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An examination of the impact of dietary lipids on behaviour and neurochemistry

Thesis presented by

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under the supervision of

Prof. Timothy G. Dinan
Prof. John F. Cryan

for the degree of

Doctor of Philosophy
July, 2015



Department of Psychiatry

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Prof. Timothy G. Dinan

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Declaration

This thesis submitted is my own work and has not been submitted for any other degree, either at University College Cork or elsewhere.

Author Contributions

All of the work conducted in this thesis was performed independently by the author with the following exceptions.

Chapter 2

Nurbazilah Ariffin performed the high performance liquid chromatography (HPLC) analysis on prefrontal cortex and brain stem along with the author.

Chapter 2

Dr. Gerard Moloney performed the meso scale discovery (MSD) analysis on plasma samples to generate the TNF-α data along with the author.

Chapter 2

Dr. Rachel Moloney & Dr. Richard O'Connor carried out the maternal separation along with the author.

Chapter 3

Dr. Holly Green performed the methyl thiazolyl tetrazolium (MTT) analysis and the immunocytochemistry on cortical cell cultures along with the author.

Chapter 4

Dr. Sahar El Aidy carried out the statistical analysis on faecal samples to generate the microbiota data along with the author.

Chapter 5

Dr. Cristina Torres-Fuentes performed the cell identification analysis on cortical astrocytes cultures along with the author.

Signed

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Matteo Pusceddu

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Publications and presentations

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2. O'Connor, R. M., Pusceddu, M. M., Dinan, T. G., Cryan, J. F. (2013). Impact of early-life stress, on group III mGlu receptor levels in the rat hippocampus: effects of ketamine, electroconvulsive shock therapy and fluoxetine treatment. *Neuropharmacology 66, 236-41.*

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1. Pusceddu, M. M., Kelly, P., Ariffin, N., Cryan J. F., Clarke G., Dinan, T. G. (2015). Early life stress blunts the effects of polyunsaturated fatty acids following exposure to maternal separation. *Psychoneuroendocrinology.*

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2. Pusceddu, M.M., El Aidy S, Kelly P, Cryan JF, Dinan TG. n-3 Polyunsaturated Fatty Acids (PUFAs) Reverse the Impact of Early-Life Stress on the Gut Microbiota. *Plos One, under revision.*
3. Pusceddu, M. M., Kelly, P., Cryan J. F., Dinan, T. G. n-3 polyunsaturated fatty acids through the lifespan: Implication for psychopathology. *Manuscript in preparation.*

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Corticosterone-induced Changes in Cortical Neurons. *Society for Neuroscience (SfN), Washington D.C., November 2014*

3. Pusceddu, M. M., Nolan, Y. M., Green, H. F., Kelly, P., Dinan, T. G., Cryan J. F. (2014). The Omega-3 Polyunsaturated Fatty Acid Docosahexaenoic acid (DHA) as a Novel Strategy for Stress-related Psychiatric Disorders: Reversal of Corticosterone-induced Changes in Cortical Neurons. *Neuroscience Ireland Symposia, Dublin, Ireland, September 2014.*
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6. Pusceddu, M. M., Kelly, P., Cryan J. F., Dinan, T. G. (2013). Impact of chronic administration of eicosapentaenoic fatty acid and early-life stress in anxiety-related behaviour. Young Life Scientists Ireland (YLSI), *Cork, Ireland, September 2013.*

Abstract

The molecular and cellular basis of stress pathology remains an important research question in biological science. A better understanding of this may enable the development of novel approaches for the treatment of stress-related mental illness. Currently available therapies for many psychiatric disorders are of limited efficacy; many patients do not experience full remission of symptoms. Furthermore, many patients report troublesome side effects, which reduce the likelihood of compliance. There is a considerable body of scientific evidence suggesting that dietary lipids, namely phospholipids and omega-3 polyunsaturated fatty acids (n-3 PUFAs), have therapeutic potential for certain psychiatric disorders.

To this end, we proposed n-3 PUFAs as a novel strategy for the prevention or amelioration of stress-related disorders. We hypothesised that these compounds would improve behavioural and neurobiological responses and alter gut microbial composition. Furthermore, we proposed a new mechanism of action exerted by n-3 PUFAs using an in vitro model of stress. Lastly, we explored the protective effects of both phospholipids and n-3 PUFAs against neuroinflammation, which has been shown to contribute to the development of stress-related disorders.

We provide further evidence that glucocorticoids, inflammation and early-life stress induce vulnerability to psychopathologies. Specifically, we have demonstrated that corticosterone (CORT) alters cortical neuron and astrocyte percentage composition, reduces brain-derived-neuronal factor (BDNF) expression, and induces glucocorticoid receptor (GR) down-regulation in mixed cortical cultures.

Interestingly, we found that lipopolysaccharide (LPS) treatment resulted in an over-expression of pro-inflammatory cytokines in cortical astrocyte cultures. Moreover, we demonstrate that early-life stress induces changes to the monoaminergic and immune systems as well as altered neuroendocrine response to stressors later in life. In addition, we found that early-life stress alters the gut microbiota in adulthood. These data demonstrate that n-3 PUFAs can attenuate CORT-induced cellular changes, but not those caused by LPS, within the cerebral cortex. Similarly, phospholipids were unable to reverse LPS-induced inflammation in cultured astrocytes. In addition, this thesis proposes that n-3 PUFAs may prevent the development or lessen the symptoms of mental illnesses, ameliorating anxiety- and depressive-like symptoms as well as cognitive effects, particularly when administered during neurodevelopment. Such effects may be mediated by GR activation as well as by modification of the gut microbiota composition.

Taken together, our findings suggest that n-3 PUFAs have therapeutic potential for stress-related disorders and we provide evidence for the mechanisms by which they may exert these effects. These findings contribute to an exciting and growing body of research suggesting that nutritional interventions may have an important role to play in the treatment of stress-related psychiatric conditions.

Chapter 1

General Introduction

1.1. The concept of Stress

Since the dawn of the last century the scientific community has sought to answer the following questions: What is stress? How do we deal with it? "Fight or flight response" and "homeostasis" constitute the most exemplified definitions of stress coined by Walter B. Cannon (October 19, 1871 – October 1, 1945) (Cannon, 1963). Hans Selye, who was influenced by the work of Cannon, introduced the concept of stress into popular as well as medical discussion (Selye, 1998, McEwen, 2005) formulating his classic theory of "General adaptation syndrome" which describes the body's short-term and long-term reactions to stress, divided into three different stages: the alarm reaction; the stage of resistance; and the stage of exhaustion. Although this theory is considered as a stereotyped response of our body to stress, it has been of great help in understanding what stress is and how we cope with it (McEwen, 2005). Indeed, diversity in the perception of stressors in the population and numerous mediators of the stress response should also be taken into consideration. For instance, Taylor et al have underlined a response to stress more prevalent in females designed to protect the self and offspring, promoting safety and reducing distress, termed "tend-and-befriend" (Taylor et al., 2000b). In addition to the recognition of differences between male and female responses during the occurrence of stress, the stage of exhaustion of defensive mechanisms when confronted with a prolonged stressful event needs to be reinterpreted. Indeed, rather than an exhaustion of mediators of stress, it is the allostatic overload that can turn on the body and cause problems. Primary mediators involved in maintaining the internal equilibrium of a system, called allostasis, include, but are not confined to, the hypothalamic-pituitary-adrenal (HPA) axis, sympathoadrenal medullary system and cytokines. They act to maintain equilibrium in essential systems for life, such as pH,

body temperature, glucose levels and oxygen tension; this equilibrium is referred to as homeostasis (McEwen, 2005).

1.1.1. Stress and HPA axis

The HPA axis, together with catecholamines and cytokines are considered to be primary mediators of allostasis (McEwen, 2005). Acting in concert, they coordinate emotional, cognitive, neuroendocrine, immune and autonomic inputs which together determine the magnitude and specificity of an individual's behavioural, neural and hormonal responses to stress. The HPA axis represents a key system in the stress response, whose activation is triggered by corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the hypothalamus. Moreover, parvocellular neurons of the PVN synthesize and release vasopressin (AVP), a nanopeptide highly expressed in the hypothalamus (Smith and Vale, 2006). AVP potentiates CRH-induced adrenocorticotropic hormone (ACTH) release from the pituitary gland, which in turn releases glucocorticoid hormones (GCs) (corticosterone in rodents and cortisol in humans) from the adrenal cortex (Figure 1.1). In addition, the adrenal medulla releases catecholamines (adrenaline and noradrenaline) which contribute to the physiological response to stress. Regulation of the HPA axis occurs by glucocorticoids' negative feedback inhibition of ACTH and CRH release by binding to high-affinity mineralocorticoid receptors (MR) and lower affinity glucocorticoid receptors (GR) in hypothalamus (de Kloet et al., 2005). Other brain regions enriched in MR and GR, such as the hippocampus and the pre-frontal cortex, are involved in this mechanism of action in order to shut the HPA axis down and return to a set homeostatic condition. By contrast, the amygdala is involved in the process of initiation of the HPA axis in order to activate the stress response necessary to deal

with the challenge. Together MR and GR determine sensitivity of the brain to stress (Sousa et al., 2008, Harris et al., 2013) and thereby modulate attention, vigilance, behaviour and memory formation and eventually adaptation and coping with stress. Activation of the HPA axis induced by chronic stress can imbalance or down-regulate both MR and GR expression (de Kloet et al., 2005, Qi et al., 2013) which can alter HPA axis feedback and result in overexposure to glucocorticoids. Accordingly, consequent overexposure of the brain and peripheral tissues to glucocorticoids has been shown to influence hippocampal viability and compromise cognition in rats (Landfield et al., 1981). Similarly, Sapolsky et al reported that chronic stress causes a loss of pyramidal neurons in the hippocampus, accompanied by cognitive deficits in rats (Sapolsky, 1985, Sapolsky et al., 1986). Hyperactivity of the HPA axis is one of the most consistent biological findings in depressed patients (Pariante and Lightman, 2008). Depressives often have elevated plasma cortisol (Halbreich et al., 1985) and ACTH (Carroll et al., 2007) levels, and frequently fail to suppress cortisol in response to dexamethasone challenge (Carroll, 1982a, b). Therefore, there is increasing attention on the effect of chronic stress as one of the main causes for the development of mental illnesses such as depression and anxiety.

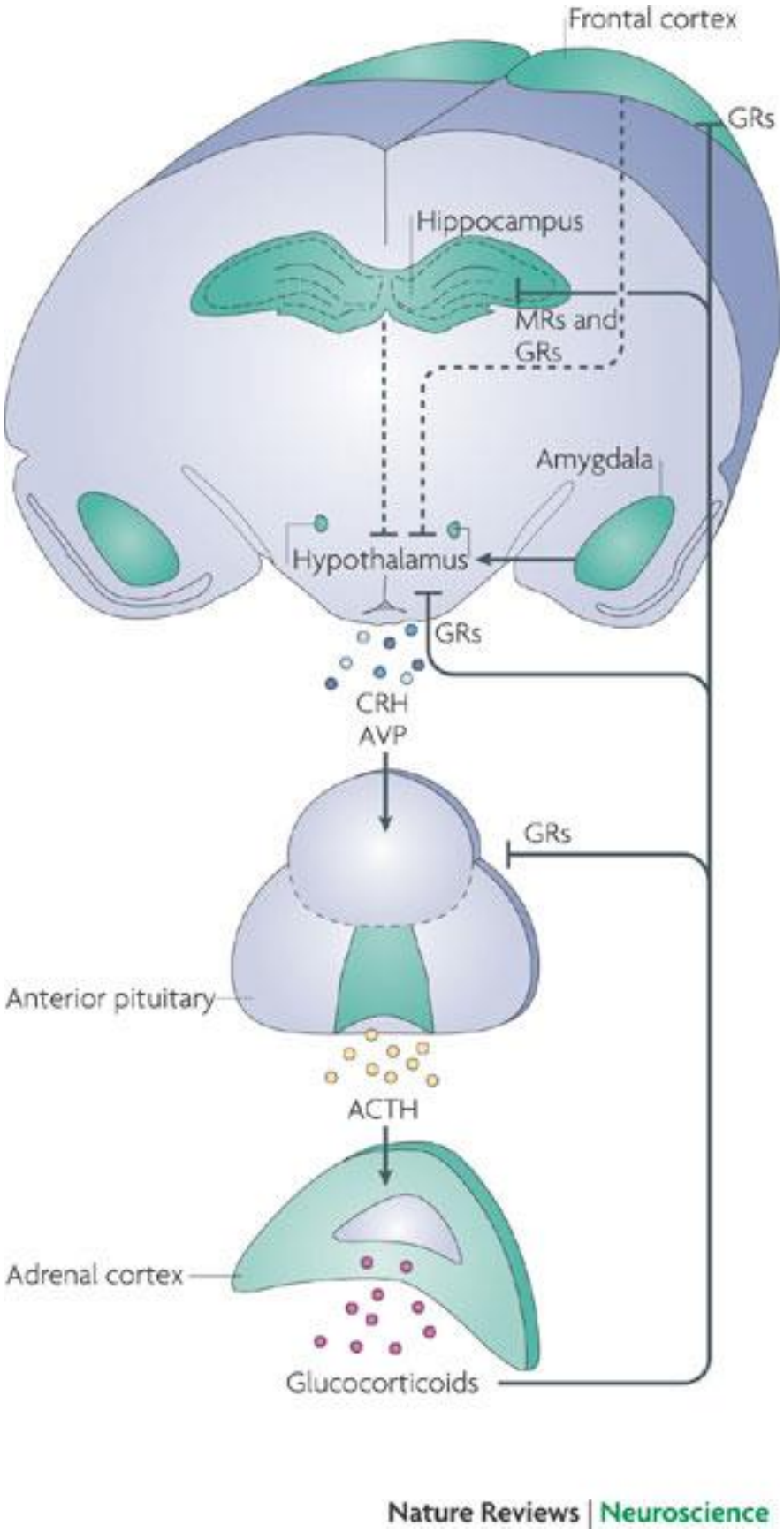


Figure 1.1: Schematic representing the regulation of the HPA axis. (Adapted from (Lupien et al., 2009)).

1.2. Depression and anxiety

Depression and anxiety are among the leading causes of total disability and economic burden worldwide afflicting about 17% of the total population of developed countries (Costello et al., 2002). Depression and anxiety significantly affect the world economy with average cost of approximately $80 billion and €106 billion per year to United States and Europe, respectively (Andlin-Sobocki and Wittchen, 2005). The World Health Organization (WHO) predicts that depression will be, after cardiovascular, the second most prevalent disease in the world by 2020 and the first by 2030 (Murray and Lopez, 1997). Using the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) it is estimated that the life-time prevalence of depression ranges from 4.6% to 20.8% (Bijl et al., 1998, Ormel et al., 1999, Andrade et al., 2003, Kessler et al., 2005) and 10.5% to 28.8% for anxiety disorders (Bijl et al., 1998, Ormel et al., 1999, Kessler et al., 2005).

Depression is a devastating illness which severely reduces an individual's quality of life with core symptoms including feelings of sadness, anxiety, emptiness, hopelessness, worthlessness, guilt, irritability, or restlessness (Barlow, 2005). Additionally, depression has been associated with physical illness, especially stroke and cardiovascular disease, as well as somatic symptoms (Goodwin, 2006). The main criteria for the diagnosis of depression, determined by the DSM-V, are reported in table 1.1. Depression is characterized by a state of low mood and aversion to activity that can affect a person's thoughts, behaviour, feelings and physical well-being. The illness is usually recurrent and about 70% of patients who have one major depressive episode will have at least one more episode, especially when the patient suspends therapeutic treatment (Fava et al., 2006, Limosin et al., 2007). Furthermore,

the depressive episode experienced during a relapse is usually far more severe than the original (Fava et al., 2006, Limosin et al., 2007). The average age of onset is about 28 years, but the first episode can occur at almost any age (Jessell et al., 2012). Although often unrecognized, depression also affects young children (Jessell et al., 2012). Depression also occurs in the elderly, and older people who become depressed often have not had an earlier episode (Jessell et al., 2012). Moreover, depression represents a risk factor for health problems including heart disease (Schroeder, 2011), metabolic syndrome (Luppino et al., 2011) and altered sleep quality (Gregory et al., 2011). Given the severity of the symptoms associated with depression and its widespread prevalence, there is a major need to unravel the pathophysiology of the disorder, and to achieve optimal treatment strategies.

Table 1.1 DSM-V Criteria for Diagnosis of Major Depression.

1	**Depressed mood most of the day, nearly every day.**
2	**Markedly diminished interest or pleasure in all, or almost all, activities.**
3	**Significant weight loss, or decrease or increase in appetite.**
4	**Insomnia or hypersomnia nearly every day.**
5	**Psychomotor agitation or retardation nearly every day.**
6	**Fatigue or loss of energy nearly every day.**
7	**Feelings of worthlessness or excessive or inappropriate guilt.**
8	**Diminished ability to think or concentrate, or indecisiveness.**
9	**Recurrent thoughts of death, suicidal ideation, or a suicide attempt.**
Note	**Five (or more) of the following symptoms present during the same 2 week period; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.**

According to the DSM-V, anxiety disorder is characterized by strong feeling of apprehension, uneasiness, fear or worry (Table 1.2). It develops when an anxiety or fear response is inappropriate or exaggerated in a given situation. Anxiety disorders are divided into subtypes of which the most common are generalized anxiety disorder (GAD), social phobia, panic disorder, obsessive-compulsive disorder and post-traumatic stress disorder (PTSD). Anxiety disorders can arise in response to life stresses such as financial worries or chronic physical illness. Indeed, chronic stress can create pathological changes in the brain which impair behavioural and physiological responses (Caldji et al., 2000, Cohen et al., 2007, Seckl, 2008). Moreover, these changes can predispose genetically susceptible individuals to psychiatric diseases such as depression and anxiety disorders (Caspi et al., 2002, Charney and Manji, 2004, Nemeroff, 2004, Jacobson and Cryan, 2007).

Today, treatments for depression are also the first choice to treat anxiety disorders (see *Treatments of depression and anxiety*). Unfortunately, they present unwanted side-effects including sedation, dependence, sexual dysfunction, headaches, nausea and insomnia.

Table 1.2 DSM-V. Anxiety disorders.

Term	Definition
Anxiety disorder	The primary symptom of anxiety disorders is excessive anxiety and/or fear. The symptoms vary considerable from one disorder to another. In general, the symptoms may be considered a disorder if they are persistent, excessive and seriously interfere with everyday life.
Generalized Anxiety disorder	The main symptoms of generalized anxiety disorders are unrealistic and excessive worry, that is difficult to control, causes significant distress and/or functional impairment, persists for 6 months, and includes at least 3 of the following additional symptoms: restlessness, feeling keyed up or on edge, fatigue, concentration problems, mind going blank, irritability, muscle tension or sleep disturbance.
Phobias	Unrealistic and excessive fears of specific objects or situations are called phobias. Phobias cause anxiety symptoms and may result in avoidance behaviours.
Specific phobia	These phobias can fall in four main categories: fear of animals or insects, fear of things in the natural environment (e.g. storms, height, and water), fear of events related to blood or injury and fear of particular situations (e.g. bridge, elevators, driving).
Social phobia	Patients that suffer of social phobia are anxious in certain kinds of social or performance situations, such as giving a speech or attending at a social gathering.
Obsessive compulsive disorder (OCD)	OCD causes people to experience unwanted, intrusive thoughts (obsessions, such as excessive fear of contamination or repeated doubts) that can prompt them to carry out repeated actions (compulsions, such as hand washing or repeated checking) to reduce the anxiety produced by those thoughts.
Post-traumatic stress disorder (PTSD)	PTSD develops in some people after extremely traumatic events, such as combat, crime, an accident or natural disaster. People with PTSD may relive the event via intrusive memories, flashbacks and nightmares; avoid anything that reminds them of the trauma; and have anxious feelings.

1.2.1. Treatments of depression and anxiety

One of the first strategies employed to pharmacologically induce an antidepressant effect was the blocking of the monoamine's oxidation by monoamine oxidase inhibitors (MAOIs), thus increasing the levels of serotonin, norepinephrine (or noradrenaline), and dopamine (Nair et al., 1993). Generally, MAOIs suffered from high levels of toxicity resulting in nausea, headaches and insomnia. As a result, these compounds are rarely used in the clinic today (Jessell et al., 2012). This high level of toxicity is mostly due to the ingestion of MAOIs in conjunction with tyramine-rich foods. In such circumstance, excess of tyramine would displace noradrenaline from the storage vesicles which would subsequently cause hypertensive crisis (Sathyanarayana Rao and Yeragani, 2009). The most important breakthrough in the history of neuropsychopharmacology was the advent of the Tricyclic Antidepressants (TCAs) in the early 1950s. TCAs act by blocking the serotonin transporter (SERT), the norepinephrine transporter (NET) and the dopamine transporter (DAT), increasing the levels of these neurotransmitters in the synaptic space, enhancing neurotransmission (Figure 1.2) (Tatsumi et al., 1997, Gillman, 2007). However, TCAs show a lot of collateral effects such as agitation, confusion, memory impairment and pacing due to non-specific effects on different receptors including the cholinergic (M1), histaminergic (H1), and adrenergic (α1) receptors (Tatsumi et al., 1997, Gillman, 2007). Not long after, in the early 1960s, benzodiazepines (BZDs), such as diazepam (Valium) were introduced into the market of psychoactive drugs by Hoffman-La Roche. BZDs enhance the effect of the neurotransmitter gamma-aminobutyric acid (GABA) at the $GABA_A$ receptor, resulting in sedative, hypnotic (sleep-inducing), anxiolytic (anti-anxiety), anticonvulsant, and muscle relaxant properties. BZDs are highly effective for the

treatment of anxiety, although chronic use of these compounds can induce several side-effects such as tolerance, dependence and withdrawal, as well as cognitive impairments (Ashton, 2005). Despite the decades of research which have been conducted on the topic, the majority of today's antidepressant and anxiolytic drugs are still based on this same mechanism, which aims to increase the levels of neurotransmitters in the brain. Selective serotonin reuptake inhibitor (SSRIs) (Barlow, 2005) represent the most widely known and prescribed antidepressants worldwide (Preskorn et al., 2004). These drugs selectively inhibit SERT (serotonin transporter) reducing serotonin reuptake and show the same potency of TCAs but less collateral effects, thanks to their better selectivity of action (Anderson, 2000).

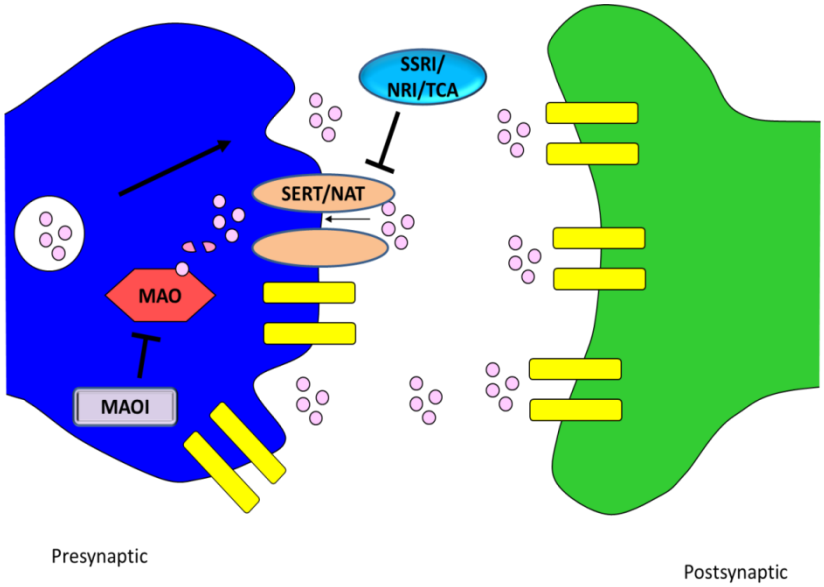


Figure 1.2: Schematic example of the molecular action employed by the majority of antidepressants.

