponzio E, Romanelli RM, D'Errico MM, Prospero E, Minelli A (2013) Fish oil supplementation reduces cortisol basal levels and perceived stress: A randomized, placebo-controlled trial in abstinent alcoholics. Mol Nutr Food Res 57: 1110–1114.

Barcelo-Coblijn G, Kitajka K, Puskas LG, Hogyes E, Zvara A, Hackler Jr L, Farkas T (2003) Gene expression and molecular composition of phospholipids in rat brain in relation to dietary n-6 to n-3 fatty acid ratio. Biochim Biophys Acta 1632: 72–79.

Bhatia HS, Agrawal R, Sharma S, Huo YX, Ying Z, Gomez-Pinilla F (2011) Omega-3 fatty acid deficiency during brain maturation reduces neuronal and behavioral plasticity in adulthood. PLoS One 6: e28451.

Bjelland I, Tell GS, Vollset SE, Konstantinova S, Ueland PM (2009) Choline in anxiety and depression: the Hordaland Health Study. Am J Clin Nutr 90: 1056–1060.

Bousquet M, Gibrat C, Saint-Pierre M, Julien C, Calon F, Cicchetti F (2009) Modulation of brain-derived neurotrophic factor as a potential neuroprotective mechanism of action of omega-3 fatty acids in a parkinsonian animal model. Prog Neuropsychopharmacol Biol Psychiatry 33: 1401–1408.

Bradbury J (2011) Docosahexaenoic acid (DHA): an ancient nutrient for the modern human brain. Nutrients 3: 529–554.

Buydens-Branchey L, Branchey M, Hibbeln JR (2008) Associations between increases in plasma n-3 polyunsaturated fatty acids following supplementation and decreases in anger and anxiety in substance abusers. Prog Neuropsychopharmacol Biol Psych 32: 568–375.

Buydens-Branchey L, Branchey M (2006) N-3 polyunsaturated fatty acids decrease anxiety feelings in a population of substances abusers. J Clin Psychopharmacol 26: 661–665.

Calabrese EJ (2008) An assessment of anxiolytic drug screening tests: hormetic dose responses predominate. Crit Rev Toxicol 38: 489–542.

Calder PC, Grimble RF (2002) Polyunsaturated fatty acids, inflammation and immunity. Eur J Clin Nutr 56:14–19.

Carrie I, Clement M, de Javel D, Frances H, Bourre JM (2000) Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. J Lipid Res 41: 473–480.

Chalon S, Delion-Vancassel S, Belzung C, Guilloteau D, Leguisquet AM, Besnard JC, Durand G (1998) Dietary fish oil affects monoaminergic neurotransmission and behavior in rats. J Nutr 128: 2512–2519.

Chalon S (2006) Omega-3 fatty acids and monoamine neurotransmission. Prostaglandins Leukot Essent Fatty Acids 75: 259–269.

Cohen-Cory S, Kidane AH, Shirkey NJ, Marshak S (2010) Brain-derived neurotrophic factor and the development of structural neuronal connectivity. Dev Neurobiol 70: 271–288.

Costa LG (2007) Contaminants in fish: risk-benefit considerations. Arh Hig Rada Toksikol 58: 367–374.

Curran HV, Schifano F, Lader M (1991) Models of memory dysfunction? A comparison of the effects of scopolamine and lorazepam on memory, psychomotor performance and mood. Psychopharmacol Berl 103: 83–90.

Delion S, Chalon S, Guilloteau D, Lejeune B, Besnard JC, Durand G (1997) Age-related changes in phospholipid fatty acid composition and monoaminergic neurotransmission in the hippocampus of rats fed a balanced or an n-3 polyunsaturated fatty acid-deficient diet. J Lipid Res 38: 680–689.

Farkas E, de Wilde MC, Kiliaan AJ, Luiten PG (2002) Systemic effects of dietary n-3 PUFA supplementation accompany changes of CNS parameters in cerebral hypoperfusion. Ann N Y Acad Sci 977: 77–86.

Farooqui AA, Horrocks LA, Farooqui T (2000) Glycerophospholipids in brain: their metabolism, incorporation into membranes, functions, and involvement in neurological disorders. Chem Phys Lipids 106: 1–29.