Despite their selectivity and their reduced collateral effects, SSRIs are not perfect compounds. Almost all SSRIs commonly cause one or more secondary symptoms, such as anhedonia, apathy, insomnia, weight loss, tremors, renal impairment, sexual dysfunction and diminished libido (Chouinard and Steiner, 1986, Lavin et al., 1993,

Hines et al., 2004). Many side effects disappear after the adaptation phase. Others, like apathy and tachyphylaxis, appear after years of administration (Byrne and Rothschild, 1998, Solomon et al., 2005, Rothschild, 2008, Amsterdam et al., 2009). Since the introduction of the SSRIs, newer medications have been developed such as NARIs (Noradrenaline reuptake inhibitors), SNRIs (Serotonin and Noradrenaline reuptake inhibitors) and NASSAs (noradrenergic and specific serotoninergic antidepressant). However, like the SSRIs, they work via the same basic mechanism of altering monoamine neurotransmission, although one exception is the recently approved drug agomelatine which represents the first melatoninergic antidepressant (de Bodinat et al., 2010). Furthermore, the intrinsic efficacy and range of patients for whom treatment is successful with these drugs remain almost the same (Machado and Einarson, 2010). It is important to understand that despite the immediate biochemical effect of antidepressant drugs (altering monoamine levels in the synapse) occurring very rapidly, the therapeutic benefit of these drugs does not occur for weeks or months. This indicates that the therapeutic mechanisms are downstream effects such as activation of intraneuronal transductional mechanisms that induce a return to cellular tropism, in particular the synthesis of transcriptional factors such as CREB (Chen et al., 2001a) and tropic factors such as BDNF (Chen et al., 2001b) that are involved in neurogenesis, spinogenesis and synaptogenesis (Malberg et al., 2000, Santarelli et al., 2003). In addition, recent research has found that chronic antidepressant treatment with drugs such as imipramine and fluoxetine induces epigenetic changes (Mill and Petronis, 2007) involved in antidepressant-like effects that can be at the base of the principal therapeutic mechanism of action of the antidepressant drugs (Santarelli et al., 2003, Sales et al., 2011). A list of currently available antidepressant treatments has been reported in Table 1.3.

Table 1.3 List of currently available antidepressant treatments.

Class	Antidepressants
Selective Serotonin Reuptake Inhibitors (SSRIs)	Citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, setraline.
Serotonine Norepinephrine Reuptake Inhibitors (SNRIs)	Desvenlafaxine, duloxetine, milnacipram, venlafaxine.
Norepinephrine Dopamine Reuptake Inhibitors (NDRIs)	Bupropion.
Tricyclic Antidepressants (TCAs)	Amitriptyline, clomipramine, desipramine, imipramine, nortriptyline.
Tetracyclic Antidepressants (TetCAs)	Mianserin, mirtazapine.
Monoamine Oxidase Inhibitors (MAOIs)	Phenelzine, selegiline.

1.2.2. Animal models of depression

Understanding the biological and chemical processes of the neurobiology of depression in clinical investigations has its ethical and practical limitations (Cryan and Holmes, 2005, O'Leary and Cryan, 2013). Today, the large cost of clinical trials represents an important barrier for the development of new drugs aimed at the CNS (Frantz, 2004). Thus, the need to develop well-validated animal models in order to prioritise specific molecular targets for follow up clinical investigation has become a clear priority in this field (Cryan and Holmes, 2005). However, some symptomatology of depression, such as suicidal thoughts, low self-esteem or feelings of worthlessness cannot be modelled in animals. Hence, the complexity of its pathogenesis has impeded the development of appropriate *in vivo* model systems in which to explore novel treatment strategies and to elucidate the neurobiological mechanisms underlying depression (Cryan and Holmes, 2005, O'Leary and Cryan, 2013). Moreover, in light of the substantial differences in brain anatomy between humans and rodents, animal models have drawn scepticism from both clinicians and research scientists. Indeed, many compounds which have displayed antidepressant-like activity in rodent models of depression have not proven to be clinically useful, although some others have become widely prescribed antidepressants (O'Leary and Cryan, 2013). Thus, despite their limitations, translational animal models are vital to develop a greater understanding of the disorder. Ideally animal models offer an opportunity to understand molecular, genetic and epigenetic factors that may lead to depression. By using animal models, the underlying molecular changes and the causal relationship between genetic or environmental alterations and depression can be examined, which would afford a better insight into pathology of depression. Today, we have many heuristic behavioural paradigms for the study of

depression, and several have been undeniably useful in deepening our understanding of the neurobiology of depression (Table 1.4).

Currently, the criteria for identifying animal models of depression rely on four main principles proposed by McKinney and Bunney (1969): the symptomatology or manifestations are 'reasonably analogous' to human depression; behavioural changes are amenable to objective monitoring; behavioural changes are reversible by treatment with clinically effective antidepressant drugs; the model is reproducible between investigators (Cryan and Holmes, 2005).

One of the most widely used rodent models of depression and antidepressant-like activity is the forced swim test (FST) (Cryan and Holmes, 2005, O'Leary and Cryan, 2013). Originally developed by Porsolt and colleagues (Porsolt et al., 1977), this test is based on the principle that rodents placed in an inescapable cylinder containing tepid water initially engage in vigorous, but futile, escape-oriented movements eventually giving way to larger spells of passive immobility (Figure 1.3a). Pre-administration of antidepressant drugs reduce the time spent immobile, so-called "behavioural despair" (Cryan and Holmes, 2005). Moreover, other factors related to the pathology of depression, such as exposure to stress or genetic predisposition can alter the immobility behaviour in the FST (Cryan and Mombereau, 2004, Cryan and Holmes, 2005). Based on the same principle as the FST, the tail suspension test (TST) is another well-known behavioural test where a mouse hung upside down by its tail will also display initial escape oriented movements before developing increasing period of immobility (Figure 1.3b). Despite the importance of both FST and TST in the discovering of new antidepressant drugs, there are some controversies among researchers as to their validity. For instance, clinical

administration of antidepressants reveal their therapeutic effect after a chronic course of treatment, whereas FST and TST are sensitive to single dose of antidepressant drugs. However, some studies have recently revealed sensitivity of these tests to chronic antidepressant administration (Dulawa et al., 2004, Cryan et al., 2005). Moreover, the interpretation of the immobility behaviour constitute another point of contention. In fact, immobility is either interpreted as reflective of behavioural despair or as an adaptive mechanism to conserve energy (West, 1990).

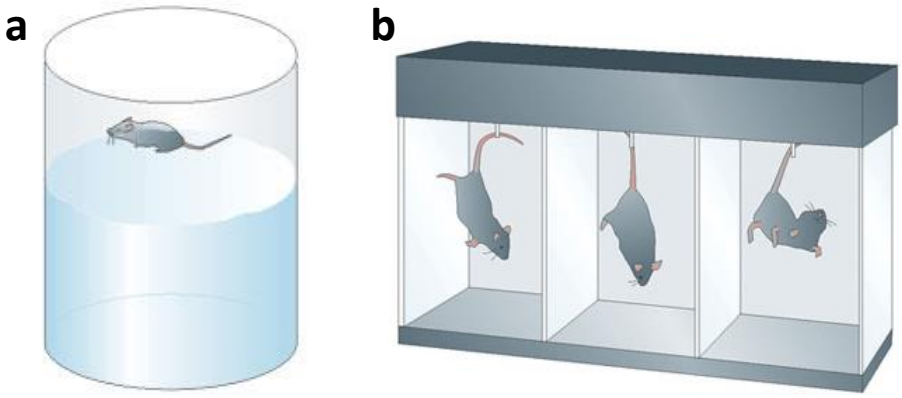


Figure 1.3: Schematic illustrating (a) the forced swim test (FST) and, (b) the tail suspension test (TST). (Adapted from (Cryan and Holmes, 2005)).

Other animal models of depression include the learned helplessness (LH) and chronic mild stress (CMS) models, both of which are based on the concept of maladaptive coping responses to uncontrollable stresses (Cryan and Holmes, 2005, O'Leary and Cryan, 2013), and the olfactory bulbectomy (OB) model (Song and Leonard, 2005). Chronic treatment with antidepressant drugs reverses some of the

behavioural deficits in these models, thus providing pharmacological validity (Cryan and Holmes, 2005, Song and Leonard, 2005). In particular, the CMS and OB models result in behavioural, neurochemical, neuroimmune and neuroendocrine alterations resembling those seen in depressed patients, some of which are responsive only to chronic, but not acute, antidepressant treatment (Cryan and Holmes, 2005, Song and Leonard, 2005). Additional paradigms, such as the sucrose preference test or intracranial self-stimulation can be employed to investigate depression-related behaviours (Cryan and Holmes, 2005). These tests are useful in assessing anhedonic aspects of animal models of depression, such as LH, CMS and OB listed above.

Table 1.4: Current rodent models used in depression research (adapted from O'Leary and Cryan, 2013).

Model	Description
Acute stress-based model	
Forced swim test (FST)	When placed in an inescapable container of water, rats and mice will adopt an immobile posture. The time spent immobile is reduced by antidepressant treatment.
Modified FST	As above but swimming and climbing behaviours are also assessed and are increased by serotonergic or noradrenergic antidepressants, respectively.
Tail suspension test (TST)	Mice when suspended from a bar by their tail will adopt an immobile posture. The time spent immobile is reduced by antidepressant treatment.
Chronic stress-based model	
Maternal separation	When separated from their mother, rodents (mainly rats) during early postnatal life can develop depression-like phenotypes in adulthood.
Prenatal stress	Exposing a pregnant dam to a stressor induces depression-like behaviour and persistent physiological changes in the offspring.
Unpredictable chronic mild stress	Rodents are exposed to a variety of unpredictable stressors leading to a constellation of symptoms that are prevented by antidepressant treatments.
Social defeat stress	Involves daily exposure to a novel aggressive counterpart. Following repeated defeat, the test subject exhibits social avoidance behaviours when subsequently exposed to an unfamiliar counterpart (an effect reversed by chronic antidepressant treatment) as well as depression-like behaviours.
Learned helplessness	Rodents exposed to inescapable shocks subsequently fail to escape when able to, an effect reduced by chronic antidepressant treatment.
Genetic predisposition studies	
Selective breeding	Independent lines of mice and rats that differ in depression-like behaviour have been generated from large heterogeneous populations of animals, e.g., Finders sensitive line (FSL) rats, Rouen helpless mice, congenital

	helpless rats.
Strain differences	Differences in behavioural responses in models of depression and antidepressant drug-like activity have been reported across various rat and mouse strains.
Directed gene targeting	Genetically modified mice with disrupted expression of proteins thought to play a role in depression.
Dysfunction of limbic circuitry	
Olfactory bulbectomy (OB)	Removal of olfactory bulbs in rodents causes a constellation of behavioural and neurochemical alterations that are reversed by chronic antidepressant treatment.
Focal lesions	Transient inactivation of the rat infralimbic cortex has antidepressant-like effects.
Optogenetics	Inhibition or activation of specific groups of neurons in limbic areas of the brain have differential effects on depression-like behaviour.
Other	
Drug withdrawal	Withdrawal from amphetamine in rodents is characterised by anhedonia, learned helplessness and increased immobility in the FST and TST.
Neonatal drug treatment	Neonatal administration of clomipramine induces a number of depression-related behaviours in adulthood
DRL-72	Reinforcement responses with inter-responses longer than 72s in rats. Antidepressant drugs improve the number of reinforced trials.

1.2.3. Animal models of anxiety

Due to its impact on the society (one-eighth of the world population has an anxiety disorder), anxiety has become a very important area of research interest in psychopharmacology in recent decades (Rabbani et al., 2003). Similar to the pathology of depression, animal models have become an invaluable tool in the analysis of the multitude of causes, genetic, environmental or pharmacological, that might extend our understanding of the psychopathology and onset of anxiety disorders (Shekhar et al., 2001).

One of the most used behavioural tests for research on anxiety, suitable for both rats and mice, is the elevated plus maze (EPM) (Pellow et al., 1985). The EPM consists of a maze raised off the ground (generally 40/50cm above the floor) composed of two crossed long arms which include a middle square. Therefore, two opposite open arms and two opposite closed arms extend from the central platform. The EPM is based on the natural aversion of rodents to heights as well as conflict between exploration and aversion to elevated open places and open spaces. Provoked behaviour profiles in the EPM appear to include elements of neophobia, exploration and approach/avoidance conflict (Bourin et al., 2007); furthermore, it is sensitive to both anxiolytic and anxiogenic treatments (Lister, 1987, Dawson and Tricklebank, 1995). The parameters used to assess anxiety-like behaviour include time spent in the open arms and the number of crossings into the open arms. It has been validated that anxiolytic drugs reduce the amount of time spent by the animals in the closed arms and increase exploratory activity without a corresponding increase in general locomotion (Cryan and Holmes, 2005, Cryan and Sweeney, 2011). An additional method to assess the anxiolytic potential of compounds include the light-dark box which consists of an arena divided by a small dark box and a brightly lit open space.

Small rodents are naturally afraid of and avoid open and brightly lit spaces, a strategy possibly developed to avoid predators; however rodents are also naturally exploratory animals. Similar to the EPM, the time spent in illuminated and dark places, as well as the number of entries in each space are taken into account for interpretation of rodents' anxiolytic-like behaviour (Cryan and Holmes, 2005, Cryan and Sweeney, 2011).

Finally, the open field represents another widely used test to observe general motor activity, exploratory behaviour and measures of anxiety (Whimbey and Denenberg, 1967, Asano, 1986, Mechan et al., 2002). It consists of an open field area (bigger than the standard rodent's house cage) on which several parameters are taken into consideration: the number of entries and time spent in the centre; periphery and corners of the field; the number of crossings into a delimited centre area of the arena; rearing (number of times the animal stands on its hind legs) and assisted rearing (forepaws touching the walls of the apparatus). Moreover, to measure anxiety-like behaviour the arena is illuminated by a 60W bulb placed at a height of 100cm. Although measurements can be done with a single behavioural test, it is better to use a battery of these tests (for instance, the EPM, the dark/light box and the open field) to assess each individual's behavioural phenotype, since these tests measure anxiety under different conditions, see table 1.5 (van Gaalen and Steckler, 2000).

Table 1.5: Current rodent models used in anxiety research (adapted from Cryan and Sweeney, 2011).

Model	Description	References
Elevated plus maze	Rodents are placed on an elevated plus-shaped maze consisting of two open arms and two enclosed arms. Rodents naturally display aversion to the open areas. Anxiolytic drugs increase number of entries and time spent in the open arms.	(Lister, 1987)
Light/ dark box	Rodents are placed in an apparatus consisting of a light and a dark compartment. Rodents naturally display aversion to illuminated areas. Anxiolytic drugs increase the time spent in the light box and the number of entries and reduce freezing behaviour.	(Bourin et al., 1996)
Open field	Rodents are placed in a bright arena and are allowed to explore it. Mice avoid anxiety-provoking areas such as the centre of the arena. Anxiolytics drugs increase the time spent in anxiety-provoking areas.	(Lucki et al., 1989)
Novelty induced hypophagia	Rodents are trained to consume a desirable food (e.g. sweetened milk) before being presented with the food in a novel environment. Animal hesitation on consuming the highly palatable food in the novel environment is considered a measure of both anxiety and anhedonia.	(Dulawa and Hen, 2005)
Novelty suppressed feeding	Food-deprived rodents are introduced to a novel anxiogenic environment where food is presented. Anxiolytic drugs reduce the latency of the animal to approach the food.	(Bodnoff et al., 1988)
Defensive marble burying test	Rodents are placed in a cage containing bedding and a number of novel marbles. Burying of the marbles is interpreted as anxiety-like behaviour. Anxiolytic drug treatment reduces the number of marble burying.	(Treit et al., 1981)
Stress induced hyperthermia	Temperature is measured twice at an interval of 15 minutes. The difference between the first and the second measurements is a physiological measure of anxiety. Anxiolytic drugs reduce the magnitude of this temperature increase.	(Adriaan Bouwknecht et al., 2007)
Ultrasonic vocalization measurements	Rodent pups are separated from their mothers and the frequency of ultrasonic distress calls is recorded. A reduction in the number of USVs emitted from separated pups is regarded as an anxiolytic effect.	(Nastiti et al., 1991)

1.3. The impact of dietary interventions on mental health

Despite the improved pharmacological approaches which moderately buffer the worldwide burden of poor mental health, recent finding suggests that this burden will continue to rise worldwide during the coming decades (Baxter et al., 2013, Whiteford et al., 2013).

Rapid urbanisation, and the general transition to more calorically dense, processed diets and reduced physical activity have had a significant impact upon the overall health of individuals in developed nations and are associated with an increased incidence of mental disorders such as anxiety and depression (Logan and Jacka, 2014).

Accumulating translational evidence has proposed that the quality of diet is a crucial and common determinant for mental disorders (Jacka et al., 2014, McNamara et al., 2015). The consumption of nutrient-poor, energy-dense, highly processed foods has indisputably increased worldwide in both developed and emerging economies contributing to a globally overfed and undernourished society. Obesity and malnutrition represent, indeed, two important risks factor for the development of mental illnesses (Kinley et al., 2015). Observational studies on nutritional status in the United States of America have reported that the intake of several brain-essential nutrients, such as vitamins, magnesium and omega-3 polyunsaturated fatty acids (n-3 PUFAs) are far lower than recommended (Bowman et al., 2014, Parker et al., 2014). Importantly, associations between healthy dietary patterns and a reduced prevalence of mental illnesses throughout the lifespan have been shown (Barre et al., 2011, Gould et al., 2013, Nanri et al., 2013, Psaltopoulou et al., 2013, Lai et al., 2014). Indeed, cross-sectional relationships between unhealthy dietary patterns and poorer

mental health in children and adolescents have recently been found (O'Neil et al., 2014). Moreover, such inadequate dietary intake during prenatal and early-life nutrition can contribute to the pathogenesis of both depressive and psychotic disorders (Susser and Lin, 1992, Brown et al., 2000, McGrath et al., 2011, Jacka et al., 2013, Steenweg-de Graaff et al., 2014). Additionally, subjects with schizophrenia show a poor diet, mainly characterized by a high intake of saturated fat and a low consumption of fibre and fruit, that partly accounts for their higher incidence of metabolic abnormalities (Dipasquale et al., 2013). Benefits from adequate dietary intake have been observed in adults with depression as well. A Mediterranean diet supplemented with nuts showed a strong trend towards a reduced risk for incident depression after three years of intervention, and this was particularly evident in individuals with type 2 diabetes (Sanchez-Villegas et al., 2013). Likewise, older adults receiving dietary coaching experienced a low incidence of major depressive episodes and exhibited a 40%-50% decrease in depressive symptoms, as well as enhanced well-being, during the initial 6-week intervention; these gains were sustained over 2 years (Stahl et al., 2014). Dietary habits exert an important influence on the function of several systems involved in the maintenance of mental health. An example is the immune system which represents an emerging risk factor for the onset of the pathology of depression (Berk et al., 2013, Cattaneo et al., 2015). Moreover, nutrition can affect the antioxidant defence system which, together with high pro-inflammatory cytokines levels, constitutes another factor involved in the pathology of depression (Maes, 2008). Additionally, nutritional factors can contribute to the mechanisms of cellular development and neuronal plasticity throughout the release of neurotrophic factor (Molendijk et al., 2011).

Nutrition-based supplements which research has highlighted as potentially beneficial in the management of mental disorders include dietary lipids such as phospholipids and n-3 PUFAs. Indeed, dietary phospholipids supplements have been reported to improve cognitive function both in animals (Corwin et al., 1985) and humans (Delwaide et al., 1986). Although mechanisms underlying their benefits are still unfolding, phospholipids seem to facilitate the activation of signalling proteins and receptors that are critical for neuronal survival, differentiation and synaptic neurotransmission. Being enriched in fatty acids, especially n-3 PUFAs, their membrane levels can be altered according to n-3 PUFAs status (Hamilton et al., 2000). Therefore, transition to more calorically dense, processed diets, poor in n-3 PUFAs, may induce alteration in phospholipids levels and in brain function. As for n-3 PUFAs, they have been under the spotlight for decades. However, only recently has research looked into the critical role of n-3 PUFAs in brain function and structure throughout the lifespan. n-3 PUFAs constitute key regulating factors of neurotransmission, neurogenesis, cell survival and neuroinflammation and are thereby fundamental for development, functioning and ageing of the central nervous system (Chalon, 2006, Rao et al., 2007b, Sarris et al., 2012, Mischoulon and Freeman, 2013, Chang et al., 2015, Dehkordi et al., 2015). It is noteworthy that these processes are altered in various neurological disorders, including attention deficit hyperactivity disorder (ADHD), schizophrenia, major depression disorder and Alzheimer's disease (Su et al., 2008, Sinn et al., 2010, Balanza-Martinez et al., 2011, Mischoulon and Freeman, 2013, Stonehouse, 2014, Su et al., 2014). Despite this evidence the concept of n-3 PUFAs as a clear therapeutic compound for mental disorders still needs further clarification.

The purpose of this thesis is to further explore the role of dietary lipids, especially phospholipids and n-3 PUFAs, which have been gaining increasing credence as potential targets for the development of novel strategies for the maintenance of mental health, in the prevention and amelioration of psychopathology.

1.4. Phospholipids in the brain

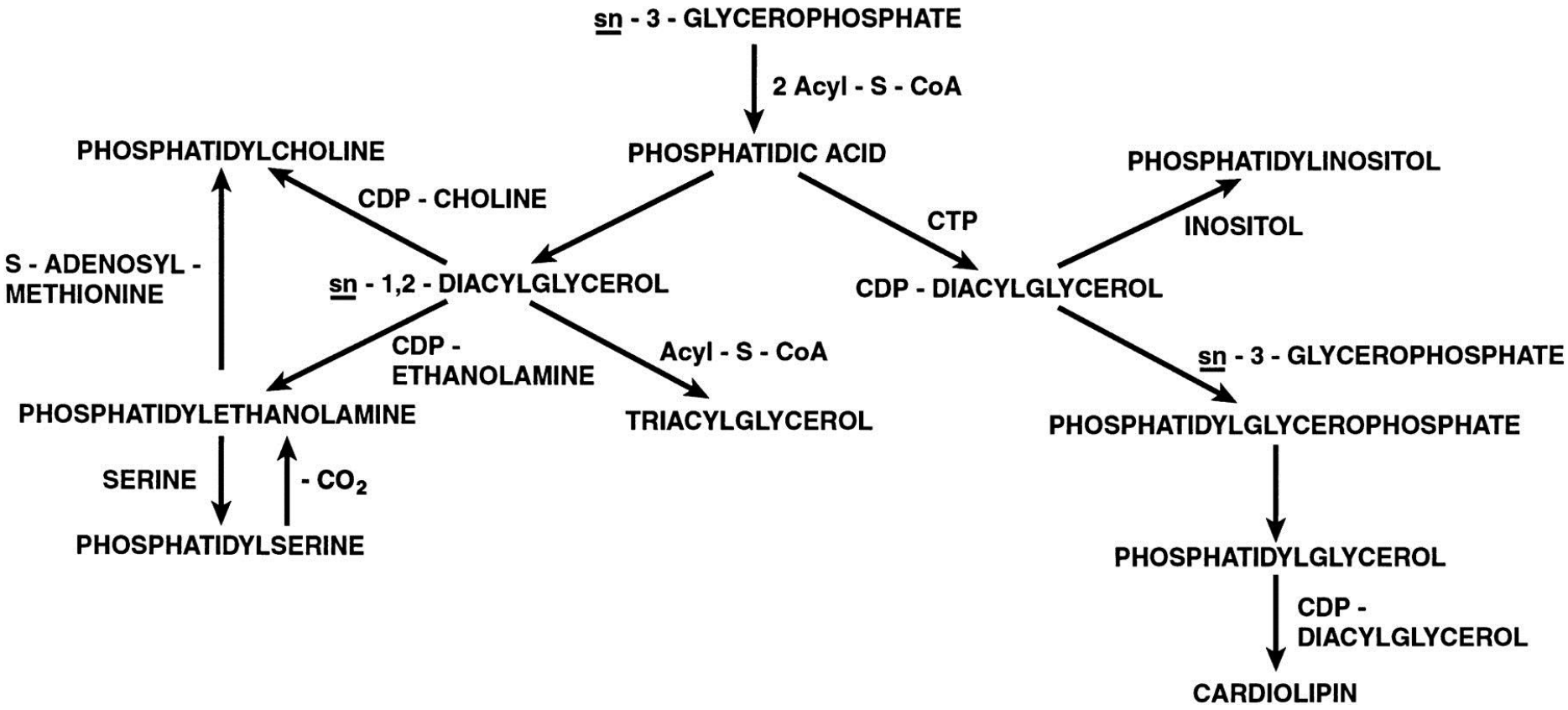
1.4.1. Metabolism and function

Phospholipids constitute the major component of all cell membranes. As such, they confer the property of fluidity on the membrane, and thus determine and influence the behaviour of membrane-bound enzymes and receptors. Within the phospholipids, it is the phosphatidylserine (PS) which has received the most attention from researchers. PS is the most abundant phospholipid in the brain, making up 13–15% of the phospholipids in the human cerebral cortex (Svennerholm, 1968). Moreover, it is enriched in docosahexaenoic acid (DHA, 22:6n-3) which is the most abundant n-3 PUFA in the brain. Serine is required by the brain for the synthesis of proteins and three classes of lipids: PS, sphingolipids, and N-acylserines. It is obtained either by uptake from the cerebral circulation or by synthesis from glucose. Moreover, serine is synthesized by astrocytes from glucose taken up by the brain, whereas neurons cannot convert glucose into serine due to lack of enzymes of synthesis.

At the level of the brain, PS has been observed to be necessary for the activation of several key signalling pathways. These include the phosphatidylinositol 3-kinase (PI3K)/Akt, protein kinase C (PKC) and Raf-1 signalling, which are known to stimulate neuronal survival, neurite growth and synaptogenesis (Newton and Keranen, 1994, Huang et al., 2011a). Hence, modulation of the PS level in the plasma membrane of neurons has significant impact on these signalling processes

and brain function. Furthermore, PS is involved in the SNARE-assisted membrane fusion between neurotransmitter-containing synaptic vesicles and synaptic plasma membrane which is necessary for exocytosis. Indeed, adequate levels of PS in the cellular membrane have been observed to be crucial in the fusion process and exocytosis of synaptic vesicles (Bhalla et al., 2005, Hui et al., 2009). A schematic summarizing the synthesis and metabolism of phospholipids has been reported in Table 1.5.

Table 1.5: Schematic summarizing the synthesis and metabolism of phospholipids.



1.4.2. Phospholipids and Cognition

The most interesting findings on the effects of dietary phospholipids supplementation have been observed on cognitive function. Aged rats given daily doses of krill PS orally for 7 days showed improvement in the Morris water maze test. There was less loss of choline acetyltransferase and acetylcholine esterase immunoreactivity, and less muscarinic acetylcholine receptor type 1 and choline transporter mRNA in the hippocampus. This is of importance since acetylcholine has been reported to improve cognition and attention (Sarter and Bruno, 1997). The neuroprotective activity of 20 mg/kg krill PS was equivalent to that of 50 mg/kg soy PS in these aged rats (Lee et al., 2010). Normal young rats given 100 mg/kg krill PS orally for 30 days also showed improvement in the Morris water maze test (Park et al., 2013). Similarly, cognitive improvement was reported in humans given oral PS supplements (Delwaide et al., 1986). Human subjects treated for 42 days with 200mg of soy-based PS showed a more relaxed state before and after mental stress as measured by electroencephalography (Baumeister et al., 2008), and no adverse effects were evident at this dose given three-times a day for 6–12 weeks (Jorissen et al., 2002). The ability to recall words increased by 42% in male and female subjects who were older than 60 years and complained of subjective memory loss when they were treated with 300 mg/day of PS containing 37.5 mg of eicosapentaenoic acid (EPA, 20:5n-3) and DHA (Richter et al., 2010). Improved verbal immediate recall was also observed in a double-blind, placebo controlled clinical trial in a large group of elderly subjects with memory complaints when treated with a daily dose of 300 mg PS containing DHA and EPA in a 3:1 ratio. A subset with relatively good cognitive performance at baseline showed the greatest improvement (Vakhapova et al., 2010). In a similar double blind study in Japanese subjects between the ages of 50 and 69 years with memory complaints, subjects having low scores at baseline showed greater

improvement in delayed verbal recall after treatment for 6 months with soybean PS (Kato-Kataoka et al., 2010).

Several biochemical responses to PS administration that have been reported in experimental animals could be involved in the mechanism of PS-mediated improvement in cognition. Stimulation of dopamine-dependent adenylate cyclase activity was observed in mouse brain following an intravenous infusion of a sonicated preparation of bovine brain PS (Leon et al., 1978). Indeed, dopamine has been shown to play an important role in some aspects of cognition, such as attention (Nieoullon, 2002). Sonicated suspensions of PS injected intravenously also increased calcium-dependent acetylcholine output from the cerebral cortex in urethane anesthetized rats (Casamenti et al., 1979). Likewise, intravenous injection of purified bovine brain PS for 8 days attenuated the decrease in acetylcholine release from the parietal cortex in aged rats, possibly by providing more choline for acetylcholine synthesis (Casamenti et al., 1991). Furthermore, orally administered krill PS in normal young rats for 30 days produced an increase in neurons positive for brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF-1) in the hippocampal CA1 region (Park et al., 2013). Of importance, both BDNF and IGF-1 play an important role in cognition (Lu et al., 2014, Pellecchia et al., 2014).

A current hypothesis is that these biochemical responses and the resulting cognitive improvements are due to PS mediated effects on neuronal membrane properties (Delwaide et al., 1986). However, experimental evidence indicating that orally or intravenously administered PS actually alters neuronal membrane properties is lacking. How the administered PS is transported in the plasma, how much enters the brain, whether it is taken up intact, and whether it is incorporated into neurons or glia is not known. Dietary phospholipids are hydrolysed during digestion, so orally administered PS most likely is not absorbed intact. PS preparations are rich in DHA, and DHA supplementation is known to

improve hippocampal function (Kim, 2007). Because the administered PS probably undergoes partial or complete hydrolysis, the beneficial effects of PS on cognition, particularly from krill or bovine sources, are possibly produced by DHA released from the PS rather than the intact PS itself. These issues will have to be investigated in order to obtain some mechanistic insight into how dietary or intravenously administered PS supplements function to produce cognitive improvement.

1.5. Introduction to polyunsaturated fatty acids (PUFAs)

1.5.1. Structure and metabolism of n-3 and n-6 PUFAs

Polyunsaturated fatty acids (PUFAs) are important constituents of all cell membranes. PUFAs confer on membranes properties of fluidity, and thus determine and influence the behaviour of membrane-bound enzymes and receptors. PUFAs are essential for survival of human and other mammals, and they cannot be synthesized in the body, hence, they have to be obtained in our diet and, thus, are also defined as essential fatty acids (EFAs). There are two types of naturally occurring EFAs in the body, the n-3 series derived from α-linolenic acid (ALA, 18:3n-3) and the n-6 series derived from cis-linoleic acid (LA, 18:2n-6). The n-3 PUFAs family is defined by a double bond beginning at the third carbon from the methyl end. Because mammals cannot insert double bonds more proximal to the methyl end than the ninth carbon atom (Δ-9 desaturase), n-3 PUFAs cannot be synthesized *de* novo, consequently n-3 PUFAs have to be present in the diet. These features are shared by the n-6 PUFAs family which are defined by a double bond beginning at the sixth carbon from the methyl end. Because neither of these fatty acids can be synthesized *de novo*, ALA and LA are referred to as essential fatty acids for mammals. Hence, ALA and LA serve as the precursor molecules from which the rest of fatty acids belonging to the n-3 or n-6 PUFAs family can be synthesized through a series of elongation and desaturation reactions. All the reactions are

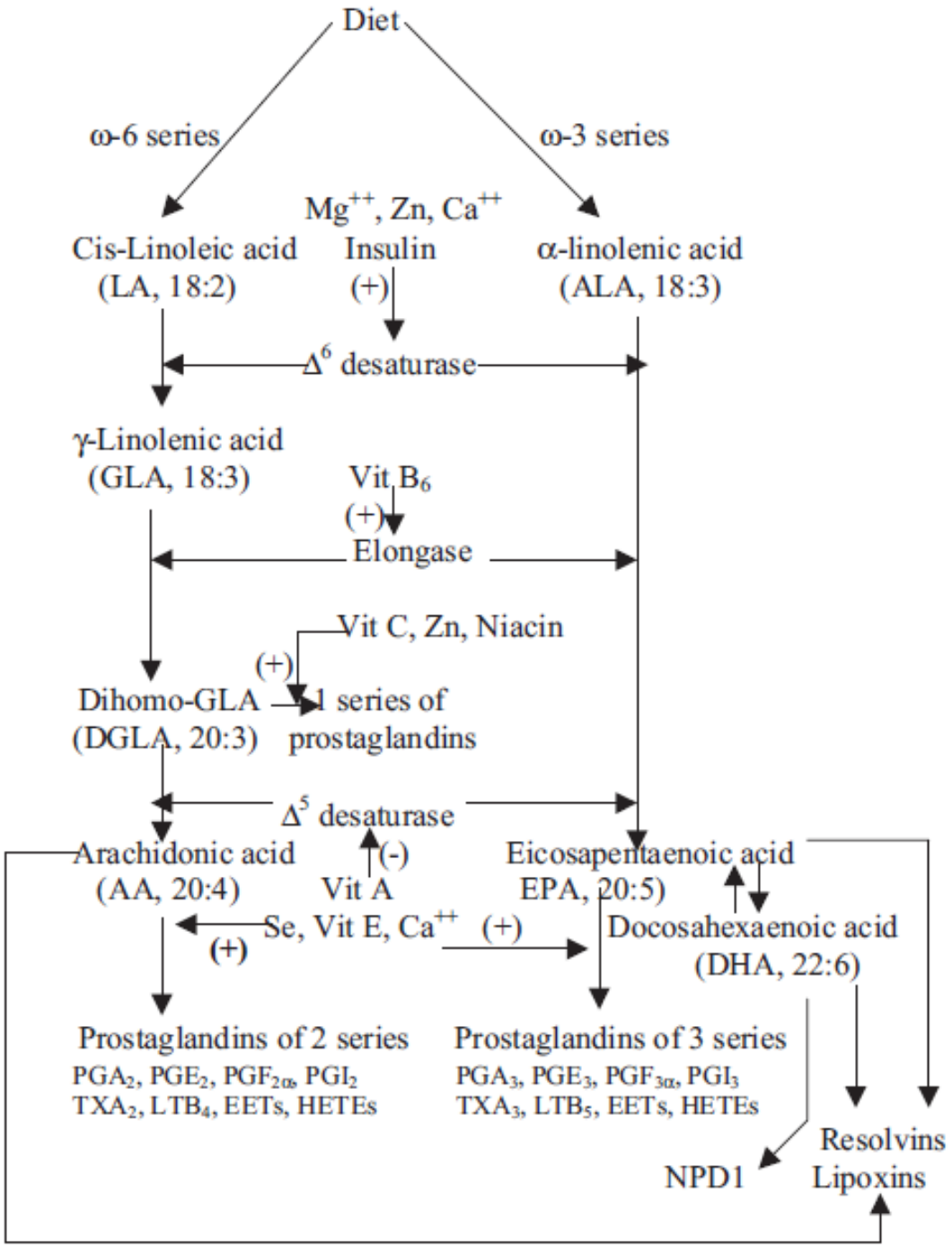
catalysed by an enzymatic system consisting of fatty acyl-CoA synthetases, Δ-6 and Δ-5 desaturases and respective elongases (Green and Yavin, 1998, Cho et al., 1999, Tvrdik et al., 2000, Horton et al., 2003, Wang et al., 2005). Evidence from several studies *in vivo* and *in vitro* indicate that these two fatty acid families not only share these enzymes, but also compete for the same enzyme (Brenner and Peluffo, 1966, Mohrhauer et al., 1967). Therefore, excessive supplementation of either n-3 or n-6 PUFAs in the diet may cause a deficiency in the other group by shutting down the synthetic pathway shared by n-3 and n-6 PUFAs. Although lower older animals have enzymes to convert n-6 to n-3 PUFAs (Spychalla et al., 1997), mammals do not possess the enzymatic machinery necessary to convert an n-3 PUFAs into an n-6 PUFAs or vice versa. However, a mouse expressing the gene encoding FAT-1 from C. elegans, was recently made that converts n-6 PUFAs into n-3 PUFAs, thereby increasing endogenous docosahexaenoic acid (DHA, 22:6n-3) via the conversion of n-6 PUFAs, resulting in a reduction in arachidonic acid (AA, 20:4n-6) levels (Kang et al., 2004). FAT-1 encodes for an n-3 PUFAs desaturase in C. elegans that recognizes 18 and 20 carbon n-6 PUFAs and inserts a double bond to form an n-3 PUFAs (Spychalla et al., 1997). Hence, these mice are the only known mammals able to convert n-6 PUFAs into n-3 PUFAs and are a useful model to elucidate the role of n-3 PUFAs in health and disease.

There are two metabolic fates for LA which is converted to γ-linolenic acid (GLA, 18:3n-6) by the action of the enzyme Δ-6 desaturase, and GLA is elongated to form dihomo-GLA (DGLA, 20:3n-6), the precursor of the 1 series of prostaglandins (PGs). DGLA can also be converted to AA by the action of the enzyme Δ-5 desaturase. AA forms the precursor of 2 series of prostaglandins, thromboxanes and the 4 series of leukotrienes. ALA is converted to eicosapentaenoic acid (EPA, 20:5n-3) by the Δ-6 desaturase and the Δ-5 desaturase. EPA forms the precursor of the 3 series of prostaglandins and the 5 series of leukotrienes.

As discussed above, the influence of LA and ALA levels interact in producing metabolic outcomes. However, ALA appears to be a much stronger suppressor of n-6 PUFAs elongation and desaturation than LA is of n-3 PUFAs elongation and desaturation. In fact, 10 times more LA is required to have an equal effect on n-3 PUFAs metabolism as ALA does on LA elongation (Holman, 1998). For this reason, and because mammals cannot interconvert n-3 and n-6 PUFAs, the dietary ALA to LA ratio is an important issue for study and discussion (Yehuda and Carasso, 1993, Emken et al., 1994, Barcelo-Coblijn et al., 2005). In addition, fatty acid elongation and desaturation is subjected to feedback regulation because both AA and DHA suppress endogenous conversion of LA and ALA into longer chain fatty acids, respectively (Enser et al., 1998, Emken et al., 1999). However, fatty acid modulation of these reactions appears to be tissue-selective (Demar et al., 2005, Igarashi et al., 2006, Rapoport et al., 2007, Igarashi et al., 2008). Reductions in dietary n-3 PUFAs uptake results in an increased ability of the liver to elongate and desaturate n-3 PUFAs, whereas the brain is not responsive to dietary levels (Demar et al., 2005) and the heart does not have the capacity to elongate and desaturate ALA into longer chain n-3 PUFAs (Igarashi et al., 2008). Thus we know that n-3 and n-6 PUFAs metabolism is more complex than a mere competition at the substrate level because this process is regulated at the genetic level as well (Lauritzen et al., 2001, Moon et al., 2001, Kitajka et al., 2002, Barcelo-Coblijn et al., 2003b). Moreover, the impact of the amount of dietary n-3 and n-6 PUFAs on their own accumulation and conversion to longer chain PUFAs is an important issue. In fact, the ratio of LA to ALA is increasingly being considered as one of the factors that influence ALA conversion into longer n-3 PUFAs. There have been a number of attempts to establish the optimal ratio in various animals in order to find the optimal ratio of LA to ALA that leads to maximal tissue DHA accumulation (Yehuda and Carasso, 1993, Blank et al., 2002, Barcelo-Coblijn et al., 2003b). By varying the LA to ALA ratios from 0.5: 1 to 10: 1, there is an effect on plasma and brain

DHA levels, but there is apparently some degree of complexity in this process (Blank et al., 2002). The highest levels of DHA are not found in diets containing the highest ALA content, but rather maximal DHA accumulation occurs when the LA to ALA ratio is within the range of 3: 1 -4: 1. Interestingly, a similar optimal ratio was obtained by other groups both from adult and aged rats (6 month old), respectively (Yehuda and Carasso, 1993, Barcelo-Coblijn et al., 2003b). This is of importance considering that especially in the western countries, the consumption of n-6 PUFAs has increased disproportionately, moving from an optimal n-6: n-3 ratio of 1-4: 1 to 20: 1 (Molendi-Coste et al., 2011). In parallel, there are coinciding increases in the incidence of neurodegenerative and psychiatric illnesses (Kiecolt-Glaser et al., 2007, Corsinovi et al., 2011), and n-3 PUFAs have been shown to be crucial for normal neurological development (Carlson and Neuringer, 1999, Moriguchi et al., 2000). A schematic summarizing the synthesis and metabolism of PUFAs has been reported in Table 1.6.

Table 1.6: Schematic summarizing the synthesis and metabolism of PUFAs.



1.5.2. Dietary sources of PUFAs

The main dietary sources of PUFAs are, for LA: cereals, eggs, poultry, most vegetable oils, whole-grain breads, baked goods, and margarine; sunflower, saffola, and corn oils are also rich in LA (Ollis et al., 1999); for ALA: canola oil, flaxseed oil, linseed and rapeseed oils, walnuts, and leafy green vegetables such as purslane. Moreover, fish provide varying amounts of n-3 PUFAs in the form of DHA and EPA. The average daily intake of PUFAs varies from country to country and again from region to region. However, in general, the intake is around 7–15 g/day in Europe and USA (Ollis et al., 1999, Das, 2006). Human milk contains 0.3–1.0% of its fat as GLA. Thus, breast fed babies get significant amounts of GLA (Harzer et al., 1983, Ollis et al., 1999). Fresh cow's milk contains small amounts of GLA (0.25% of the total fats). Evening primrose oil, borage oil, black currant oil, and hemp seed oil contain substantial amounts of GLA. GLA is present in evening primrose oil at concentrations of 7–14% of total fatty acids; in borage seed oil it is 20–27%; and in black currant seed oil at 15–20%. GLA is also found in some fungal sources (Harzer et al., 1983, Horrobin, 1983, Ollis et al., 1999). Moderate amounts of DGLA are found in human milk (Harzer et al., 1983), liver, testes, adrenals, and kidneys (Takayasu et al., 1970, Ollis et al., 1999). Small amounts are present in cow's milk. AA is found in modest amounts in human milk and in small amounts in cow's milk. Meat, egg yolks, some seaweeds, and some shrimps contain substantial amounts of AA (Bezard et al., 1994). The average daily intake of AA is estimated to be in the region of 100–200 mg/day (Meyer et al., 2003), more than enough to account for the total daily production of various PGs, which is estimated to be about 1 mg/day. The main sources of adrenic acid (22:44n-6) are adrenals, kidneys, testes, and brain. The major source of EPA and DHA in the diet is from marine fish. Fresh water fish are unlikely to contain substantial amounts of EPA and DHA. It is important to note that because of their instability, substantial loss of PUFAs occurs during food processing and

hydrogenation. Some of these fatty acids may be denatured and converted into trans fats that are harmful to the body (Cantwell et al., 2005, Lopez-Garcia et al., 2005). It has been argued that the fall in the intake of biologically active n-3 PUFAs, especially that of EPA and DHA, has been one of the major changes in Western nutrition in the last 50 years, and has contributed to the increasing incidence of atherosclerosis, coronary heart disease, hypertension, metabolic syndrome X, obesity, collagen vascular diseases and, possibly, cancer.

1.6. n-3 PUFAs in early-life

1.6.1 Embryogenesis and post-natal stages of brain development

Brain development is a sequential anatomical process characterized by specific well-defined stages of growth and maturation. It has become more evident that this process is influenced by n-3 PUFAs. Indeed, DHA levels increase sharply along the perinatal period. In rats, the first important step of acquisition of DHA takes place in the embryonic phase and in the first three postnatal weeks of life, whereas in humans this period occurs between the last trimester of gestation and the first 6-10 months after birth (Clandinin et al., 1980b, a). This is due to a general requirement for DHA, not only from the brain, but also from different organs of the foetus, such as retina and liver (Clandinin et al., 1980b). At these stages of life the foetal metabolic capability to convert ALA to DHA is extremely limited (less than 0.2% in children). Indeed, it is the mother that guarantees an adequate delivery of DHA to the foetus through the placenta. It has been shown that higher dietary intakes of DHA during pregnancy are proportional to higher maternal-foetal transfer of DHA (Innis, 2005, 2007). This process has been elegantly described by following labelled-ALA and its conversion to labelled-DHA with further migration into the baboon placenta (Innis, 2006). During this period, DHA is preferentially accumulated in phospholipids such as phosphatidylethanolamine and

phosphatidylserine. In fact, DHA perinatal reduction in the hippocampus of adult rats (<75%) is associated with lower levels of phosphatidylserine (<30%) (Murthy et al., 2002). Moreover, inadequate intake of n-3 PUFAs reduces hippocampal and cortical dimensions and morphology in the embryonic rat (Coti Bertrand et al., 2006). Conversely, significant elevations (>15%) in brain DHA concentrations are associated with increased (>30%) phosphatidylserine in the frontal cortex of rats (two months old) after perinatal fish oil supplementation (Chalon et al., 1998). This translates into increased cortical dopaminergic levels together with improved cognition and learning ability in adult rats (Chalon et al., 1998). The evidence suggests the importance of n-3 PUFAs on normal development starting from the early stages of life. Indeed, being components of cell membranes, n-3 PUFAs are involved in membrane lipid biosynthesis including myelination, synaptogenesis, and dendritic arborisation (Wurtman, 2014). Hence, the presence of DHA in membrane lipids raise the possibility that inadequate DHA in the brain alters neuronal and glial cellular surface as well as branches and dendrites that are in continuous flux during development and learning. The Perinatal Lipid Nutrition Project (PeriLip) and The Early Nutrition Programming Project (EARNEST) have recently developed consensus recommendations concerning dietary fat intake for pregnant and lactating women. They recommend a minimum DHA intake of 200 mg/day (Koletzko et al., 2007). Interestingly, supplementation of n-3 PUFAs during pregnancy not only increases breast milk DHA content but results also in slightly longer gestation as well as reduced risk of preterm delivery (Olsen et al., 1993, Singh, 2005). Accordingly, infants born prematurely show less accumulation of DHA and of the n-6 PUFA arachidonic acid (ARA) in the brain (Clandinin et al., 1980b, a, Innis, 1991, Barcelo-Coblijn and Murphy, 2009). However, the increased supply of DHA to the developing foetal nervous system leads to a progressive depletion of maternal plasma DHA (Uauy et al., 2000, Smuts et al., 2003). Of relevance, Hibbeln et al, in a cross-national

ecological analysis, reported reduced levels of maternal milk DHA and lower seafood consumption correlated with higher rates of postpartum depression (Hibbeln, 2002). Therefore, diets enriched in omega-3s are recommended to both restore the depletion of DHA in the mother as well as to look after the needs of the foetus and suckling infant.