Ferraz AC, Delattre AM, Almendra RG, Sonagli M, Borges C, Araujo P, Andersen ML, Tufik S, Lima MM (2011) Chronic ω-3 fatty acids supplementation promotes beneficial effects on anxiety, cognitive and depressive-like behaviors in rats subjected to a restraint stress protocol. Behav Brain Res 219: 116–122.

File SE, Cheeta S, Kenny PJ (2000a) Neurobiological mechanisms by which nicotine mediates different types of anxiety. Eur J Pharmacol 393: 231–236.

File SE, Gonzalez LE, Andrews N (1998) Endogenous acetylcholine in the dorsal hippocampus reduces anxiety through actions on nicotinic and muscarinic1 receptors. Behav Neurosci 112: 352–359.

File SE, Kenny PJ, Cheeta S (2000b) The role of the dorsal hippocampal serotonergic and cholinergic systems in the modulation of anxiety. Pharmacol Biochem Behav 66: 65–72.

Fux M, Benjamin J, Nemets B (2004) A placebo-controlled cross-over trial of adjunctive EPA in OCD. J Psychiatr Res 38: 323–325.

Green P, Hermesh H, Monselise A, Marom S, Presburger G, Weizman A (2006) Red cell membrane omega-3 fatty acids are decreased in nondepressed patients with social anxiety disorder. Eur Neuropsychopharmacol 16: 107–113.

Hibbeln JR, Bissette G, Umhau JC, George DT (2004) Omega-3 status and cerebrospinal fluid corticotrophin releasing hormone in perpetrators of domestic violence. Biol Psychiatry 56: 895–897.

Hossain MA (2011) Fish as source of n-3 polyunsaturated fatty acids (PUFAs), which one is better-farmed or wild? Adv J Food Sci Technol 3: 455–466.

Innis SM, De La Presa Owens S (2001) Dietary fatty acid composition in pregnancy alters neurite membrane fatty acids and dopamine in newborn rat brain. J Nutr 131: 118–122.

Kiecolt-Glaser JK, Belury MA, Andridge R, Malarkey WB, Glaser R (2011) Omega-3 supplementation lowers inflammation and anxiety in medical students: a randomized controlled trial. Brain Behav Immun 25: 1725–1734.

Kobayashi K, Shimizu E, Hashimoto K. Mitsumori M, Koike K, Okamura N (2005) Serum brain-derived neurotrophic factor (BDNF) levels in patients with panic disorder: as a biological predictor of response to group cognitive behavioral therapy. Prog Neuropsychopharmacol Biol Psych 29 : 658–663.

Languille S, Aujard F, Pifferi F (2012) Effect of dietary fish oil supplementation on the exploratory activity, emotional status and spatial memory of the aged mouse lemur, a non-human primate. Behav Brain Res 235: 280–286.

Larrieu T, Madore C, Joffre C, Layé S (2012) Nutritional n-3 polyunsaturated fatty acids deficiency alters cannabinoid receptor signaling pathway in the brain and associated anxiety-like behavior in mice. Physiol Biochem 68: 671–681.

Lewis NM, Seburg S, Flanagan NL (2000) Enriched eggs as a source of n-3 polyunsaturated fatty acids for humans. Poult Sci 79: 971–974.

Magil SG, Zeisel SH, Wurtman RJ (1981) Effects of ingesting soy or egg lecithins on serum choline, brain choline and brain acetylcholine. Nutr 111: 166–170.

McCall N, Mahadevia D, Corriveau JA, Glenn MJ (2015) Adult emotionality and neural plasticity as a function of adolescent nutrient supplementation in male rats. Pharmacol Biochem Behav 132:125-135.

Messamore E, McNamara RK (2016) Detection and treatment of omega-3 fatty acid deficiency in psychiatric practice: Rationale and implementation. Lipids Health Dis 15:25 -38.

Millan MJ (2003) The neurobiology and control of anxious states. Prog Neurobiol 70: 83–244.

Newhouse PA, Sunderland T, Tariot PN, Weingartner H, Thompson K, Mellow AM, Cohen RM, Murphy DL (1988) The effects of acute scopolamine in geriatric depression. Arch Gen Psychiatry 45: 906–912.

Ouagazzal AM, Kenny PJ, File SE (1999) Stimulation of nicotinic receptors in the lateral septal nucleus increases anxiety. Eur J Neurosci 11: 3957–3962.