1.6.2. Early post-natal life

Animal studies reveal n-3 PUFAs as guarantors of proper brain development in early post-natal life (Zhang et al., 2010, Lei et al., 2013). n-3 PUFAs supplementation has been shown to protect from sevoflurane-induced neuronal loss and decreased neurogenesis in the cerebral cortex and hippocampus of neonatal rats (Lei et al., 2013). Furthermore, this effect can be long-lasting and show further benefits in neurocognitive function later in adulthood (Lei et al., 2013). In humans, Jensen et al have shown a higher Psychomotor Development Index in breastfed infants of mothers who underwent DHA administration (200mg/day) for a period of 4 months (Jensen et al., 2005). Psychomotor development, eye-hand coordination and visual acuity were all improved after DHA algal-oil treatment. However, these improvements were limited to infants 30 months old and no further advantages on mental development were found. This study has limitations in terms of treatment duration and assessment instruments. A different study measured IQ in children whose mothers underwent maternal supplementation with cod liver oil (1183 mg/10 mL DHA, 803 mg/10 mL EPA) started from week 18^{th} until 3 months after delivery (Helland et al., 2003). The scoring of the Mental Processing Composite of the Kaufman Assessment Battery for Children (K-ABC) was increased by 4 points in 4 year old children who were born to mothers who had taken cod liver oil enriched in DHA and EPA. However, only 84 children of the 590 pregnant women enrolled in the study completed the K-ABC at 4 years of age. Another similar study has shown higher mental processing scores, high degree of stereopsis and stereo acuity in 3.5

years old children whose mothers underwent a DHA-rich diet (Williams et al., 2001). Therefore, in mammals the correct acquisition of DHA represents a critical step in embryogenesis and in the early post-natal stages of development. Given the importance to maintain proper DHA levels during the neonatal period of life, the content intake and the kind of fatty acids introduced in the body are of importance. In fact, infants fed formula with 0.4 or 2.4% energy from ALA had 2.3 ± 0.2 and 2.2 ± 0.3 g/100 g fatty acids as DHA in plasma phospholipids, respectively, despite the large difference in ALA intake, whereas infants fed formula with only 0.12% energy from DHA had plasma phospholipid DHA levels of 5.2 ± 0.2 g/100 g (Ponder et al., 1992, Innis et al., 1996, Innis, 2007).

1.6.3. Adolescence: brain activity, learning and cognition

n-3 PUFAs maintain their importance in brain development and functioning during adolescence (Table 1.7). At this stage of life, the consumption of an n-3 PUFAs deficient diet may alter the composition of brain lipids and result in impairment in cognition and emotional state. Adolescent rats fed with an n-3 PUFAs deficient diet tend to have decreased n-3 PUFAs mass (82% less) compared to control (Bondi et al., 2014). Such animals showed less exploratory behaviour, weaker memory performance and increased tyrosine hydroxylase expression (dopamine precursor) in the dorsal striatum. Moreover, Weisser et al have found decreased BDNF levels in the hippocampus and the hypothalamus of adolescent rats which underwent DHA-deficient diet (Weiser et al., 2015). The hippocampus and hypothalamus represent two brain regions that exhibit a high degree of plasticity and are essential elements in the neuroendocrine response to stress. This is of importance as youth and early adulthood are the most vulnerable periods for the development of most major mental illnesses. Thus, increases in the levels of hypothalamic and hippocampal BDNF content may provide an

adolescent enhanced resiliency to stressors and negative mood states during a time of great physical, mental, and hormonal change.

In humans, Kuratko et al provided a systematic review based on 15 publications regarding the influence of DHA in learning and behaviour in healthy children (Kuratko et al., 2013). The studies differed in purpose and design, and some of them did not achieve consistent conclusions regarding DHA effect on specific cognitive tests. Despite that, Kuratko et al reported benefits of DHA supplementation in brain activity and in school performance from over half of the considered studies. A landmark study from 2010 demonstrated improved brain activity after DHA supplementation (McNamara et al., 2010a). McNamara et al, showed for the first time, regulation of cortical metabolic function and cognitive development exerted by DHA concentrations in the grey matter of healthy boys during sustained attention (McNamara et al., 2010a). The study was conducted in thirty three subjects (9 years old) who were assigned to receive placebo or one of the two doses of DHA (400 mg/d; 1200 mg/d) for 8 weeks. Moreover, a longitudinal study in Arctic Quebec has revealed a relation within school-age children with higher DHA cord plasma concentration and memory function (Boucher et al., 2011). However, a study conducted in DHA-fed healthy children in the UK did not show any improvement on cognitive performance and learning (Kennedy et al., 2009). Other studies have observed benefits of n-3 PUFAs in malnourished subjects. For instance, improvements in learning and cognitive performance have been shown in n-3 PUFA supplemented malnourished 7–9 year old South Africans (Dalton et al., 2009) and 8–12 year old Mexican children (Portillo-Reyes et al., 2014). Although, no benefits were found in 6–10 year old malnourished children from India (Muthayya et al., 2009) and Indonesia (Osendarp et al., 2007). The discordance between these studies may be accounted for by differences in the extent of malnutrition. Moreover, further discrepancies can be due to differences in the experimental plan such as supplements used, different dosages, duration of the trials. Overall,

the current research supports the view that adequate intake of DHA from the prenatal period onwards may have a positive impact on brain activity, learning and cognition in healthy children. Moreover, this stage of life coincides with important phases of brain development such as neurogenesis, neuroblast migration and differentiation, synaptogenesis and axonal myelination (Green et al., 1999). Thus, PUFAs are of relevance for the development and function of the CNS and they may have a beneficial effect on health including mental health (Das, 2006, McNamara and Carlson, 2006).

Table 1.7: n-3 PUFAs impact in healthy adolescents (adapted from Stonehouse, 2014).

Double-blind randomized placebo-controlled trials of n-3 PUFAs in healthy adolescents.					
Participants	**Treatment**	**Length of trial**	**Measurements**	**Outcomes**	**References**
6–11 years, low-income iron deficient children (*n* = 321, analysed *n* = 288).	(1) Iron + fish oil; (2) Iron + placebo; (3) Fish oil + placebo; (4) Placebo + placebo. Fish oil = 0.5 g/day LC omega-3 (0.42 g DHA + 0.08 g EPA).	8.5 months.	HVLT.	LC omega-3 PUFA without iron had negative effects on working memory in children with iron deficiency anaemia and on long-term memory and retrieval in girls with iron deficiency, whereas boys with iron deficiency performed better.	(Baumgartner et al., 2012)
7–9 years, low-income, marginally nourished indigenous children (*n* = 183, analysis on *n* = 155).	Fish flour bread spread provided at school (~0.89 g/week DHA (0.13 g/day)) *vs*. control bread spread.	6 months.	HVLT, Spelling test, Reading test.	Improved verbal learning ability and memory. Prevented decline in spelling. Effects more pronounced in children with lower baseline performance scores.	(Dalton et al., 2009)
10–12 years (*n* = 90, analysis on *n* = 86).	(1) Low dose algal oil: 0.4 g DHA; (2) High dose algal oil: 1.0 g/day DHA; (3) Placebo (vegetable oil).	8 weeks.	Cognitive performance.	Word recognition task: Low dose: faster performance; High dose: slower performance.	(Kennedy et al., 2009)
8–10 years (*n* = 450, analysis on *n* = 348).	Fish oil (0.4 g DHA + 0.06 g EPA)/day + micronutrients *vs*. placebo (olive oil).	16 weeks.	KBIT-2, WIAT-2, WMTB-C, creature counting, MFFT, comPET, SNAP-	No treatment effects.	(Kirby et al., 2010)

			IV, SDQ.		
8–10 year boys (*n* = 38, analysis on *n* = 33).	(1) Low dose algal oil: 0.4 g/day DHA; (2) High dose algal oil: 1.2 g/day DHA; (3) Placebo (corn oil).	8 weeks.	Sustained attention test, fMRI.	Both dosages increased activation of the dorsolateral prefrontal cortex during sustained attention task. No effect on attention or reaction time of attention.	(McNamara et al., 2010a)
6–10 years, low income, marginally nourished (*n* = 598, analysis on *n* = 550).	(1) High micronutrients + 0.93 g ALA + 0.10 g DHA/day; (2) High micronutrients + 0.14 g/day ALA; (3) Low micronutrients + 0.93 g ALA + 0.10 g DHA/day; (4) Low micronutrients + 0.14 g/day ALA.	12 months.	Cognitive test battery.	No treatment effects.	(Muthayya et al., 2009)
6–10 years. Australia: well nourished, (*n* = 396, analysed *n* = 276) Indonesia: marginally nourished, (*n* = 384, analysed *n* = 367).	(1) High micronutrients; (2) DHA + EPA (0.09 g DHA + 0.02 g EPA)/day; (3) Micronutrients + DHA + EPA (as above); (4) Placebo.	12 months.	Cognitive test battery.	No treatment effects.	(Osendarp et al., 2007)
3–13 years, indigenous children with low literacy ability (n = 408).	Fish oil 0.75 g LC omega-3 per school day (0.56 g EPA + 0.17 g DHA) plus 0.06 g/day gamma linolenic acid vs. placebo (palm oil).	20 weeks.	WRAT4, DAP, MAP, CBRS.	Non-verbal cognitive development (Draw-A Person): Improvements with strongest effects in 7–12 year olds.	(Parletta et al., 2013)

8–12 years, mild-moderately malnourished (*n* = 59, analysis on *n* = 50).	Fish oil 0.45 g/day LC omega-3 (0.18 g EPA + 0.27 g DHA) *vs*. placebo (soybean oil).	3 months.	Anthropometric measures, neuropsychological battery test.	Improved processing speed, visual-perceptive capacity, attention, executive function.	(Portillo-Reyes et al., 2014)
7–9 years, underperforming in reading (≤33rd centile) (*n* = 362).	Algal oil: 0.6 g/day DHA *vs*. placebo (corn/soybean oil).	16 weeks.	Age-standardized measures of reading, working memory, and parent- and teacher-rated behaviour.	Treatment improved reading.	(Richardson et al., 2012)

Abbreviations: HVLT, Hopkins Verbal Learning Test; KBIT-2, Kaufman Brief Intelligence Test, Second Edition; WIAT-2, Wechsler Individual Achievement Test, Second Edition; WMTB-C, Working Memory Test Battery for Children; MFFT, Matching Familiar Figures Task; SNAP-IV, Swanson, Nolan, and Pelham rating scale for ADHD; SDQ, Strengths and Difficulties Questionnaire; WRAT-4, Wide Range Achievement Test: Fourth Edition; DAP, The Draw-A-Person; MAP, Matrix Analogies Test; CBRS, Comprehensive Behaviour Rating Scales.

1.6.4. Early-life and psychopathology

The Western diet has been associated with increased odds of hyperactivity, and attention/hyperactivity disorder (ADHD) (Howard et al., 2011). Deficits in n-3 PUFAs have been associated with higher risk of development of ADHD, dyslexia, dyspraxia, and autistic spectrum disorders (Richardson and Puri, 2000, Richardson and Ross, 2000, Richardson, 2004). Moreover, typical physical signs linked with ADHD, such as excessive thirst, frequent urination, rough, dull, or dry hair and skin, and soft or brittle nails are consistent with deficit in PUFAs (Colquhoun and Bunday, 1981, Stevens et al., 1995, Stevens et al., 1996, Bell et al., 2000, Richardson et al., 2000, Taylor et al., 2000a). In a cross-sectional study, Burgess et al observed that 40% of recruited ADHD subjects (53 ADHD 6–12 years old boys) had significantly lower proportions of plasma DHA, EPA, and AA and greater frequency of PUFAs deficiency syndrome such as thirst, frequent urination, and dry hair (Burgess et al., 2000). However, the remaining 60% of ADHD subjects did not show significantly lower proportions of fatty acids plasma levels compared to the controls. Other studies have related lower plasma levels of n-3 PUFA with ADHD in children (Mitchell et al., 1987, Stevens et al., 1995, Bekaroglu et al., 1996, Stevens et al., 1996, Burgess et al., 2000). Behavioural and learning problems, such as anomalous visual, motor, attentional, or language processing have been linked to lower plasma phospholipid concentrations of n-3 PUFA (Burgess et al., 2000, Richardson, 2004). While the cause or causes of ADHD are not obvious in most children, there is evidence that deficits in PUFA levels are related with ADHD, and in other cases the lack of adequate PUFAs may worsen the pathological condition. The reason for lower levels of PUFAs in a subpopulation of children exhibiting ADHD can be attributed to inadequate consumption of PUFAs, inefficient metabolic conversion of PUFAs as well as enhanced metabolism (Burgess et al., 2000). It has been reported that testosterone can impair the biosynthesis of n-3 PUFA whilst oestrogens are positively related with the conversion of

DHA from ALA both in rodents (Huang and Horrobin, 1987, Marra and de Alaniz, 1989) and in humans (Childs et al., 2008). This finding is consistent with the higher prevalence of ADHD in males than in females (Ramtekkar et al., 2010).

In animal studies the spontaneously hypertensive rat (SHR) is generally used as a model of ADHD with memory deficits, as described elsewhere (Meneses et al., 2011). Despite the relation between n-3 PUFAs deficiency and ADHD in children, there is a distinct paucity of information describing the effects of n-3 adequate diet in ADHD animal models. It has been shown that diets enriched in PUFAs (n-6/ n-3 PUFAs ratio of 1: 2.7) can partially ameliorate ADHD-like behaviour by improving reinforcer-controlled activity, impulsiveness and inattention in SHR (Dervola et al., 2012). Dervola et al have also highlighted an increased dopamine (DA) and serotonin (5-HT) turnover ratio together with decreased levels of glutamate in the striatum of the same animals (Dervola et al., 2012). Indeed, ADHD has been linked with deficits in the DA system, inducing modulation of the glutamatergic transmission and hyperactivity in mice (Gainetdinov et al., 2001) as well as dysfunction of the dopaminergic reward-processing circuitry in young subjects (Paloyelis et al., 2012). A recent study has shown reduction in locomotor activity in n-3 PUFAs-fed SHR compared to rats deficient in n-3 PUFAs (Hauser et al., 2014). However, an SHR control group with an adequate diet was not included.

In humans, Stevens and colleagues showed that supplementation of PUFAs in ADHD-children was able to improve oppositional defiant behaviour from a clinical to a nonclinical range (Stevens et al., 2003). In this study, both parents and teachers rated improvement in conduct problems and attentional difficulties after 4 months of treatment. However, more than half of the patients were on medication for ADHD. Richardson and Puri demonstrated that n-3 PUFAs benefit some but not most ADHD rating scales. Twelve weeks of supplementation of a mixture of PUFAs (providing 480 mg DHA, 186 mg EPA, 96 mg GLA,

42 mg AA, and 60 IU vitamin E as DL-a tocopherol per day) showed improvement in anxiety/shyness tests, cognition (CPRS-L), inattentiveness, hyperactivity/impulsiveness and Conners total global index (Richardson and Puri, 2002). However, none of the subjects were formally diagnosed with ADHD and the sample size was small. Although the studies reported above seem to be encouraging, three recent systematic reviews have reported only minor n-3 PUFA effects in reducing ADHD symptoms (Bloch and Qawasmi, 2011, Gillies et al., 2012, Grassmann et al., 2013). Grassman et al have reported mild behavioural and cognitive improvement in ADHD children after treatment with low doses of n-3 PUFAs (Grassmann et al., 2013). However, the studies included in their analysis were heterogeneous, had small sample sizes, and only a limited number were placebo controlled. Interestingly, Bloch and Qawasmi have reported modest effects in the treatment of ADHD after treatment with high doses of n-3 PUFAs (especially EPA) in comparison with current pharmacotherapies such as psychostimulants, atomoxetine, or α(2) agonists (Bloch and Qawasmi, 2011). Furthermore, Gillies et al reported improvement after combined n-3 PUFAs and n-6 PUFAs supplementation only in a minority of the studies that met the inclusion criteria of their review (Gillies et al., 2012). Given its evidence of modest efficacy, it may be reasonable to investigate n-3 PUFAs as a supplement to traditional pharmacologic interventions. Future studies need to be adequately powered, placebo controlled and use adequate dosage. A summary regarding the implication of n-3 PUFAs in mental illnesses in early-life & adolescence has been reported in table 1.8.

Table 1.8: n-3 PUFAs impact on mental illnesses in early-life & adolescence (adapted from Sinn et al., 2010).

Double-blind randomized placebo-controlled trials of n-3 PUFAs and mental illness in early-life & adolescence.					
Participants	**Treatment**	**Length of trial**	**Measurements**	**Outcomes**	**References**
N = 54; 6-12 year old (78% boys); idiopathic ADHD diagnosis; were being treated successfully with medication.	345 mg DHA (algae-derived) or undefined placebo.	16 weeks.	CPRS; CBC; TOVA; CCT.	Treatment = placebo on all measures.	(Voigt et al., 2001)
N = 50; 6-13 year old (78% boys); ADHD diagnosis; high FADS; some on medication (equally allocated to conditions).	96 mg GLA, 40 mg AA, 80 mg EPA, 480 mg DHA, 24 mg Vit E or olive oil placebo.	16 weeks.	DBD; ASQ; CPT; WJPEB-R; FADS.	Treatment > placebo: DBD-Conduct (parents); DBD-Attention (teachers). Other 14 outcome measures non-significant.	(Stevens et al., 2003)
N = 40; 6-12 year old (80% boys); ADHD diagnosis; 15% medicated; 82% comorbid conditions.	100 mg EPA, 514 mg DHA or olive oil placebo (supplied in soymilk & bread).	8 weeks.	DTVP; STM; CPT; Other.	Treatment = placebo on all measures (except that placebo > treatment on CPT and STM).	(Hirayama et al., 2004)
N = 29; 8-12 year old (62% boys); normal IQ; low reading ability; above average ADHD scores on Conners' Index; no participants in treatment for ADHD.	864 mg LA, 42 mg AA, 96 mg ALA, 186 mg EPA, 480 mg DHA, 60 iμ Vit E or olive oil placebo.	12 weeks.	CPRS.	Treatment > placebo: CPRS; Cognitive problems/inattention; Anxious/shy; Conners' global index; DSM inattention; DSM hyperactive/impulsive; Conners' ADHD Index.	(Richardson and Puri, 2002)
N = 117; 5-12 year old (77%	60 mg AA, 10 mg GLA, 558	12 weeks	MABC; WORD;	Treatment > placebo:	(Richardson and

boys); Developmental Coordination Disorder, 1/3 with ADHD symptoms in clinical range, not in treatment; IQ > 70.	mg EPA, 174 mg DHA, 9.6 mg Vit E or olive oil placebo.	active vs. placebo; one-way crossover to active treatment for 12 weeks.	CTRS.	WORD; CTRS Oppositional behaviour; Cognitive problems/inattention; Hyperactivity; Anxious/shy; Perfectionism; Social problems; Conners' index; DSM-IV inattention, hyperactive/impulsive.	Montgomery, 2005)
N = 132 (questionnaire data available for 104); 7-12 year old (74% boys); ADHD symptoms in clinical range; unmedicated.	60 mg AA, 10 mg GLA, 558 mg EPA, 174 mg DHA, 9.6 mg Vit E, or palm oil placebo.	15 weeks active vs. placebo; one-way crossover to active treatment for 15 weeks.	CPRS, CTRS Vocabulary, subtests from WISC-III & TEA-ch, Stroop.	Treatment > placebo CPRS: Cognitive problems/inattention; Hyperactivity; ADHD Index; Restless/Impulsive; DSM-IV Hyperactive/Impulsive; Oppositional. Treatment = placebo on other subscales and CTRS. Treatment > placebo on Creature Counting & vocabulary. Treatment = placebo on other cognitive tests.	(Sinn and Bryan, 2007, Sinn et al., 2008)
N = 75, 8-18 year old children with diagnosed ADHD, unmedicated (85% males).	60 mg AA, 10 mg GLA, 558 mg EPA, 174 mg DHA, 9.6 mg Vit E or olive oil placebo.	3 months active vs. placebo; one-way crossover	Investigator-rated ADHD Rating Scale-IV; CGI.	Treatment = placebo overall Treatment > placebo in subgroups with inattentive subtype & comorbid neurodevelopmental	(Johnson et al., 2009)

		to active treatment for 3 months.		disorders.	
N = 54 (45 with bloods), 7-12 year old (79% male) with ADHD/ADHD symptoms (50% diagnosed).	1g EPA-rich oil, 1g DHA rich oil or sunflower oil placebo.	3 x 3 crossover (4 months on each treatment).	CPRS, reading, writing, vocabulary, TEA-ch.	Treatment = placebo in 12-month crossover. Over 4 months erythrocyte DHA increases associated with improvements on CPRS - oppositional behaviour, anxiety/shyness – divided attention & reading. In subgroup with learning difficulties (n = 16 with blood) also on CPRS hyperactivity/impulsivity and spelling.	(Milte et al., 2011)
N = 13 (5-17 years) with Autistic Disorder (81.9% male).	1.5g/d n-3 PUFA (0.84g EPA, 0.7g DHA), Vit E ; or coconut oil placebo.	6 weeks parallel design.	Aberrant Behavior Checklist.	Treatment > placebo for stereotypy & hyperactivity (trends with large effect sizes). Treatment = placebo on 3 other subscales.	(Amminger et al., 2007)
N = 20 (6-12 year old; 25% girls); children with major depressive disorder.	2g ethyl-EPA (96% from fish oil) or placebo, Vit E.	4 weeks parallel design, adjunctive therapy.	HDRS.	Treatment > placebo at weeks 2, 3 and 4 on HDRS score and on core depressive symptom subscales.	(Nemets et al., 2006)

N = 81 individuals (27 males, 54 females; mean -age=16.4) with ultra-high risk (UHR) for psychosis.	~1.2 g ω-3 PUFAs (0.7g EPA, 0.48g DHA, 7.6 mg vitamin E).	12-weeks.	Gaussian Process Classification.	Treatment > placebo on GPC	(Sinn et al., 2010)
N = 80 boys aged 8-14 (41 ADHD, 39 controls).	10 g of margarine daily, enriched with either 650 mg of EPA/DHA or placebo.	16 weeks.	CBCL, SWAN, TRF, fMRI.	Treatment > parent-rated attention in both ADHD control.	(Bos et al., 2015)

Abbreviations*:* ASQ, Conners' Abbreviated Symptom Questionnaires; CBC, Child Behaviour Checklist; CCT, Children's Colour Trails test; CGI-S, Clinical Global Impression-Severity; CPRS, Conners' Parent Rating Scales; CPT, Conners' Continuous Performance Test; CTRS, Conners' Teacher Rating Scales; DBD, Disruptive Behaviour Disorders rating scale; FADS, fatty acid deficiency symptoms; HDRS, Hamilton Depression Rating Scale; MABC, Movement Assessment Battery for Children; Stroop, Stroop color-word test; STM, Short-term memory; TEA-ch, Test of Everyday Attention for children; TOVA, Test of Variables of Attention; WISC-III, Wechsler Intelligence Scale for Children, version 3; WORD, Wechsler Objective Reading Dimensions; WJPEB-R, Woodstock-Johnston Psycho-Educational Battery – Revised.

1.7. n-3 PUFAs in adulthood

1.7.1. n-3 PUFAs: mechanisms and animal studies

In adulthood, full brain development is already achieved. However, CNS functionality can be negatively affected by experience of stress (Lupien et al., 2009). At this stage of life, prolonged stressful events can impair hippocampal neurogenesis, induce cortical neuronal atrophy and alter the monoaminergic system (McEwen, 2001, Lupien et al., 2007, Ahmad et al., 2010, Mondelli et al., 2011). Such impairments are associated with altered cognitive behaviour as well as increased susceptibility to stress-related pathologies, such as anxiety and depression (Baumeister et al., 2014a, Cattaneo et al., 2015). In light of these CNS alterations it is interesting to investigate whether n-3 PUFAs exert any beneficial role in face of stress and stress related-pathologies. Studies conducted in rodents fed with diets deficient in n-3 PUFAs have shown impairment in hippocampal functions (Aid et al., 2003) as well as altered monoaminergic neurotransmission (Chalon, 2006). Moreover, Rao et al have shown lower cortical BDNF protein levels proportional to deprivation of n-3 PUFAs in rats which underwent n-3 PUFAs deficient diet (Rao et al., 2007b). These impairments have been observed in conjunction with increased anxiety- and depressive-like behaviour (DeMar et al., 2006, Larrieu et al., 2012, Larrieu et al., 2014a). Indeed, DHA supplementation completely reverses the anxiety-like behaviour induced by an n-3 PUFA-deficient diet and attenuates the freezing behaviour in conditioned-fear stress responses (Takeuchi et al., 2003). Furthermore, animals supplemented with n-3 PUFAs are more resilient to anxiety and depressive-like behaviour than neurodevelopmentally normal animals (Park et al., 2012, Mizunoya et al., 2013). Interestingly, it has been proposed that n-3 PUFAs deficiency may induce a chronic stress state by disruption of a GR-mediated signalling pathway along with HPA axis hyperactivity (Larrieu et al., 2014a). Controversially, the same authors observed that supplementation of n-3 PUFAs prevented detrimental chronic social defeat stress-induced

emotional and neuronal impairments by impeding HPA axis dysfunction (Larrieu et al., 2014a). Similarly, n-3 PUFAs supplementation decreased stressed plasma corticosterone levels and improved animal performance in the elevated plus maze and forced swim test, as well as in the Morris water maze in rats that underwent restraint stress (Ferraz et al., 2011). Interestingly, EPA supplementation abrogates IL-1β-induced corticosterone secretion in rats. This anti-inflammatory effect exerted by EPA ameliorated anxiety-like behaviour induced by IL-1β treatment (Song et al., 2004). Of note, psychopathologies, such as depression and bipolar disorder, seem to be involved with altered inflammatory response (Baumeister et al., 2014b, Cattaneo et al., 2015). N-3 PUFAs decrease inflammation initiated through the NFκB cascade via actions at the peroxisome proliferator-activated receptor γ (PPARγ) and modulation of toll-like receptors (Lee et al., 2003, Pascual et al., 2005). Another DHA metabolite, neuroprotectin D1, decreases the production of tumor necrosis factor-α (TNF-α) and interferon-γ (IF-γ) by activated T cells (Ariel et al., 2005, Bazan, 2005). Moreover, in cultured BV-2 microglia, both DHA and EPA increased expression of heme oxygenase-1, and decreased expression of TNF-α, interleukin-6 (IL-6), nitric oxide synthase, and cyclo-oxygenase 2 (COX-2) (Lu et al., 2010). Finally, rats that consumed inadequate n-3 PUFAs from birth had higher plasma levels of IL-6, C-reactive protein, and TNF-α, which was reversed by subsequently feeding an ALA -containing diet (McNamara et al., 2010b).

Other mechanisms showing the potential benefits of n-3 PUFAs on stress-related pathologies have been proposed. For instance, DHA-regulation of BDNF protein levels has attracted the attention of several researchers. This is of importance since stress-related pathologies such as depression are strongly associated with decreased levels of BDNF (Schmidt et al., 2011). Of note, lower levels of hippocampal BDNF were observed in postmortem samples from suicide completers than in normal controls (Dwivedi et al., 2003, Karege et al., 2005). Moreover, hippocampal neurogenesis is supported by BDNF (Duman and Monteggia, 2006); decreased

expression levels of BDNF appear to contribute to neuronal atrophy, as has been found postmortem in the hippocampus of people with depression (Sheline et al., 1996). As mentioned previously, decreased BDNF mRNA and protein levels are also found in rats fed a diet containing inadequate n-3 PUFAs, suggesting a direct mechanism of regulation of BDNF that is n-3 PUFAs dependent (Rao et al., 2007b). Indeed, DHA supplementation to cortical astrocytes cells increases CREB and BDNF protein levels via a p38 MAPK dependent mechanism *in vitro* (Rao et al., 2007b). Moreover, diet supplemented with DHA enhances synaptic plasticity, memory and learning through an increase of calcium–calmodulin protein kinase II (CaMKII) levels, CREB and BDNF proteins in the hippocampus of rats (Wu et al., 2008). In addition, the volume of the hippocampus is increased in mice fed an α-linoleic acid-enriched diet (Venna et al., 2009). Likewise, increased hippocampal neurogenesis has been observed in rats fed diets supplemented with DHA and EPA, as well as in mutant mice expressing fat-1 that are able to metabolize n-6 PUFA into n-3 PUFA, and thus do not require n-3 PUFAs in the diet (He et al., 2009, Dyall et al., 2010). Taken together, these findings suggest that higher levels of tissue and dietary n-3 PUFAs support BDNF expression in the hippocampus, which in turn fosters hippocampal neurogenesis and supports neurotrophic and neuroprotective properties (Wu et al., 2004, Blondeau et al., 2009, Venna et al., 2009, Cysneiros et al., 2010, Vines et al., 2012).

Other similarities have been found between monoaminergic dysfunction in stress-related pathologies and lower dietary levels of n-3 PUFAs. Decreased activity of the monoamine neurotransmitters serotonin and norepinephrine has been hypothesized to contribute to the pathophysiology of psychiatric diseases, with dopamine also playing a minor role. Important changes in the serotonergic system observed in depression include lower brainstem concentrations of serotonin of post-mortem depressives and suicide completers (Shaw et al., 1967, Lloyd et al., 1974, Beskow et al., 1976). The densities of 5-HT_{1A} and 5-HT_{2A} serotonin

receptors were higher in the prefrontal cortex as well, suggesting decreased serotonergic neurotransmission (Arango et al., 1990, Yates et al., 1990). The increased availability of serotonin produced by antidepressant drugs causes these receptors to down regulate, further suggesting a role of serotonin in both the disease process and the mechanisms of action of antidepressants (Baldessarini, 2001). In animal studies, lower serotonin concentrations in the frontal cortex were detected in adult female rats fed with an n-3 deficient diet. This was related to reduction in brain DHA content of about 25%, similar to that observed in depression (McNamara et al., 2007, Levant et al., 2008). Male rats with a 61% decrease in brain DHA, induced by feeding them a diet deficient in n-3 PUFA from birth, exhibited lower expression of the serotonin synthesizing enzyme tryptophan hydroxylase in the midbrain, and higher serotonin turnover in the prefrontal cortex, compared to controls (McNamara et al., 2009, McNamara et al., 2010b). In contrast, a diet containing ALA reversed the effect on serotonin turnover (McNamara et al., 2010b). Consistent with this, adult rats supplemented with DHA and EPA exhibited increased concentrations of serotonin in the frontal cortex and hippocampus (Vines et al., 2012). Similarly, in mice, the decreases in brain serotonin levels induced by unpredictable chronic mild stress, were reversed by n-3 PUFAs diet supplement (Vancassel et al., 2008).

The role of n-3 PUFAs in modulation of noradrenergic neurotransmission has received relatively little attention. Studies in cultured SH-SY5Y neuroblastoma cells suggest that either brief exposure to, or incorporation of, DHA increased basal, but not KCl-evoked release of [3H]- norepinephrine by a mechanism involving enhanced exocytosis (Mathieu et al., 2010). DHA treatment also increased the density of β-receptors on rat astrocytes in primary culture (Joardar et al., 2006). In animal studies, decreased levels of norepinephrine were observed in the cortex, hippocampus, and striatum of rats raised from conception on a diet containing inadequate n-3 PUFA (Takeuchi et al., 2002). The dopamine system is also

affected by variation in dietary n-3 PUFA content. Virgin females with lower tissue DHA levels had altered abundancy of D1 and D2 dopamine receptors in the caudate nucleus compared to virgin females with normal DHA (Davis et al., 2010). These receptor alterations are similar to those found in several rodent models of depression, and are consistent with the proposed hypodopaminergic basis for anhedonia and motivational deficits in depression (Papp et al., 1994, Kram et al., 2002, Bjornebekk et al., 2007). Reduction of dietary n-3 PUFA intake also led to increased basal dopamine release, decreased vesicular monoamine amine transporter VMAT2 density, and lower levels of tyramine-stimulated dopamine release in rats (Zimmer et al., 2000). The behavioural outcomes related to the n-3 PUFAs mechanisms of action described above have been reported in table 1.9.

Table 1.9: Effect of n-3 PUFAs on adult rodents' behaviour.

Effect of n-3 PUFAs in adult rodents behaviour					
Animals	**Treatment**	**Length of treatment**	**Measurements**	**Outcomes**	**References**
C57Bl6/J mice 2nd generation.	(1) control; (2) n-3 def diet; (3) n-3 suppl diet.	3 months old.	Chronic social defeat test, open field, forced swim test.	(3) ameliorated chronic social defeat stress-induced emotional and neuronal impairments by impeding HPA axis hyperactivity.	(Larrieu et al., 2014b)
C57BL6/J female mice.	(1) n-3 def diet; (2) n-3 suppl diet.	After weaning, both groups were fed with a control diet.	Social investigation, forced swim test, open field.	Anxiety-like behaviour induced by (1) was abolished by the cannabinoid agonist WIN55,212-2.	(Larrieu et al., 2012)
C57Bl/6 mice.	(1) control; (2) n-3 def diet + (1) after weaning.	Until 14 weeks old.	Open-field, object recognition, light-dark transition, elevated plus maze, social interaction tests.	(2) reduced anxiety-like behaviour compared to (1).	(Palsdottir et al., 2012)
Wistar rats 2nd generation.	(1) n-3 adeq diet; (2) n-3 def diet.	Until 60 days old.	Inhibitor avoidance task, flinch-jump task, open-field, elevated plus maze.	(1) improved inhibitor avoidance task and elevated plus maze performances compared to (2).	(Moreira et al., 2010)

Long Evans rats.	3 generations (F): (1) n-3 adeq diet; (2) n-3 def diet; (3) n-3 def till birth of 3F; (4) n-3 def till weaning of 3F; (5) n-3 def till 7 weeks of 3F.	Till 9 or 13 weeks of age.	MWM, motor activity.	(3), (4) similar MWM outcomes and DHA brain levels to (1).	(Moriguchi and Salem, 2003)
Wistar rats, 2nd generation.	(1) Control; (2) n-3 def diet; (3) same as (2) + DHA/AA at weaning.	After lactation all groups received (1).	Passive-avoidance test.	(3) reversed learning impairments observed in (2).	(Garcia-Calatayud et al., 2005)
Wistar Imamichi rats, 2nd generation.	(1) Control; (2) n-3 def diet; (3) same as (2) + DHA (300mg/kg/day).	DHA was administrated 1 week prior behavioural test.	Elevated plus maze, fear conditioning.	(3) reversed behavioural impairments observed in (2).	(Takeuchi et al., 2003)
Wistar rats, 2nd generation.	(1) Control; (2) fish oil.	2 months.	Elevated plus maze, ambulatory activity test.	(2) improved animal behaviours.	(Chalon et al., 1998)
Long Evans rats.	(1) def diet; (2) adeq diet.	15 weeks.	Forced swim test, resident intruder test, open field.	(2) Improved forced swim and resident intruder test.	(DeMar et al., 2006)
Long Evans rats.	(1) Control; (2) artificial rearing: n-3 def diet; (3) artificial rearing: n-3 adeq diet.	Till 9 weeks.	Motor activity, elevated plus-maze, Morris water maze.	(3) Improved spatial learning compared to (2).	(Lim et al., 2005)

Long Evans rats.	(1) Control; (2) n-3 def diet; (3) same as (2) + DHA enriched diet after weaning.	56 days.	Locomotor activity, thermal stimulus.	(3) reversed behavioural impairment compared to (2).	(Levant et al., 2004)
Long Evans rats, 2nd & 3rd generation.	(1) n-3 def diet; (2) n-3 adeq diet.	8 weeks.	Motor activity, elevated plus maze, Morris water maze.	(2) reversed behavioural impairment compared to (1).	(Moriguchi et al., 2000)
Donryu rats, 2nd generation.	(1) Control; (2) n-3 def diet; (3) same as (2) + DHA after weaning.	7 wks.	Brightness-discrimination learning test.	(3) reversed behavioural impairment compared to (2).	(Ikemoto et al., 2001)

1.7.2. Stress & cognition in adult healthy humans

N-3 PUFAs' benefits find their validation in clinical studies, as summarized in table 1.10, albeit there is a paucity of information. Delarue et al, for example, evaluated the effect of a diet enriched in n-3 PUFAs in seven healthy subjects that underwent 30 minutes of mental arithmetic and Stroop test (Delarue et al., 2003). Three weeks of 7.2g/ day of fish oil administration was able to reduce plasma epinephrine, plasma cortisol and energy expenditure elicited by thirty minutes of mental stress. The data support the hypothesis that fish oil supplementation prevents the adrenal activation elicited by mental stressors. However, an effect of acclimatization due to the repetition of the testing procedure over time may have interfered with the final results. However, four weeks of fish oil supplementation has been shown to protect from an exaggerated neuroendocrine response elicited by LPS (2 ng/kg) induced cytokine release (Michaeli et al., 2007). Indeed, both ACTH and cortisol plasma levels were blunted by 7g/ day of fish oil but not the high levels of cytokines induced by LPS. This is in contrast with the n-3 PUFAs anti-inflammatory effect (Grimm et al., 2002), perhaps due to the content of EPA (17%) and DHA (11%) in the fish oil supplement being too low. Accordingly, another study recruiting healthy medical students receiving 2496g/ day of n-3 PUFA (7:1 EPA/DHA ratio) supplementation showed reduction of stimulated IL-6 and TNF-α plasma levels. Moreover, the administration of n-3 PUFA reduced anxiety symptoms in healthy students without an anxiety disorder diagnosis (Kiecolt-Glaser et al., 2011). Albeit data are promising, further studies are required to better investigate the role of n-3 PUFAs in the regulation of the stress response. This evidence requires more interest since stress can exert a pivotal role in memory and cognition (McEwen, 2007). Indeed overall few studies exploring the role of n-3 PUFAs in cognition in healthy subject have been carried out. Researchers at the University of Pittsburgh have determined that n-3 PUFAs can enhance cognition in young individuals. Healthy young

adults (18-25 years of age) experienced an improvement in working memory after six months of n-3 PUFAs supplement, mostly DHA (750 mg/d) and EPA (930 mg/d) (Narendran et al., 2012). Working memory assessment was performed using a verbal n-back task that used three loads of working memory (1-back, 2-back and 3-back) (Abi-Dargham et al., 2002). In addition, prior to supplementation of n-3 PUFAs, higher red blood cells DHA levels were significantly positively correlated with working memory test performance in a group of young adults. Although further investigation in larger samples is needed to understand the relationship, this study is consistent with some but not all clinical trials that have evaluated the pro-cognitive effects of n–3 PUFA in humans. Muldoon et al previously showed that high plasma levels of DHA are associated with cognitive function during middle age (Muldoon et al., 2010). Two hundred and eighty healthy subjects (30–54 year of age) not taking fish oil supplements were exposed to a neuropsychological test battery. DHA was related with better scores on tests of nonverbal reasoning, mental flexibility, working memory, and vocabulary but no other n-3 PUFAs. Interestingly, another study conducted in Australia observed a fish oil adaptogenic role in stressed university staff (Bradbury et al., 2004). Six weeks of 6g/ day fish oil intake (1.5g DHA 360g EPA) were enough to ameliorate the Perceived Stress Scale (PSS) scoring. However, the study was able to find further improvements in comparison with the untreated control but not the placebo group (olive oil). Perhaps, 6g of olive oil intake might have had a protective effect against perceived stress. Indeed, half of the placebo recruiters reported stress reduction and believed themselves to be taking the active treatment. Moreover, n-3 PUFAs affect cardiovascular reactivity to mental stress, blunting calf vascular conductance (CVC), attenuating heart rate (HR), and muscle sympathetic nerve activity (MSNA) (Carter et al., 2013). Due to a lack of investigation, further research is warranted to elucidate the mechanisms by which n–3 PUFAs enhance cognitive performance in healthy individuals.

Table 1.10: n-3 PUFAs impact in adult healthy humans (adapted from Stonehouse, 2014).

Double-blind randomized placebo-controlled trials of n-3 PUFAs in healthy adults.					
Participants	**Treatment**	**Length of trial**	**Measurements**	**Outcomes**	**References**
University students, mean age ~22 years (*n* = 56, analysed *n* = 54).	2.3 g/day fish oil (1.74 g EPA + 0.25 g DHA) *vs*. placebo (olive oil).	4 weeks.	Mini International Neuropsychiatric Interview, Neutral and emotional information processing tests.	No effects on attention, memory or reaction time of attention.	(Antypa et al., 2009)
22–51 years (*n* = 33).	2.8 g/day fish oil (1.6 g EPA + 0.8 g DHA).	35 days.	Zimmermann & Fimm Attention Test procedure, EEG.	Improvements in sustained attention and reaction time of sustained attention.	(Fontani et al., 2005)
18–35 years (*n* = 159, analysed *n* = 140).	(1) DHA-rich fish oil (0.45 g DHA + 0.09 g EPA)/day; (2) EPA-rich fish oil (0.2 g DHA + 0.3 g EPA)/day; (3) Placebo (olive oil).	12 weeks.	Cognitive performance and mood battery test.	No treatments effects.	(Jackson et al., 2012a)
18–29 years (*n* = 65).	(1) Low dose DHA fish oil (0.45 g DHA + 0.09 g EPA)/day; (2) High dose DHA fish oil (0.9 g DHA + 0.18 g EPA)/day; (3) placebo (olive oil).	12 weeks.	COMPASS, spatial working memory, Numeric working memory, 3-Back task, Simple reaction time, Choice reaction time, Stroop task, RVIP.	Increased cerebral blood flow Cognitive tasks only assessed at end of study using comprehensive computerized cognitive test battery (episodic memory, working memory, attention, reaction time, executive function). Both dosages improved reaction	(Jackson et al., 2012b)

				times on two attention tasks, but effects were lost when correcting for multiple testing.	
College students (mean age ~20 ± 2 years) (*n* = 43, analysed *n* = 41).	Fish oil (0.72 g EPA + 0.48 g DHA)/day *vs*. placebo (coconut oil).	4 weeks.	RAVLT, SCWT, TMT, PANAS.	No effects on verbal learning and memory, inhibition and executive control.	(Karr et al., 2012)
Mildly depressed adults, 18–70 years (average ± SD age 38 ± 14 years) (*n* = 218, analysed *n* = 190).	Fish oil 1.5 g/day LC omega-3 (0.85 g DHA + 0.63 g EPA) *vs*. placebo (olive oil).	12 weeks.	DASS, BDI, GHQ, STAXI-2.	No treatment effects.	(Rogers et al., 2008)
18–45 years (*n* = 228, analysed *n* = 176).	Fish oil (1.2 g DHA + 0.17 g EPA)/day *vs*. placebo (high oleic acid sunflower oil).	6 months.	Computerized cognitive test battery (episodic and working memory, attention, reaction time (RT) of episodic and working memory, and attention and processing speed).	Improvement in reaction times and working memory. RBC DHA increased by 2.6% (to ~7.9%); RBC EPA increased by 0.2% (to ~0.81%).	(Stonehouse et al., 2013)

Abbreviations: BDI, Beck Depression Inventory; DAS, Differential Ability Scales; GHQ, General Health Questionnaire; PANAS, Positive and Negative Affect Schedule; RAVLT, Rey Auditory Verbal Learning Test; RVIP, Rapid Visual Information Processing; SCWT, Stroop Color Word Test; STAXI-2, State-Trait Anger Expression Inventory-2; TMT, treadmill test.

1.7.3. The role of n-3 PUFAs in the treatment of Major Depressive Disorder

Since the last century the incidence of major depressive disorder (MDD) in western countries has increased. In contrast, the dietary intake of n-3 PUFAs has dramatically declined in favour of n-6 PUFAs intake (Molendi-Coste et al., 2011). However, the role of n-3 PUFAs in MDD is unclear, and deserves further investigation. Several studies suggest that frequent consumption of n-3 PUFAs may decrease the incidence of depression and suicidal ideation in the general population (Tanskanen et al., 2001, Silvers and Scott, 2002, Logan, 2003). Joseph Hibbeln has been one of the first investigators to draw attention to the importance of n-3 PUFAs in psychiatric disorders. In 1998, Hibbeln showed a cross-national significant negative correlation between worldwide fish consumption and prevalence of MDD (Hibbeln, 1998). Moreover, higher n-6/n-3 ratios, such as AA/EPA ratio, have been detected in blood samples (Adams et al., 1996, Peet et al., 1998, Maes et al., 1999, Tiemeier et al., 2003, Lin et al., 2010) and red blood cell (RBC) phospholipids (Adams et al., 1996, Logan, 2003) of depressed patients. Accordingly, n-3 PUFAs concentrations in the blood reflect an accurate, but not identical, representation of n-3 PUFAs levels in the brain (Horrobin, 2001). Lower DHA levels have also been found in the post-mortem orbitofrontal cortex (OFC) of MDD patients (McNamara et al., 2007). However, these findings do not show that fish consumption can cause differences in the prevalence of MDD or that eating fish or fish oils are useful in treatment. Other studies did not find any correlation between n-3 PUFA levels in the amygdala (Hamazaki et al., 2012) and entorhinal cortices (Hamazaki et al., 2013) of MDD subjects. One double-blind, placebo-controlled study investigated the effect of EPA as adjunct to antidepressant therapy in a group of twenty MDD diagnosed patients. Although this was a small study (17 women, 3 men), the addition of 2g of EPA to standard antidepressant medication showed highly significant benefits by week three of treatment. Primarily, EPA showed effects on insomnia, depressed mood, and feelings of guilt and

worthlessness (Nemets et al., 2002). In 2002, Peet and Horrobin observed that a specific EPA dosage (1g/ day) was effective in ameliorating depressive symptoms in subjects with persistent depression despite ongoing treatment with antidepressant. In this twelve-week, randomized, double-blind, placebo-controlled trial, 53% of the subjects who received EPA (17 subjects), achieved a 50% reduction on the Hamilton Depression Rating Scale score. In addition, the EPA had a broad-spectrum positive effect leading to improvements in anxiety, sleep, lassitude, libido and suicidal ideation (Peet and Horrobin, 2002). Although clinical outcomes on the effect of EPA on major depression seem to be promising, trials using DHA are inconclusive. Thirty-six subjects with major depression assigned to receive DHA (2g/ day) for 6 weeks did not show differences in the score of the Montgomery-Asberg Depression Rating Scale compared to the placebo treated group (Marangell et al., 2003). A recent meta-analysis focused on the hypothesis that EPA represents the key compound of the n-3 PUFA family with regards to effects in the treatment of major depression. Fifteen trials (916 total participants) using n-3 PUFAs as either a mono- or adjunctive-therapy were analysed. Studies were selected based on prospective, randomized, double-blinded, placebo-controlled study design, if depressive episode was the primary complaint with or without comorbid medical conditions and, if appropriate outcome measures were used to assess depressed mood. This meta-analysis concluded that n-3 PUFAs supplements with more than 60% of EPA (in a dose range of 200 to 2200 mg/ day in excess of DHA) ameliorated the clinical condition. However, doses containing primarily DHA, or less than 60% EPA, were not effective against primary depression. Moreover, trial duration (4 to 16 weeks) was not a predictor of outcomes, suggesting that EPA improvements may not be limited to the initial treatment period (Sublette et al., 2011). Although it is not yet possible to recommend n-3 PUFAs as either a mono- or adjunctive-therapy in major depression, the current research is strong enough to justify further studies.