Pandey SC, Zhang H, Roy A, Misra K (2006) Central and medial amygdaloid brain-derived neurotrophic factor signaling play a critical role in alcohol-drinking and anxiety-like behaviors. J Neurosci 26: 8320–8331.

Pandey SC, Zhang H, Ugale R, Prakash A, Xu T, Misra K (2008) Effector immediate-early gene arc in the amygdala plays a critical role in alcoholism. J Neurosci 28: 2589–2600.

Pérez MA, Terreros G, Dagnino-Subiabre A (2013) Long-term omega-3 fatty acid supplementation induces anti-stress effects and improves learning in rats. Behav Brain Funct 9: 25–36.

Poo MM (2001) Neurotrophins as synaptic modulators. Nat Rev Neurosci 2: 24-32.

Raison CL, Capuron L, Miller AH (2006) Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol 27: 24–31.

Rapoport SI, Rao JS, Igarashi M (2007) Brain metabolism of nutritionally essential polyunsaturated fatty acids depends on both the diet and the liver. Prostaglandins Leukot Essent Fatty Acids 77: 251–261.

Ross BM (2009) Omega-3 polyunsaturated fatty acids and anxiety disorders. Prostaglandins Leukot Essent Fatty Acids 81: 309–312.

Santos Vaz J, Kac G, Emmett P, Davis JM, Golding J, Joseph R. Hibbeln JR (2013) Dietary patterns, n-3 fatty acids intake from seafood and high levels of anxiety symptoms during pregnancy: findings from the avon longitudinal study of parents and children. PLoS One 8: e67671.

Schipper P, Kiliaan AJ, Homberg JR (2011) A mixed polyunsaturated fatty acid diet normalizes hippocampal neurogenesis and reduces anxiety in serotonin transporter knockout rats. Behav Pharmacol 22: 324–334.

Suliman S, Hemmings SM, Seedat S (2013) Brain-Derived Neurotrophic Factor (BDNF) protein levels in anxiety disorders: systematic review and meta-regression analysis. Front Integr Neurosci 7: 55–65.

van de Rest O, Geleijnse JM, Kok FJ, van Staveren WA, Hoefnagels WH, Beekman AT, de Groot LC (2008) Effect of fish-oil supplementation on mental well-being in older subjects: a randomized, double-blind, placebo-controlled trial. Am J Clin Nutr 88: 706–713.

Vancassel S, Durand G, Barthelemy C, Lejeune B, Martineau J, Guilloteau D, Andrès C, Chalon S (2001). Plasma fatty acid levels in autistic children. Prostaglandins Leukot Essent Fatty Acids 65: 1–7.

Vaz Jdos S, Kac G, Emmett P, Davis JM, Golding J, Hibbeln JR (2013) Dietary patterns, n-3 fatty acids intake from seafood and high levels of anxiety symptoms during pregnancy: findings from the Avon Longitudinal Study of Parents and Children. PLoS One 8: e67671.

Vinot N, Jouin M, Lhomme-Duchadeuil A, Guesnet P, Alessandri JM, Aujard F, Pifferi F (2011) Omega-3 fatty acids from fish oil lower anxiety, improve cognitive functions and reduce spontaneous locomotor activity in a non-human primate. PLoS One 6: e20491.

Wu A, Ying Z, Gomez-Pinilla F (2004) Dietary omega-3 fatty acids normalize BDNF levels, reduce oxidative damage, and counteract earning disability after traumatic brain injury in rats. J Neurotrauma 21: 1457–1467.

Yehuda S, Rabinovitz S, Carasso RL, Mostofsky DI (2002) The role of polyunsaturated fatty acids in restoring the aging neuronal membrane. Neurobiol Aging 23: 843–853.

Zeisel SH, Blusztajn JK (1994) Choline and human nutrition. Annu Rev Nutr 14: 269–96.

Zeisel SH, Mar MH, Howe JC, Holden JM (2003) Concentrations of choline-containing compounds and betaine in common foods. J Nutr 133: 1302–1307.

Zimmer L, Hembert S, Durand G, Breton P, Guilloteau D, Besnard J C, Chalon S (1998) Chronic n-3 polyunsaturated fatty acid diet-deficiency acts on dopamine metabolism in the rat frontal cortex: a microdialysis study. Neurosci Lett 240: 177–1781.