1.7.4. The role of n-3 PUFAs in the treatment of Bipolar disorder

To date the link between n-3 PUFAs levels and bipolar disorder is poorly understood. In summary, n-3 PUFAs can have a slight beneficial effect on depressive symptoms when added to an existing psychopharmacological maintenance treatment for bipolar disorder. However, there is no clear evidence that this combination has a beneficial effect on manic symptoms. Two controlled studies have improved bipolar symptoms with a large dose combination of EPA and DHA over four months (Stoll et al., 1999) and 1-2g/day ethyl-EPA over 12 weeks (Frangou et al., 2006). However, current data on the efficacy of DHA and EPA in the treatment of bipolar disorder are insufficient for us to draw definite conclusions that can guide clinical practice. Further investigations are required into the effects of n-3 PUFAs on the various phases of bipolar disorder.

Overall, well-designed and executed randomised controlled trials in this field are clearly lacking and the need for such high-quality primary research is acute. Study duration should be long enough to ensure that the n-3 PUFAs can be fully absorbed into brain cell membranes, and therefore should ideally be three months at a minimum. Finally, the dose and composition of the n-3 PUFAs supplement should be modelled on current evidence, which suggests that 1-2 g/day of EPA or majority EPA supplement may be the most effective form of the treatment, although further research into the efficacy of varied compositions and doses of n-3 PUFAs treatment is also necessary. A summary of the implication of n-3 PUFAs in psychopathology has been reported in table 1.11.

Table 1.11: n-3 PUFAs' impact on mental illnesses in adulthood (adapted from Sinn et al., 2010).

Double-blind randomized placebo-controlled trials of n-3 PUFAs and mental illness in adulthood.					
Participants	**Treatment**	**Length of trial**	**Measurements**	**Outcomes**	**References**
N = 70 (18-70 years), depressed (>15 on HDRS), medicated.	Ethyl-EPA – 1, 2 or 4 g/day or placebo.	12 weeks parallel design, adjunctive therapy.	HDRS, MADRS, BDI.	Treatment > placebo on all 3 rating scales with 1g/day EPA – strong effects for core depressive symptoms. Treatment = placebo on 2g and 4g/day (non-significant trends).	(Peet and Horrobin, 2002)
N = 20 (28-73 years), diagnosed major depression disorder (85% women) HDRS score > 18.	2g ethyl-EPA (96% from fish oil) or placebo, Vit E.	4 weeks parallel design, adjunctive therapy.	HDRS.	Treatment > placebo at weeks 2, 3 and 4 on HDRS score and on core depressive symptom subscales.	(Nemets et al., 2002)
N = 22 (18-60 years), outpatients with major depressive disorder; HDRS score > 18, medicated.	3.3g/day n-3 PUFA (2.2g DHA, 1.1g EPA).	8 weeks parallel design, adjunctive therapy.	HDRS.	Treatment > placebo on HDRS.	(Su et al., 2003)
N = 35 (18-65 years), major depressive disorder diagnosis; HDRS score > 16 (80% female).	2g/day DHA or placebo.	6 weeks parallel.	MADRS, HDRS, GAFS.	Treatment = placebo on outcome measures.	(Marangell et al., 2003)
N =77 (18-65 yrs recruited, mean age 38), being treated	3g/day n-3 PUFA (2.4g DHA; 0.6g EPA) + Vit E or	12 weeks parallel,	HDRS short form, BDI.	Treatment = placebo on outcome measures	(Silvers et al., 2005)

for current depressive episode (53% female).	olive oil placebo.	adjunctive therapy.		(improvements in both groups at week 2).	
N = 83 (18-72 years, M = 45), outpatients with major depression diagnosis.	3g/day n-3 PUFA (2.2g DHA, 0.6g EPA) + Vit E or olive oil placebo.	4 month parallel design, adjunctive therapy.	HDRS, BDI, GAFS.	Treatment = placebo on outcome measures (improvements in both groups).	(Grenyer et al., 2007)
N = 24 (18-40 years); with major depressive disorder during pregnancy.	2.2g EPA + 1.2g DHA or placebo, both with tocopherols & orange flavour.	8 weeks, parallel design.	HDRS, EPDS, GDI.	Treatment > placebo on outcome measures.	(Su et al., 2008)
N = 190 (18-70 years recruited, mean age = 38); people from GP surgeries or public with mild-moderate depression (77% female).	630mg EPA, 850mg DHA, 870mg olive oil, or olive oil placebo (both with tocopherols & orange oil).	12 weeks parallel design.	DASS, BDI, STAEI, mood using diary and visual probe task, cognitive function.	Treatment = placebo on outcome measures (improvements in both groups).	(Rogers et al., 2008)
N = 120 (recruited 40-55 yrs; mean age 49) post-menopausal women with psychological distress & depressive symptoms.	1.5g ethyl-EPA, 0.5g ethyl-DHA.	8 weeks parallel design.	PGWB, HSCL-D-20, HDRS.	Treatment = placebo on all measures (improvements in both groups). Treatment > placebo in women without MDE (major depressive episode diagnosis).	(Lucas et al., 2009)
N = 122; major depression + coronary heart disease.	930mg ethyl-EPA + 750mg ethyl DHA/day or corn oil placebo.	10 weeks parallel design, adjunctive therapy.	BDI-II, HDRS.	Treatment = placebo on outcome measures (improvements in both groups).	(Carney et al., 2009)

N = 30 (18-65 years); inpatients with bipolar disorder.	9.6g/day n-3 PUFA (6.2g EPA, 3.4g DHA) or olive oil esther placebo.	4 month parallel design; adjunctive therapy.	HDRS, YMRS, CGI-S, GAS.	Treatment > placebo on GAS, HDRS and CGI; Treatment = placebo on YMRS.	(Stoll et al., 1999)
N = 116 (n= 57 bipolar depressed; n = 59 rapid cycling), mean age: 45; 51% male.	6g/day ethyl-EPA or liquid paraffin placebo.	4 month parallel design; adjunctive therapy.	IDS, YMRS, CGI-BP (bipolar disorder).	Treatment = placebo on outcome measures.	(Keck et al., 2006)
N = 75 (mean age: 47); outpatients with bipolar depression + scores > 17 on HDRS (76% female).	1g/day ethyl EPA (n = 24) ; 2g/day ethyl EPA (n = 25) or paraffin placebo.	12 week parallel design, adjunctive therapy.	HDRS, YMRS, CGI.	Treatment > placebo on HDRS & CGI on 1g and 2g/day. Treatment = placebo on YMRS.	(Frangou et al., 2007)
N = 49 (16-64 years, M = 30); presenting after act of repeated self-harm (65% women).	1.2g/day EPA + 0.9g DHA or corn oil placebo (with 1% EPA/DHA).	12 weeks parallel design in addition to standard care.	BDI, HDRS, OAS-M, IMT/DMT, PSS, DHUS.	Treatment > placebo on BDI, HDRS, PSS, DHUS. Treatment = placebo on OAS-M & IMT/DMT (hostility/aggression, memory).	(Hallahan et al., 2007)
Study 1: N = 45 (mean age: 44 yrs); schizophrenic patients, PANSS score > 40. Study 2: N = 30 (mean age 35 years); diagnosed schizophrenia, untreated.	2g/day EPA or corn oil placebo.	3 months parallel, single therapy unless drugs needed.	PANSS; need for antipsychotic medication.	Treatment > placebo, particularly on positive subscale; 12/12 placebo and 8/14 EPA patients took medication.	(Peet et al., 2001)
N = 87 (18-65 years, M = 40;	3g/day ethyl EPA + Vit E or	16 weeks	PANSS, CGI,	Treatment = placebo on	(Fenton et al., 2001)

61% male) diagnosed schizophrenia or schizoaffective disorder.	mineral oil + Vit E placebo.	parallel design, adjunctive therapy.	MADRS, RBANS, AIMS, SARS.	outcome measures (some showed improvements in both groups).	
N = 40 (18-55 years, M = 45); schizophrenic, treatment resistant patients, PANSS score > 10.	3g/day ethyl-EPA or liquid paraffin placebo.	12 weeks parallel design, adjunctive therapy.	PANSS, ESRS.	Treatment > placebo on PANSS and dyskinesia subscale of ESRS. Treatment = placebo on other ESRS subscales.	(Emsley et al., 2002)
N = 115 (20-62 years, M = 37; 66% male), treatment-resistant schizophrenia; PANSS > 50.	1, 2 or 4g/day ethyl-EPA or liquid paraffin placebo.	12 weeks parallel design, adjunctive therapy.	PANSS, LUNSERS, MADRS, AIMS, BAS, SARS.	Treatment = placebo on all rating scales; 2g treatment > placebo for patients on clozapine (associated with ↑AA).	(Peet et al., 2002)
N = 69 (mean age 21 ± 4; 76% male) first episode psychosis patients.	2g/day ethyl-EPA or mineral oil placebo not absorbed by intestinal tract (both with Vit E).	12 weeks parallel design, adjunctive therapy.	BPRS, SANS, CDSS, CGI, GAF, SOFAS.	Treatment = placebo on all outcome measures. Treatment > placebo on CGI co-varying for duration of untreated psychosis; treatment > placebo at weeks 4-6.	(Berger et al., 2007)
N = 81 (13-25 years, M = 16 ± 2, 40% male), met defined risk factors for psychosis.	1.2-g/d n-3 PUFAs 0.7g EPA, 0.48g DHA, and 7.6mg of vitamin E).	12 weeks.	PANSS, MADRS, GAF.	Treatment > placebo on PANSS and GAF at 12 weeks, 6 and 12 months.	(Amminger et al., 2010)
N = 41 (19-30 years, 70% female); university students	1.5-1.8g/day DHA or 97% soybean oil + 3% fish oil	3 months parallel design.	P-F Study; Stroop; Dementia-detecting	Treatment > placebo on aggression (increased in	(Hamazaki et al., 1996)

(study measured aggression and executive function).	placebo capsules.		test.	placebo group during exam time); treatment = placebo on other measures.	
N = 30 (18-40 years; M = 26); females with moderately severe borderline personality disorder.	1g/day ethyl-EPA or mineral oil placebo.	8 weeks parallel design.	OAS-M; MADRS.	Treatment > placebo aggression and depressive symptoms	(Zanarini and Frankenburg, 2003)
N = 154 with MDD	(1) EPA 1 g/d; (2) DHA 1 g/d; (3) Placebo.	8 weeks.	HDRS-17, QIDS-SR-16, CGI-S.	Treatments and placebo improved HDRS-17, QIDS-SR-16, CGI-S.	(Mischoulon et al., 2015)

Abbreviations: AIMS, Abnormal Involuntary Movement Scale; BDI, Beck Depression Inventory; BPRS, Brief Psychiatric Rating Scale; CBC, Child Behaviour Checklist; CDSS, Calgary Depression Scale for Schizophrenia; CGI-S, Clinical Global Impression-Severity; DASS, Depression & Anxiety Stress Scale; DHUS, Daily Hassles & Uplifts Scale; DMT, Delayed Memory Task; ESRS, Extrapyramidal Symptom Rating Scale; GAFS, Global Assessment of Functioning Scale (revised GAS); GAS, Global Assessment Scale; HDRS, Hamilton Depression Rating Scale; HSCL-D-20, 20-item Hopkins Symptom Checklist Depression Scale; IDS, Inventory for Depressive Symptomology; IMT, Immediate Memory Task; LUNSERS, Liverpool University Neuroleptic Side-Effects Rating Scale; MADRS, Montgomery-Asberg Depression Rating Scale; OAS-M, The Overt Aggression Scale, Modified; PANSS, Positive and Negative Syndrome Scale; PGWB, Psychological General Well-Being Schedule; PSS, Perceived Stress Scale; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status; SANS, Scale for the Assessment of Negative Symptoms; SARS, Simpson-Angus Rating Scale; SOFAS, Social and Occupational Functioning Assessment Scale; STAEI, State-Trait Anger Expression Inventory; YMRS, Young Mania Rating Scale.

1.8. n-3 PUFAs in elderly

1.8.1 n-3 PUFAs: Animal studies and mechanisms

Old age represents the last stage of life where decay of the functionality and structure of the brain occurs. With ageing, oxidative stress and insertion of cholesterol into the cellular membrane augment each other. Consequently, the formation of free radicals, a toxic metabolite of the cholesterol and degradation of the phospholipids of membranes affect the fluidity and functionality of the cell membranes. Moreover, the levels of PUFAs are reduced (Barcelo-Coblijn et al., 2003a), probably due to less activity of the enzymes that regulate their pathway (Dyall, 2015), as are the incorporation of PUFAs into the cellular membrane (Engler et al., 1998) and their passage rate via the blood brain barrier (Vandal et al., 2014). Neuronal viability, synaptogenesis, regulation of membrane bound enzymes, control of the ionic channel structure and activity are strongly compromised in the mammalian brain (Das, 2006). As a consequence of such aged-related effects, major brain areas can be strongly affected. Decreased volume and function of hippocampus has been observed in association with altered processing of learning, memory and cognition in the elderly (Driscoll et al., 2003). Several experimental studies on rodents have shown a correlation between n-3 PUFAs deficiency and memory impairment (Fedorova and Salem, 2006). This evidence suggests that n-3 PUFAs status would exert an impact on age-related cognitive decline (24-26 months old) (Umezawa et al., 1995, Kelly et al., 2011) and on several brain physiological parameters that are altered during ageing (Su, 2010). Some studies have shown altered spatial learning capacities in n-3 PUFAs deficient old mouse, twenty-eight weeks and eight months old, respectively (Umezawa et al., 1995, Carrie et al., 2002) or old rats (8 months old) (Yamamoto et al., 1991) as compared to n-3 PUFAs supplied old animals, but some others failed to do so in mice (19-23 months old) (Moranis et al., 2012). Despite some contradictory results

(Barcelo-Coblijn et al., 2003a) beneficial effects of n-3 PUFAs, short or long-term supplementation, on cognitive improvement have been documented in old rats (80-100 weeks old) (Gamoh et al., 2001, Kelly et al., 2011). These effects seem to be, at least partly, attributable to their anti-oxidative and anti-inflammatory activity which has been shown to be involved in memory formation through restoration of synaptic plasticity, specifically long term potentiation (LTP) in old rats (24-26 months) (Dyall et al., 2007, Lynch et al., 2007, Kelly et al., 2011). Lynch and colleagues have attributed such effects to the anti-oxidative activity of EPA which is associated with reduced microglia aged-related activation and reduced IL-1β levels in the hippocampus of aged rats (22 months old) (Martin et al., 2002, Lynch et al., 2007, Kelly et al., 2011). Moreover, it has been observed that n-3 PUFAs can regulate the functionality of the glutamatergic system, which can undergo dysregulation during ageing. Accordingly, excess of glutamate may lead to excessive release of arachidonic acid (AA), especially when cell membranes have a high AA/DHA ratio such as in the brains of n-3 PUFAs deficient animals (Rao et al., 2007a). The release of excess AA then initiates a pro-inflammatory cascade of events involving the production of eicosanoids via the activation of inducible cyclooxygenase (COX2) and lipoxygenases (LOX), and the production of pro-inflammatory cytokines (Bazan, 2007, Farooqui et al., 2007). n-3 PUFAs moderate the onset of the AA signalling cascade by down regulating the pro-inflammatory isoforms (cytoplasmic (cPLA2) and secretory (sPLA2)) of PLA2 and COX2, and by reducing the production of eicosanoids. This counteracting effect of n-3 PUFAs on the AA signalling cascade has been described in peripheral tissues (Calder, 2005) and in the brain (Rao et al., 2007a, Rapoport, 2008). Furthermore, DHA has been shown to regulate brain cytokines expression in response to experimentally induced inflammation both in vitro and in adult mice (De Smedt-Peyrusse et al., 2008, Mingam et al., 2008). Dysregulation of the glutamatergic system also implies synaptic dysregulation due to alteration of receptors

expression and functionality (Su, 2010). n-3 PUFAs are involved in the regulation of glutamatergic receptors such as the post-synaptic 2-amino-3-propionic acid (AMPA) and N-methyl-d-aspartate (NMDA) receptors. Indeed, applying DHA directly onto isolated neurons increases probability of the NMDA-associated channels opening, thereby facilitating NMDA currents (Nishikawa et al., 1994). This suggests the possibility that n-3 PUFAs may play an important role in the genesis of LTP and in cognitive performance through regulation of the glutamatergic system. Interestingly, enhanced LTP and activation of NMDA and AMPA receptors are involved neuronal plasticity and neurogenesis (Luscher and Malenka, 2012). Accordingly, Wu et al have reported that DHA improves synaptic plasticity and water-maze learning memory performance by increasing the amounts of CaMKII, CREB, BDNF and synapsin-1 in the hippocampus of aged rats (Wu et al., 2008). In support of these findings, *in vitro* studies confirm that cellular proliferation is increased by medium supplemented with DHA (Kawakita et al., 2006, Kan et al., 2007). Moreover, DHA improves neuronal differentiation, as shown by enhanced neuritogenesis in older animals (18-20 months old) (Calderon and Kim, 2004, Robson et al., 2010). More studies have concluded that n-3 PUFAs supplementation has a beneficial effect of reversing age-related decreases in nuclear receptors such as retinoic acid receptors (RARs), retinoid X receptors (RXRs), and peroxisome proliferator-activated receptors (PPARs), and increasing neurogenesis in old rats (25-26 months old) (Kawakita et al., 2006, Dyall et al., 2010). Therefore, DHA could play a positive role in aged organisms, by regulating the glutamatergic system, and favouring neuronal maturation.

1.8.2. The role of n-3 PUFAs in cognition in aged healthy humans

In light of these observations, clinical studies have been carried out to elucidate the role of n-3 PUFAs in healthy older subjects (Table 1.12). However, there is limited investigation regarding the role of n-3 PUFAs as beneficial for cognitive function. One of the largest randomized controlled trial to date recruited 867 cognitively healthy subjects (70-79 years old) did not reveal improved cognitive functioning in the California Verbal Learning Test (CVLT) after twenty four months of treatment (200 mg EPA plus 500 mg DHA) (Dangour et al., 2010). Even though, at the end of the study, the n-3 PUFA serum levels were higher compared to the placebo group (olive oil), the n-3: n-6 PUFAs ratio was relatively high in both the groups. Moreover, all the recruited subjects showed quite a high cognitive functioning at the beginning of the study, assessed with the Mini-Mental State Examination (MMSE). Thus, the author argued that this could be a reason for no differences in the scoring of the CVLT between the groups. However, they did not assess an analysis of PUFAs composition at the beginning of the clinical trial. In another study, higher administration of n-3 PUFAs (900 mg DHA) have shown improvements in verbal recognition memory and visuospatial learning in old subject with low habitual intake of DHA after twenty four weeks (Yurko-Mauro et al., 2010). Accordingly, higher concentration (1.3 g DHA and 0.45 g EPA) and longer duration (12 months) of n-3 PUFAs showed improvement in different cognitive domains of a neuropsychological battery (Lee et al., 2013). In this study the choice of the subjects could have been critical. Thirty five healthy old women with mild cognitive impairment (MCI) from low socioeconomic background were recruited. Moreover, in this group the habitual intake of n-3 PUFAs was inadequate due to financial reasons. This might give greater scope for improvement in cognitive tests. MCI and low n-3 PUFAs consumption might give more range of improvement in cognitive tests compared to subjects with more adequate intake of seafood. These findings are in agreement with a previous double-blind

randomised control trial (Sinn et al., 2012). Forty subjects with MCI and low fish intake were divided in three experimental groups to receive a supplement rich in EPA (1.67 g EPA plus 0.16 g DHA), DHA (1.55 g DHA + 0.40 g EPA) or LA 2.2 g. After six months of n-3 PUFAs supplementation, depressive symptoms, assessed by the Geriatric Depression Scale (GDS) and self-reported physical health were improved especially in the DHA group as well as verbal fluency (Initial Letter Fluency) out of eleven different cognitive assessments. Moreover, cognitive improvement after n-3 PUFAs daily intake are associated with reduced risk factors for cardiometabolic disease. Lowered plasma triacylglycerides and systolic blood pressure f-glucose, and TNF-α levels were inversely related to improved cognitive performance (Nilsson et al., 2012) after five weeks of 3g of n-3 PUFAs (EPA 1500 mg, DHA 1050). Moreover, n-3 PUFAs supplementation have been shown to increase the activation of the dorsolateral prefrontal cortex during a working memory task (Konagai et al., 2013) and to improve brain structure (Witte et al., 2014) in healthy aged men. Despite that, a recent trial (4000 people, average age 72 years old) did not show any benefit of n-3 PUFAs supplementation for preventing cognitive decline over a period of 5 years. Among older persons affected by age-related macular degeneration (AMD), a EPA (650 mg)/ DHA (350 mg) mixture failed on improving cognitive function (Chew et al., 2015). In conclusion, at this last stage of life the supplementation of n-3 PUFAs seems to be of help particularly in subjects with MCI and low habitual fish intake. Interestingly, DHA seems to be more of benefit in both memory and cognition than EPA. These results are in contrast to what is observed in adulthood in which the EPA/DHA combination with higher concentration of EPA than DHA is favoured in most of the studies. This discrepancy could be due to the phospholipids degradation occurring at this last stage of life. Since the DHA constitutes the most abundant fatty acids in the brain and due to its importance in the formation and

functionality of the CNS during early life, it is plausible that DHA can ameliorate cognitive performance more than other PUFAs in older subjects.

Table 1.12: n-3 PUFAs' impact in healthy elderly (adapted from Stonehouse, 2014).

Double-blind randomized placebo-controlled trials of n-3 PUFAs in healthy elderly.					
Participants	**Treatment**	**Length of trial**	**Measurements**	**Outcomes**	**References**
70–75 years, cognitively healthy, MMSE ≥ 24 (median = 29) (*n* = 867, analysis on *n* =748).	Ethyl ester fish oil (0.2 g EPA + 0.5 g DHA)/day *vs*. placebo (olive oil).	24 months.	CVLT.	No effect on global cognitive function, memory, processing speed, executive function, global delay score.	(Dangour et al., 2010)
60–80 years, stable MI patients, MMSE >21 (average ± SD 28 ± 1.6 points).	(1) 0.4 g/day EPA + DHA; (2) 2 g/day ALA; (3) EPA + DHA + ALA; (4) Placebo.	40 months.	MMSE.	No effect on MMSE.	(Geleijnse et al., 2012)
60–80 years, healthy women (*n* = 57, analysed *n* = 49).	(1) 0.8 g/day DHA (algal oil); (2) 12 mg/day lutein; (3) DHA + lutein; (4) Placebo.	4 months.	Cognitive test battery measuring verbal fluency, memory, processing speed and accuracy.	Treatments (1), (2), (3) improved verbal fluency. DHA + lutein improved rate of learning and memory in 1 of 6 recall tests.	(Johnson et al., 2008)
≥60 years, MCI, MMSE = 26.4 (25–28), middle to low-socioeconomic status (*n* = 36, analysed *n* = 35).	Fish oil (1.3 g DHA + 0.45 g EPA)/day *vs*. placebo (corn oil).	12 months.	MMSE, RAVLT.	Improved memory (short-term memory, working memory, immediate visual memory, delayed recall).	(Lee et al., 2013)
51–72 years, healthy (*n* = 44, analysed *n* = 38.	Fish oil (1.05 g DHA + 1.50 g EPA)/day *vs*. placebo.	5 weeks.	Working memory.	Improved working memory. TNF-α inversely related to working memory performance.	(Nilsson et al., 2012)
>65 years, MCI, MMSE ≥ 22	(1) EPA-rich fish oil (1.67 g	6 months.	Cognitive battery.	DHA improved verbal fluency	(Sinn et al., 2012)

(average ~27 ± 2.5) (*n* = 50).	EPA + 0.16 g DHA)/day); (2) DHA-rich fish oil: (1.55 g DHA + 0.40 g EPA)/day; (3) Placebo (safflower oil).			(test of fluid thinking/semantic memory). Only one out of 11 cognitive assessments affected.	
45–77 years (average ~56 ± 8.7 years), healthy (*n* = 112, analysed *n* = 75).	Tuna oil (0.25 g DHA + 0.06 g EPA)/day *vs*. placebo (soybean oil).	90 days.	CDR, visual acuity.	No treatment effects.	(Stough et al., 2012)
≥65 years, cognitively healthy, median (25, 75 percentile) MMSE = 28, ranged from 23 to 30, (*n* = 302).	(1) Low-dose fish oil (0.26 g EPA + 0.18 g DHA)/day; (2) High dose fish oil (1.09 g EPA + 0.85 g DHA)/day; (3) Placebo (oleic acid).	26 weeks.	Cognitive test battery.	Treatment improved attention in *APOE4* allele carriers. Treatment–gender interactions: Attention improved in men.	(van de Rest et al., 2008)
50–90 years, non-demented participants with memory complaints, MMSE ≥ 27 (average ~28.5 ± 1.11), (*n* = 157, analysed *n* = 122).	PS containing LC omega-3: 300 mg PS + 0.08 g (DHA + EPA)/day.	15 weeks.	Immediate and delayed verbal recall, learning abilities, and time to copy complex figure.	Improved verbal immediate recall. A subset of participants with higher baseline cognitive status performed better on immediate and delayed verbal recall, learning abilities and time to copy a complex figure.	(Vakhapova et al., 2010)
50–75 years, MMSE < 26 (average ~29 ± 1.0, ranged from 26 to 30), (*n* = 80, analysed *n* = 65).	Fish oil 2.2 g/day LC omega-3 (1.32 g EPA + 0.88 g DHA) *vs*. placebo (sunflower oil).	26 weeks.	Stroop Color-Word test, TMT, AVLT.	Improved executive function. Sub-set who showed greatest increase in n-3 index showed improved memory. Improved white matter microstructural integrity, grey matter volume in frontal, temporal, parietal and limbic areas.	(Witte et al., 2014)

				Improvements in executive function associated with peripheral BDNF and inversely with fasting insulin.	
≥55 years (average ~70 ± 9 years), subjective memory complaints with ARCD, MMSE >26 (*n* = 485).	0.9 g/day DHA from algal oil *vs*. placebo (corn + soy oil).	24 weeks.	CANTAB PAL, MMSE.	Improved visuospatial learning and episodic memory, immediate and delayed verbal recognition memory.	(Witte et al., 2014)

Abbreviations: AVLT, Auditory Verbal Learning Test; CANTAB-PAL, Cambridge Neuropsychological Test Automated Battery - Paired Associates Learning; CDR, Clinical Dementia Rating; CVLT, California Verbal Learning Test; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; RAVLT, Rey Auditory Verbal Learning Test.

1.9. The microbiome-gut-brain axis

1.9.1. The microbiome-gut-brain axis & stress

There is increasing evidence suggesting a bi-directional communication between the central nervous system (CNS) and the gut-microbiota which is recognized as the microbiome–gut–brain axis (Cryan and Dinan, 2012, Bercik and Collins, 2014, Mayer et al., 2014a). This communication is believed to influence the parallel development of both CNS and gut microbiota which can remarkably influence health and disease (Moloney et al., 2014). Recent studies have highlighted the importance of the gut microbiota in influencing stress responses and related behaviours (Foster and McVey Neufeld, 2013). For instance, germ free mice (which have no commensal microbiota) display reduced anxiety-like behaviour compared with conventionally-reared mice and this behaviour can be reversed by colonization of the gut with microbiota from control mice (Foster and McVey Neufeld, 2013). Similarly mice treated with probiotics display reduced anxiety-like behaviour and lower corticosterone responses to stress (Foster and McVey Neufeld, 2013). In contrast treatment of mice with subclinical doses of pathogenic bacteria results in increased anxiety-like behaviour and activation of the HPA axis (Foster and McVey Neufeld, 2013). Changes in anxiety behaviour and stress responsiveness induced by manipulating the gut microbiota are associated with altered expression of GABA receptors in the brain (Bravo et al., 2011). This is important given dysfunctional GABA signalling has been linked to anxiety and depression and the $GABA_A$ receptor is a target for some anxiolytic drugs (Mohler, 2012). Importantly, stress also influences the composition of the gut microbiota such as, repeated social stress exposure disrupts commensal microbial populations in adult mice. Moreover early life stress (via post-natal maternal separation) in rats results in increased stress reactivity and anxiety-like behaviour and is associated with long-term changes in the composition and diversity of the gut microbiota (Foster and McVey Neufeld, 2013). Indeed, emerging evidence has shown the

involvement of the gut microbiota in maternal stress and maternal separation in brain and associated behaviour (Bailey and Coe, 1999). Prenatal stress has been shown to change the composition of the microbiome in adult rat (Golubeva et al., 2015) neonatal mice (Jasarevic et al., 2015) and infant humans (Zijlmans et al., 2015). Changes in the gut microbiota composition were reported in monkeys subjected to maternal separation between six and nine months of age with shedding of lactobacilli three days following separation, followed by the return of normal lactobacilli levels seven days later. Moreover, we have previously shown, albeit using somewhat crude Denaturing Gradient Gel Electrophoresis- based analysis, that adult rats that underwent maternal separation showed altered faecal microbial composition compared with normally reared control animals (O'Mahony et al., 2009). These studies provide important evidence that the microbiota can alter mental processes and reduce stress responses. Thus, future studies of how gut microbes contribute to the function of the CNS will play an important role in advancing understanding of stress-related disorders.

1.9.2. The microbiome-gut-brain axis & PUFAs

The human intestine harbours trillions of micro-organisms, containing over 100-fold more genes than the host genome, whereby the collective genome of these micro-organisms has co-evolved with the host and contributes to biochemical and metabolic functions that the host could not otherwise perform (Backhed et al., 2004). It is becoming clear that the microbiota normally has a balanced compositional signature that confers health benefits and that a disruption of this balance confers disease susceptibility (Cryan and O'Mahony, 2011). There is a growing appreciation for the impact of dietary fatty acids on the intestinal microbiota composition of the host (Patterson et al., 2014, Yu et al., 2014, Marques et al., 2015). Indeed, excessive dietary intakes of refined carbohydrates and fat can influence the intestinal microbiota composition of the host (Turnbaugh et al., 2009). For example, while a diverse

intestinal microbiota is preferable, ingestion of a high-fat diet containing palm oil has been reported to decrease bacterial diversity (Mujico et al., 2013). Similarly, reduced bacterial diversity has been reported in early-life stressed rats (omahony). Recent finding has demonstrated that a rich dietary source of n-3 PUFAs (flaxseed/fish oil) may have a bifidogenic effect on the intestinal microbiota composition of the host by increasing the levels of Bifidobacterium, while also positively influencing the composition of host tissues with n-3 PUFA-derived health-promoting fatty acids. Of note, specific Bifidobacteria strains have been shown to reduce anxiety-like behaviour in an anxious mouse strain (Savignac). However there is a paucity of information in regards of the impact of n-3 PUFAs on the microbiome-gut-brain axis. Nonetheless, these initial findings can undoubtedly open up to further investigation for a complete elucidation of the molecular processes linking bacteria, n-3 PUFAs enriched diets and stress-related disorders.

1.10. Primary hypothesis and aims of thesis

This thesis aims to interrogate the hypothesis that dietary lipids exert a positive role in the neurobiology of stress and have potential as new therapeutic strategies for stress-related disorders. In particular, it is unclear if n-3 PUFAs can prevent the effects of chronic stress in the development of stress-related pathologies. Moreover, the effect of n-3 PUFAs on gut microbiota, as a putative strategy against stress, has hitherto been poorly considered. Most importantly, the role played by n-3 PUFAs on reversing cellular changes induced by the stress hormone corticosterone (CORT) has yet to be investigated. Finally, the role of dietary lipids in neuroinflammation, which is believed to contribute to the development of stress-related pathologies, remains to be elucidated.

We aim to test this hypothesis in an animal model of early-life stress, the maternal separation paradigm, which induces long-lasting alterations in many systems which impact cognition, emotional state and gut microbiota. Moreover, such stress produces a phenotype that is comparable to depression and anxiety in humans. In addition, as outlined below we will attempt to delineate the potential neurobiological mechanisms that may underlie CORT stress-induced changes in *in vitro* models. Using another *in vitro* model, we will also investigate the potential reversal of lipopolysaccharide (LPS)-induced inflammation by dietary lipids.

Aim 1: Does DHA reverse CORT-induced cellular changes in mixed cortical cultures? Are these changes related to cellular viability, apoptosis, GR regulation, BDNF expression levels?

Aim 2: Do phospholipids and/or DHA reverse LPS-proinflammatory activity in astrocyte cortical cultures? Are these changes related to reduced pro-inflammatory cytokines expression levels?

Aim 3: Do neurodevelopmentally normal or early-life stressed female rats fed with n-3 PUFAs exhibit improvements in cognition, anxiety-like and depressive-like behaviours? Are these changes related to hypothalamic-pituitary-adrenal (HPA) axis function, glucocorticoid receptor (GR) functionality, immune activity, brain monoamines levels, and gut microbiota composition?

Chapter 2

n-3 PUFAs have beneficial effects on anxiety and cognition in female rats: Effects of Early Life Stress

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2.1. Abstract

Stressful life events, especially those in early life, can exert long-lasting changes in the brain, increasing vulnerability to mental illness especially in females. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) play a critical role in the development and function of the central nervous system (CNS). Thus, we investigated the influence of an eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) (80% EPA, 20% DHA) n-3 PUFAs mixture on stress-related behavioural and neurobiological responses.

Sprague-Dawley female rats were subjected to an early-life stress, maternal separation (MS) procedure from postnatal days 2 to 12. Non-separated (NS) and MS rats were administered saline, EPA/DHA 0.4g/kg/day or EPA/DHA 1g/kg/day, respectively. In adulthood, EPA/DHA treated animals had a dose dependent reduction in anxiety in NS rats. Furthermore, cognitive performance in the novel object recognition task (NOR) was improved by EPA/DHA treatment in NS animals only. EPA/DHA 1g/kg/day decreased behavioural despair in the forced swim test. Notably, EPA/DHA high dose increased the translocation of GRs into the nucleus of NS rat hippocampus. However, the levels of mBDNF remained unchanged in all the experimental groups. The corticosterone response to an acute stress was blunted in MS rats and this was further attenuated by pre-treatment with EPA/DHA. Immune response and monoamine neurotransmission were significantly altered by early-life stress. In conclusion, our study supports the view that n-3 PUFAs are beneficial in neurodevelopmentally normal animals but have little positive benefit in animals exposed to early life stress.

2.2. Introduction

Stressful life events, especially those in early life, can exert long-lasting changes in the brain increasing the likelihood of adverse health consequences (Cryan and Dinan, 2013; Lupien et al., 2009). The World Health Organization (WHO) predicts that by 2020, mental illness, including stress-related diseases, will be the second leading cause of disabilities globally (Lucassen et al., 2014). Women appear to have an increased risk of suffering from stressful life events and seem to be more susceptible to the development of major mental illnesses such as major depression including atypical depression (Dinan, 2005). However, it is worthy of note that currently, in contrast to men, there is a distinct paucity of information describing stress responses in women (Taylor et al., 2000). Thus, understanding the mechanisms behind the higher susceptibility of women to the pathophysiology of stress may open up novel strategies to improve the ability of women to cope with stressful life events.

Inadequate maternal care has been linked to developmental, emotional, and social deficits in human infants (Field, 1998) and in the rat (Caldji et al., 1998). In rodents, the maternal separation (MS) model is a well-known paradigm used to investigate the biological consequences of early-life stress (O'Mahony et al., 2011). MS alters response in several behavioural paradigms in adult rodents (O'Mahony et al., 2009) producing a phenotype which is comparable to depression in adult humans (Schmidt et al., 2011). Such stress also induces long-lasting alterations in many systems which impact cognition and emotional state (Maccari et al., 2014).For instance, we have found that MS increases proinflammatory cytokines in the periphery and results in exaggerated hypothalamic–pituitary adrenal (HPA) axis responses (O'Mahony et al., 2011).Moreover, the negative feedback inhibition of the HPA axis, regulated by hippocampal glucocorticoid receptors (GRs) as well as glucocorticoids secretion, are altered in adult rodents after early-life stress experience

(Levine and Wiener, 1988; Lupien et al., 2009). This is of importance considering that dysregulation of the HPA axis, which controls the physiological response to stress, is implicated in the pathogenesis of depression (Holsboer, 2000; Julio-Pieper and Dinan, 2010).

It is well recognized that changes in diet are a viable strategy for enhancing cognitive abilities, protecting the brain from damage, counteracting the effects of aging and warding off mental disorders (Logan, 2003). Polyunsaturated fatty acids (PUFAs) constitute 20% of dry weight brain and as constituents of the cellular membranes, they exert a profound impact on an organism's development, structure, and function (Yehuda et al., 1999). Growing evidence shows that n-3 PUFAs play an important role in the regulation of monoamine neurotransmission (Chalon, 2006). Rats deficient in n-3 PUFAs show dramatically reduced dopamine and serotonin levels in the frontal cortex and lower density of the synaptic vesicles in the CA1 region of the hippocampus (Chalon, 2006). This impairment can be translated to deficiencies related with learning performance as well as structural changes within the mesocorticolimbic pathway (Yoshida et al., 1997).

The effects of n-3 PUFAs on cognitive function, depressive- and anxiety-like symptoms have been linked to the action of brain-derived neurotrophic factor (BDNF) (Autry and Monteggia, 2012) which increases neuroplasticity and cell survival (Maitre, 1996). Several studies have reported BDNF as one of the primary targets for regulation by n-3 PUFAs (Rao et al., 2007). Moreover, recent findings have shown a connection between BDNF and glucocorticoid signalling that contribute to the regulation of the HPA axis, as well as to the modulation of stress response (Jeanneteau et al., 2012). Indeed, glucocorticoid receptors (GR), largely expressed in the hippocampus, have been shown to regulate the levels of BDNF through specific molecular signalling (Chen et al., 2012). Furthermore, the abundance and activity of GRs is linked with the functionality of the HPA axis, the dysregulation of which is implicated

with maladaptive responses to the stress (Pariante, 2009). Supplementation of n-3 PUFAs has been shown to impede the disruption of normal HPA axis functionality associated with the development of neuropsychiatric disorders (Larrieu et al., 2014). Moreover, n-3 PUFAs, among their biological properties, have shown anti-inflammatory effects as a possible pathway through which they can be effective both in preventing or treating stress related disorders (Grosso et al., 2014).

In the light of these observations, we hypothesized that chronic intake of n-3 PUFAs would improve the performance of adult female rats exposed to early life stress. Furthermore, we predicted that these effects would be related to molecular changes at BDNF and GR levels as well as with changes in monoamine neurotransmission.

2.3. Methods

Maternal separation

Maternal separation was performed as previously described (O' Connor et al., 2013; O'Mahony et al., 2008). Briefly, Sprague Dawley male and female rats were obtained from Harlan Laboratories UK (250g – 300g) and mated in the local animal unit. Food and water was available ad libitum and animals were maintained on a 12:12-h dark–light cycle with temperature at 21°C ± 1°C. Rat pups were separated from their dams as a whole litter for a period of 180 min between postnatal days 2 and 12. Separations were conducted between 0900h and 1200h a.m. in plastic cages placed on top of heater pads (30°C-33 °C) in a separate room to the main holding room. Control rats consisted of non-handled pups, left untouched with their respective dams. After postnatal day 12, rats were left undisturbed except for routine cage cleaning every two days and a weekly body weight measurement until they were 5 weeks old. Female rats were chosen because stress-related psychiatric disorders

are more prevalent in the female population. 60 animals were group-housed 5 per cage in plastic cages with sawdust bedding in an enriched environment with shredded paper and a cardboard roll. All experiments were in full accordance with the European Community Council Directive (86/609/EEC).

Treatments

Once the animals were 5 weeks old, both MS rats and NS controls underwent oral administration of an eicosapentaenoic acid (EPA)/docosaexaenoic acid (DHA) (80% EPA, 20% DHA) n-3 PUFAs mixture by gavage. Individual groups consisted of rats from 10 different litters to account for any confounding litter effects. Ten rats per experimental group were used in this study. Treatments consisted of 1) saline water; 2) EPA/DHA 0,4g/kg/day; 3) EPA/DHA 1g/kg/day. These EPA/DHA concentrations have been used in previous studies (Calviello et al., 2004; Yilmaz et al., 2004). EPA/DHA treatments were combined with saline water to achieve a final volume of 500 µl as in the case of the saline treatment groups. Treatments were chosen based on the Food and Agriculture Organization of the United Nations (FAO) recommendations. FAO recommends a minimum DHA intake of 10-12 mg/kg per body weight for children 6-24 months old and EPA/DHA 100-250 mg/day for children aged 2-10 years. In our study, the maximum EPA/DHA intake was 100 and 250 mg for the low dose and the high dose per body rat, respectively. Treatments were prepared freshly every day and administered between 0900h and 1100h a.m. for a period of 6 weeks prior to behavioural tests. Oral administration continued throughout behavioural testing. The same animals were used for both the behavioural and neurochemical analysis described below.

Behaviour test battery

At 11 weeks animals underwent a behavioural test battery. For all behavioural tests, animals were habituated to the testing room by placing home-cages in the test room for at least 15 minutes prior to testing. The same animals were assessed across all behavioural tests with at least 4/6 days of resting in between tests. All the behavioural tests were conducted at the stage of metestrus and diestrus to avoid that hormonal fluctuation of the estrous cycle affected the behavioural tests. Classification of the stage of the estrous cycle was based on the vaginal smear on the day of the experiment and the prior vaginal smear history.

Elevated plus maze

The elevated plus maze is one of the most commonly rodent tests of anxiety and was performed as previously described (Cryan et al., 2004). The maze consisted of two open arms (51 × 10 cm) and two enclosed arms (51 × 10 × 41 cm) that all extended from a common central platform (10 × 10 cm). The apparatus was elevated 55 cm above the floor on a central pedestal. Animals were acclimatized to the testing room for 30 min prior experiment. At week 11, animals were placed in the centre of the maze facing an open arm to begin. Animal behaviour was videotaped for the duration of 5 min test. Frequency of open and closed arms entries were scored plus percentage time in open arms compared with total time.

Open Field test

At week 12, rats were subjected to a novel stress, the open field as described previously (O'Mahony et al., 2014). At the beginning of each trial, animals were placed in the centre of a brightly illuminated white open field arena (1000 lux), measuring 0.9 m in diameter, and observed for 10 min. The trials were conducted between 0900h a.m. and 0100 p.m. Animals

were acclimatized to the testing room for 30 minutes prior experiment. The arena was cleaned with 70% ethanol to avoid cue smell between each trial. At the end of each trial, animals were returned to their cages. Distance moved, velocity, percentage of time spent in inner zone and frequency of inner zone entries were analysed as recorded using a tracking system (Ethovision XT 8.5, Noldus).

Novel object recognition task

This test of spatial working memory was conducted as described previously (Bevins and Besheer, 2006). At week 13 rats were habituated to the experimental room for 15 min. The day after, rats were placed in the middle of a white plastic rectangular box (42X62x36) under a dimly light, 74-80 Lux at the level of the arena, for 10 min. In the evening, rats were habituated to two identical objects (familiar objects) placed in the back left and right corners of the experimental box for 10 min. The day after rats were placed in the box with the two familiar object used previously for a total time of 10 min (acquisition phase). After a delay of one hour, one of the two familiar objects was substituted with a novel object and rats were replaced in the middle of the box at the mid-point of the wall opposite the sample objects for a total time of 5 min (retention phase). Animals were acclimatized to the testing room for 15 min prior each experiment. Box and objects were cleaned with alcohol 70% to avoid any cue smell between each trial. Directed contacts with the objects, include any contact with mouth, nose or paw or minimal defined distance (<2 cm), were scored using a stop watch. Any contact in which the animal is standing or leaning on the object as a way of exploring other aspects of the chamber were not interpreted as directed contact with object. Discrimination index (novel object exploration time/total exploration time)–(familiar object exploration time/total exploration time) and total time spent with novel and familiar object were scored.

Forced swim test

To assess behaviours relevant to antidepressant action at week 14 a modified rat forced swim test (FST) (Slattery and Cryan, 2012) was used to determine the antidepressant-like effect of EPA/DHA in MS and NS rats. A second scoring method was used to determine latency to become immobile (Carlezon et al., 2002). Briefly, on day one, rats were placed individually in Pyrex cylinders (46 cm tall × 21 cm in diameter) filled with water to 30 cm depth at 25 °C for habituation to the test. The rats were removed 15 min later, dried and placed in their home cage. On day two, 24 h after the first exposure, the rats were again placed in the swim apparatus for 5 min and behaviours were monitored from above by video camera for subsequent analysis. Three predominant behaviours were recorded in each 5 s period of the 300 s test. Climbing behaviour was defined as upward movements of the forepaws along the edge of the swim chamber. Swimming behaviour was identified as horizontal movements throughout the cylinder. Immobility was described when no additional activity was observed other than that required to keep the rat's head above the water. Latency to become immobile was defined as the time at which the rat first initiated a stationary posture that did not reflect attempts to escape from the water. In this characteristic posture, the forelimbs are motionless and tucked toward the body. To qualify as immobility, this posture had to be clearly visible and maintained for $\geq$ 2.0 sec.

Determination of plasma corticosterone

On day one of the FST, blood samples via tail incision were collected at three different time points; immediately before and after the test, as well as 2 hours later. For this end, rats were restrained and the end of the tails was held with two fingers. Using a single edge razor blade a diagonal incision of 2mm long was made at 15mm from the end of the tail. Approximately 200 µl blood was collected in a collecting tube containing EDTA to avoid blood coagulation by increasing the pressure of the fingers on the tail above the incision. Blood was mixed with EDTA by gently inverting the tube and centrifuged at 3500g at room temperature for 15min. Plasma was carefully aspirated and stored at -80°C. Corticosterone levels were assayed using a commercially-available ELISA kit (Corticosterone EIA Kit, ADI-900-097, Enzo Life Sciences) according to manufacturer instructions. Light absorbance was read with a multi-mode plate reader (Synergy HT, BioTek Instruments, Inc.) at 405 nm.

Determination of plasma cytokine levels

Immediately after culling, trunk blood samples were collected in a tube containing EDTA to avoid blood coagulation and centrifuged at 3500g for 15min for plasma collect. Concentrations of the cytokine tumour necrosis factor alpha (TNF-α) were analysed using a commercially available electrochemiluminescence multiplex system (MSD, Gaithersburg, MD, USA) according to the manufacturer protocol. Samples were added to pre-coated wells in duplicate and incubated for 2 h. Wells were then washed using phosphate buffered saline (PBS)-Tween 20 and incubated with α-TNFα antibody in diluent for 2 hours. Wells were then washed using phosphate buffered saline (PBS)-Tween 20. ELISA plates were analysed using the Sector 2400 imager from Mesoscale Discovery. This is an ultra-sensitive method which has a detection limit for TNFα of 0.3 pg/ml.

Neurotransmitter concentrations

Neurotransmitters concentrations of selected brain regions were determined using high-performance liquid chromatography (HPLC) technique as described previously (Clarke et al., 2013). HPLC analysis was conducted in 3 different brain areas: brain stem, hippocampus and prefrontal cortex. Briefly, brain tissues were sonicated in 500 µl of chilled mobile phase spiked with 4 ng/40 µl of N-Methyl 5-HT (Sigma Chemical Co., UK) as internal standard. The mobile phase contained 0.1 M citric acid, 5.6 mM octane-1-sulphonic acid (Sigma), 0.1 M sodium dihydrogen phosphate, 0.01 mM EDTA (Alkem/Reagecon, Cork) and 9% (v/v) methanol (Alkem/Reagecon), and was adjusted to pH 2.8 using 4N sodium hydroxide (Alkem/Reagecon). Homogenates were then centrifuged for 15 minutes at 22000 g at 4°C and 40 µl of the supernatant injected onto the HPLC system which consisted of a SCL 10-Avp system controller, LECD 6A electrochemical detector (Shimadzu), a LC-10AS pump, a CTO-10A oven, a SIL-10A autoinjector (with sample cooler maintained at 40C) and an online Gastorr Degasser (ISS, UK). A reverse-phase column (Kinetex 2.6 u C18 100×4.6 mm, Phenomenex) maintained at 30°C was employed in the separation (Flow rate 0.9 ml/min). The glassy carbon working electrode combined with an Ag/AgCL reference electrode (Shimdazu) operated a +0.8V and the chromatograms generated was analyzed using Class-VP 5 software (Shimadzu). The neurotransmitters were identified by their characteristic retention times as determined by standard injections, which run at regular intervals during the sample analysis. The ratios of peak heights of analyte versus internal standard were measured and compared with standard injection. Results were expressed as ng of neurotransmitter per g fresh weight of tissue.

Protein extraction and Western Blot analysis

Protein extracts from subcellular compartments were obtained using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem - 539790). Protein concentration from each sample was then measured using a Pierce BCA protein assay kit (Thermo scientific – 23225) and normalized. 10-40ug of protein sample was separated by 4-20% polyacrylamide gel electrophoresis (GenScript, Piscataway, NJ and Bio-Rad, Hertfordshire, U.K.) transferred onto a 0.2 μm Immobilon-P PVDF membrane (Sigma) in wet minitransfer system Hoefer TE 22 (Hoefer, Holliston, MA) and probed with primary antibodies in 5% fat-free milk in TBST overnight at 4°C. Rabbit monoclonal antibody anti-GR (D6H2L; 1:500) was from Cell Signaling (12041S); rabbit polyclonal antibody anti-BDNF (N-20; 1:500) was from Santa Cruz Biotechnology (SC546); mouse monoclonal antibody anti-alpha-tubulin (1:1000) was from Sigma (T5168). Proteins were then incubated with secondary antibodies (HRP-conjugated, Sigma) made in 5% fat-free milk in TBST for 1 h at room temperature. Blots were visualized using Amersham ECL Prime reagent (GE Healthcare Life Sciences, Buckinghamshire, U.K.) using the LAS-3000 Imager (FujiFilm, Tokyo, Japan) and Image Reader LAS-3000 2.2 software. Quantitative image analysis was done in ImageJ software (NIH, Bethesda, MD). The density of the protein band in each sample was normalized to alpha-tubulin levels (endogenous control).

Sample collection

Animals were sacrificed by decapitation and trunk blood was collected in EDTA-coated tubes and centrifuged for 15 min at 10000 g. Plasma supernatant was collected and stored on dry ice. The brain was quickly excised and dissected and each brain region was snap-frozen on dry ice. All samples were frozen at −80°C for later analysis.

Statistics

All data are presented as mean ± SEM. Data were analyzed using a two-way ANOVA followed by Fisher's LSD post-hoc test using the statistical software package SPSS 21.0 (IBM). The temporal dynamic of stress-induced corticosterone levels and weekly body weight, were analysed with two-way repeated measure ANOVA. A p-value of 0.05 was selected as the threshold of statistical significance. The statistical significance was indicated as follows: * indicates $p<0.05$; ** indicates $p<0.01$ and *** indicates $p<0.001$.

2.4. Results

Body weight

Animals did not differ in terms of body weight across the various treatment groups across the duration of the treatment. (Data not shown).

Elevated plus maze

No differences were found between saline treated NS and MS animals during the elevated plus maze test. However, administration of EPA/DHA n-3 PUFAs mixture exerted a positive effect on anxiety-like behaviour displayed by NS rats in the EPM. Indeed, *post-hoc* analysis revealed EPA/DHA 1g/kg/day significantly increased % of time spent in the open arms (OA) ($p<0.05$) as well as number of entries in the open arms (OA) ($p<0.05$) in NS group only (fig. 1B-E).

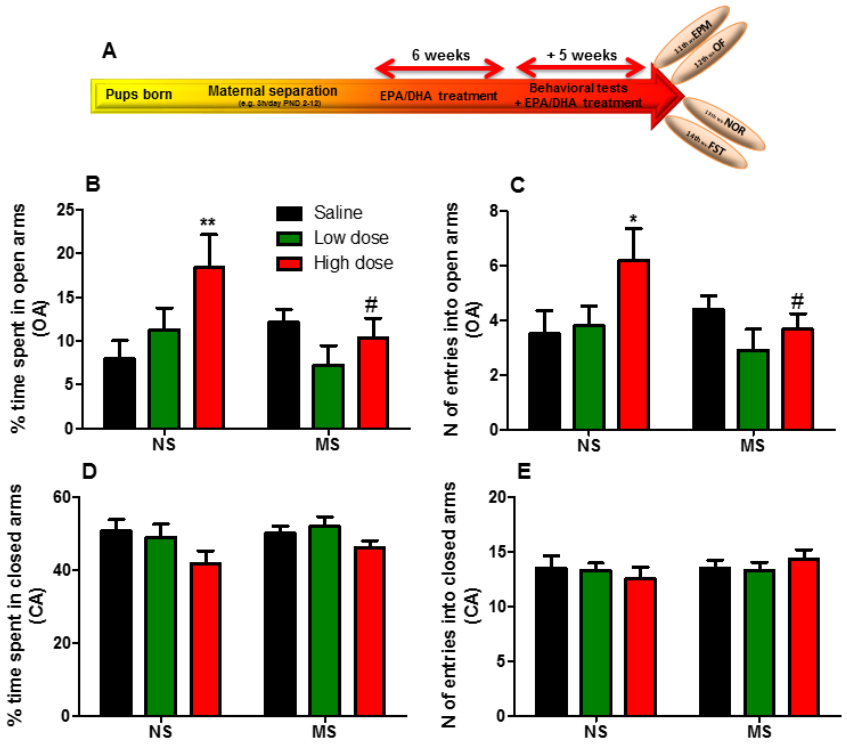


Figure 1. Experimental time line and effects of maternal separation (MS) and EPA/DHA administration on behavioural response in the elevated plus maze. (A) Schematic representing the time course of the maternal separation procedure, EPA/DHA treatment and behavioral tests. (B) non separated (NS) rats treated with EPA/DHA high dose spent significantly more time (C) and showed higher crossing into the open arms. (D) and (E) represented animal behaviour on % of time and number of entries into the closed arms of the elevated plus maze, respectively. Data are presented as mean ± S.E.M. ($n = 10$ per group). Intergroup comparisons: * and **: $P < 0.05$ and $P < 0.01$ respectively compared to NS saline; #: $P < 0.05$ compared to NS high dose.

Open field

Neither early-life stress nor EPA/DHA treatments revealed any significant differences between groups in the open field (Table 1).

Open Field	Non separated (NS)			Maternally separated (MS)		
	Saline	Low dose	High dose	Saline	Low dose	High dose
Nr. entries inner zone	18.30 ± 3.18	19.25 ± 3.51	23.80 ± 3.41	17.00 ± 2.39	21.50 ± 3.99	20.67 ± 2.54
% time inner zone	9.90 ± 1.68	9.95 ± 1.38	8.58 ± 1.50	7.01 ± 1.07	10.15 ± 1.74	7.08 ± 1.34
Total distance (cm)	5803.46 ± 262	6026.12 ± 306	6062.54 ± 323	5884.86 ± 304	6222.60 ± 187	6401.15 ± 137
Velocity (cm/sec)	9.71 ± 0.44	10.05 ± 0.60	10.12 ± 0.54	9.82 ± 0.51	10.08 ± 0.37	10.68 ± 0.23

Table 1. Summary of the effects of maternal separation (MS) and EPA/DHA administration on behavioural response in the open field. Data are presented as the average mean of each experimental group ± S.E.M. (n = 10 per group).

Novel object recognition task (NOR)

Cognitive behaviour assessed by NOR task did not differ between MS saline treated animals and their NS counterpart. However, EPA/DHA 1g/kg/day treatment increased the ability to discriminate the novel object from the familiar one (p<0.05) as well as decreased the time spent exploring the familiar object (p<0.05) during the 5 min retention phase in NS group only as revealed by *post hoc* analysis (Figure 2E-F).

Forced swim test

As shown in figure 2 (A-D), there was no difference between saline treated NS and MS animals. However, *post hoc* analysis revealed EPA/DHA high dose delayed the latency to become immobile in the NS animals only, (p<0.05). Moreover, EPA/DHA 1g/kg/day significantly reduced immobility time as revealed by *post hoc* analysis (p<0.05) (Figure 2A-D). However, no significant effects were observed on swimming frequency or on climbing scores in any of the groups.

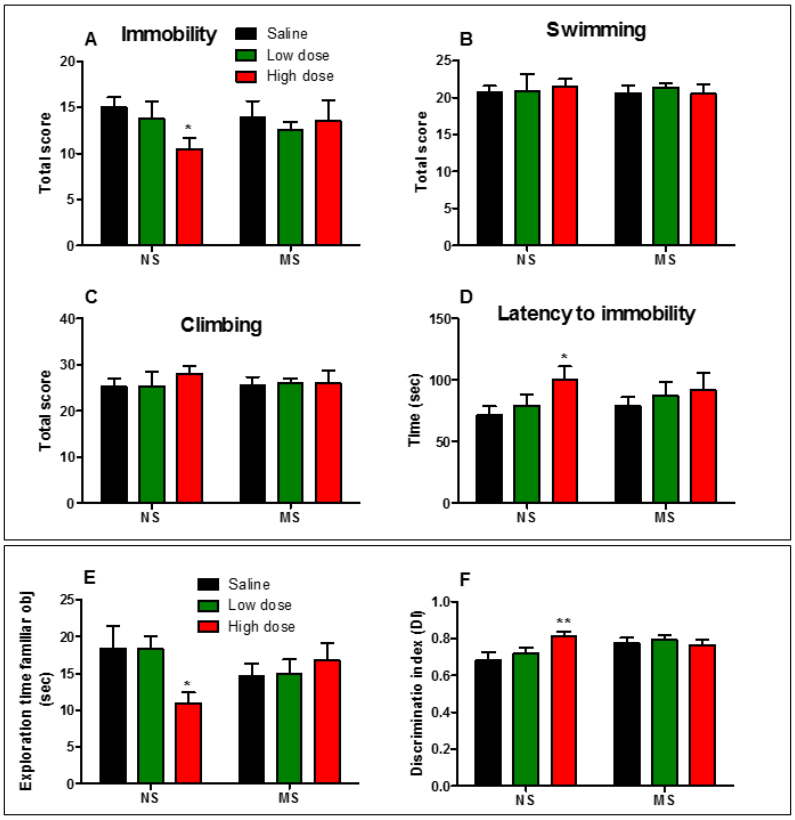


Figure 2. Effects of maternal separation (MS) and EPA/DHA administration on behavioural response during the forced swim test and the novel object recognition task. (A) EPA/DHA high-dose significantly reduced immobility time. (B) and (C) There was no significant differences on swimming frequency and climbing scores. (D) Latency to become immobile was increased by EPA/DHA high-dose. (E) Interaction with the familiar object was significantly reduced by the non separated (NS) animals treated with EPA/DHA high dose. (F) EPA/DHA high dose animals were able to better discriminate the novel object from the familiar one in the NS group only. Data are presented as mean ± S.E.M, (n = 10 per group). Intergroup comparisons: * and **: $P < 0.05$ and $P < 0.01$ respectively compared to NS saline.

Temporal dynamics of stress-induced corticosterone levels & immune response

Early-life stress significantly decreased the plasma corticosterone response ($p<0.001$) when compared to the NS group (figure 3E). In contrast, the plasma levels of the pro-inflammatory cytokine TNF-α were significantly increased by early-life stress in the MS saline group only when compared to the control group ($p<0.05$). See figure 3F.

Protein extraction and Western Blot analysis

GR protein expression levels: nuclear and cytosolic fraction

MS did not influence the nuclear and cytosolic GR protein expression levels in the hippocampus when compared to the NS group. However, EPA/DHA 1g/kg/day decreased levels of GR protein into the hippocampal cytosolic fraction of NS rats ($p<0.05$) as represented in figure 3A. Furthermore, EPA/DHA high dose showed increased nuclear GR protein levels in the NS animals ($p<0.05$; figure 3B). GR activation, expressed by GR nucleus/cytosol ratio, was significantly higher in the EPA/DHA high dose group when compared to its respective NS control group ($p<0.01$; figure 3C).

mBDNF protein expression levels:

Hippocampal mBDNF protein expression levels were not influenced either by MS or by EPA/DHA treatments in any of the experimental groups (figure 3D).

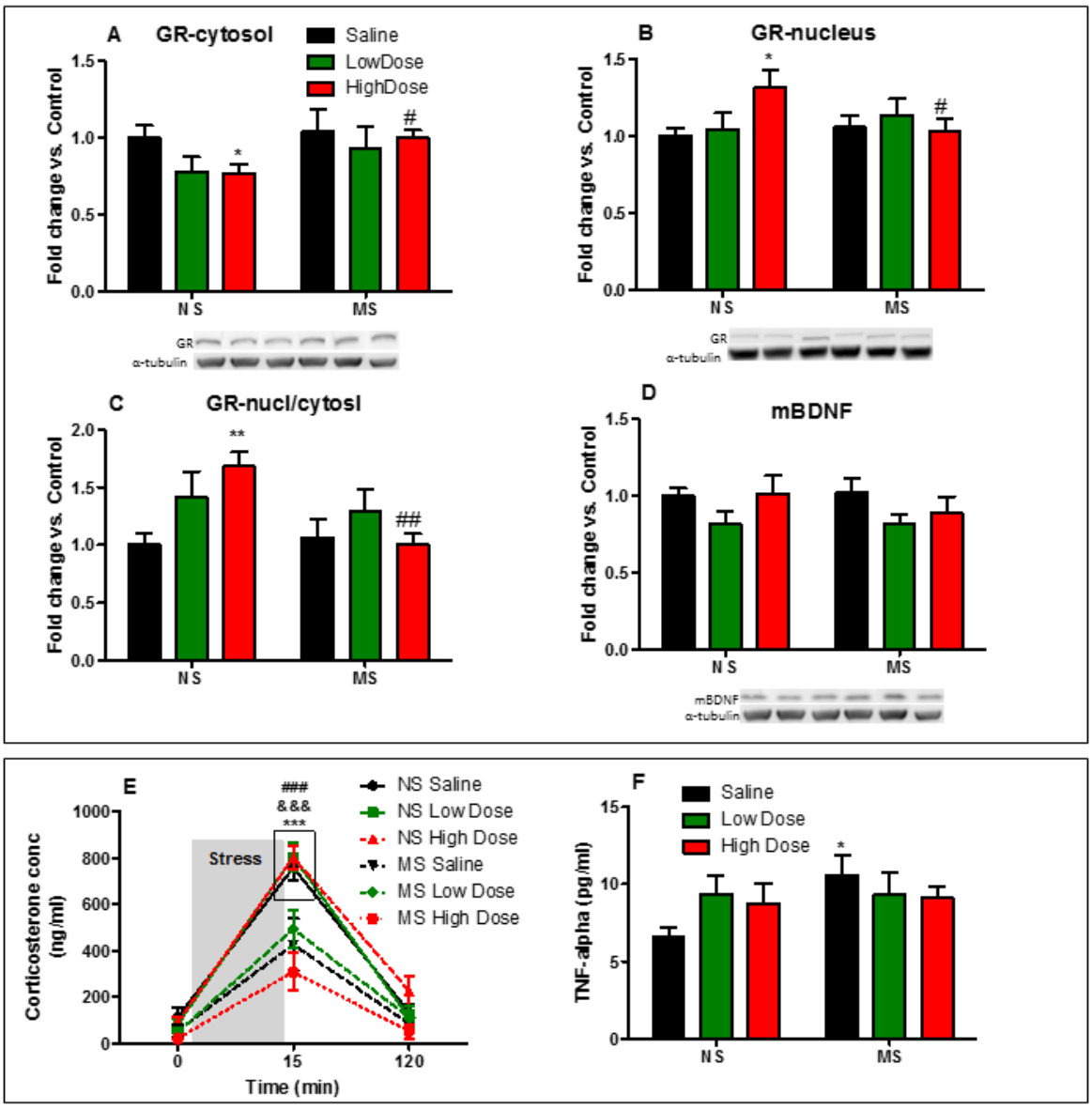


Figure 3. Effects of maternal separation (MS) and EPA/DHA administration on GR and BDNF protein expression levels, neuroendocrine and immune responses. (A) GR levels were reduced in the cytosol of non separated (NS) rats that were administered with EPA/DHA high dose. However, EPA/DHA effect was blunted by MS. (B) EPA/DHA high dose increased the levels of GR in the nucleic fraction of the hippocampus of non separated (NS) rats. Nonetheless, MS rats treated with EPA/DHA high dose showed lower levels of GR in the nucleus if compared to the NS counterpart. (C) GR nucleus/cytosol ratio was increased by EPA/DHA high dose when compared to its NS control group. (D) mBDNF protein levels remained unchanged after maternal separation and EPA/DHA treatment. (E) *S*tress-induced corticosterone levels were reduced in the MS groups when compared to the respective controls at time point 120min only. In contrast, no significant effect was exerted by EPA/DHA treatment in any of the groups. (F) MS increased the plasma levels of TNF-α when compared to the NS group. Data are expressed as mean ± S.E.M. ($n = 10$ per group). Intergroup comparisons: *, ** and ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively compared to NS saline; &&&: $P < 0.001$ compared to NS low dose; #, ## and ###: $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively compared to NS high dose.

Neurotransmitter concentrations

Early life stress influenced the levels of 5-HT and 5-HT turnover. 5-HT levels were elevated in the hippocampus and pre-frontal cortex of MS saline rats when compared to the NS group ($p < 0.05$). A consistent effect of MS was found in 5-HT turnover across all the three regions analysed with lower 5-HT turnover ratio found in the brain stem, hippocampus and pre-frontal cortex of MS saline group ($p < 0.05$). Whereas, EPA/DHA 1g/kg/day was able to increase 5HIAA levels in the brain stem of NS animals only ($p < 0.05$). See table 2.

Pre frontal cortex	**Non separated (NS)**			**Maternally separated (MS)**		
	Saline	Low dose	High dose	Saline	Low dose	High dose
NA (ng/g)	2019.13 ± 75.57	1898.39 ± 300.7	1886.22 ± 135.8	1892.33 ± 95.70	1962.82 ± 140.3	2178.38 ± 69.83
5-HT (ng/g)	521.55 ± 32.24	485.9 ± 72.95	607.8 ± 55.36	669.8 ± 30.69*	614.7 ± 39.52	641.6 ± 30.88
5-HIAA (ng/g)	657.9 ± 40.89	590.4 ± 73.75	672.5 ± 41.81	606.6 ± 30.50	700 ± 47.30	693.4 ± 32.12
5-HIAA\5-HT	1.33 ± 0.15	1.35 ± 0.16	1.14 ± 0.06	0.96 ± 0.06*	1.18 ± 0.11	1.09 ± 0.06
Hippocampus	**Non separated (NS)**			**Maternally separated (MS)**		
	Saline	Low dose	High dose	Saline	Low dose	High dose
NA (ng/g)	3307 ± 199.9	2914 ± 179.9	2832 ± 161.4	2552 ± 165.9**	2337 ± 141$^{\&}$	2141 ± 216.1$^{\#}$
5-HT (ng/g)	63.87 ± 10.98	80.68 ± 10.79	79.16 ± 14.20	127 ± 22.77*	137.1 ± 26.23$^{\&}$	137.1 ± 23.75$^{\#}$
5-HIAA (ng/g)	643.1 ± 23.67	649.5 ± 31.94	667.1 ± 34.19	612.5 ± 29.98	587.2 ± 34.34	625.6 ± 91.67
5-HIAA\5-HT	12.24 ± 1.51	9.12 ± 1.16	10.52 ± 1.62	6.76 ± 1.62*	5.75 ± 1.06	4.00 ± 0.77$^{\#}$

Brain stem	Non separated (NS)			Maternally separated (MS)		
	Saline	Low dose	High dose	Saline	Low dose	High dose
NA (ng/g)	3417 ± 118.2	3223 ± 115.1	3182 ± 104.7	3194 ± 125.3	2654 ± 94.4$^{\&,\dagger}$	2839 ± 101.5†
5-HT (ng/g)	573.3 ± 31.75	538.5 ± 24.13	609 ± 24.86	647.5 ± 29.57	655 ± 36.22$^{\&}$	660 ± 63.62$^{\#}$
5-HIAA (ng/g)	936.4 ± 40.23	978.6 ± 45.46	1071 ± 40.93*	969.5 ± 25.84	920.7 ± 37.05	995.5 ± 42.11
5-HIAA\5-HT	1.72 ± 0.10	1.82 ± 0.05	1.73 ± 0.10	1.46 ± 0.05*	1.43 ± 0.07$^{\&\&}$	1.6 ± 0.15

Table 2. Summary of the effects of maternal separation (MS) and EPA/DHA treatment on monoamine levels detected by HPLC in the prefrontal cortex, hippocampus and brain stem. Data are presented as the average mean of each experimental group ± S.E.M. (n = 10 per group). Intergroup comparisons: * and **: $P < 0.05$ and $P < 0.01$ respectively compared to NS saline; & and &&: $P < 0.05$ and $P < 0.01$ respectively compared to NS low dose; #: $P < 0.05$ compared to NS high dose; †: $P < 0.05$ compared to MS saline.

2.5. Discussion

The impact of lifetime dietary habits and their role on health, especially mental health represents an important topic for the discovery of alternative potential approaches for the prevention of stress-related psychiatric disorders (Jacka et al., 2014). In this study, we show that n-3 PUFAs exert some emotional and cognitive improvements on the reaction to stress exposure in normal animals. However, these benefits are not observed in animals who were exposed as pups to a maternal separation paradigm.

Our behavioural results show that a specific EPA/DHA dose (1g/kg/day) increased resilience to psychological stress as indicated by the elevated plus maze paradigm and antidepressant type effects in the forced swim test. Moreover, improvement in long term memory was shown by NS female rats treated with n-3 PUFAs during the novel object recognition task. In accordance with our observations, it has been already shown that rats deficient in n-3 PUFAs exhibit impaired performance on cognition and mood disorders as well as neurobiological effects associated with depression (Levant et al., 2008). Therefore, it is plausible to conclude that EPA/DHA as employed by the current study, can exert positive outcomes on stress-related behavioural responses following normal neurodevelopment. That n-3 PUFAs had little impact on animals subjected to MS is perhaps not surprising. Separation from the dam represents one of the most potent stressors for pups inducing alterations at different levels: animal behaviour (Berman et al., 2014), the HPA axis (Liu et al., 1997), the immune system (O'Mahony et al., 2009) and the brain-gut axis (O'Mahony et al., 2011). Furthermore, these previous studies were conducted in male animals, but we used female animals as females are more prone to depression and anxiety disorders. Results in human depressives indicate that n-3 PUFAs alone do not have antidepressant properties but may augment antidepressants (Su et al., 2003).

The responsivity of the HPA axis plays a pivotal role in behavioural abnormalities induced by stress (Lupien et al., 2009). In this study, evaluation of the HPA axis response to a novel stressful environment was assessed by measuring corticosterone levels via tail incision during behavioural testing. This is a well validated method for use during acute stress studies without confounding the measurements (Vahl et al., 2005). Moreover, the procedure is considered stress-free as indicated by the low, basal levels of corticosterone following repeat sampling in the absence of a stressor (Fluttert et al., 2000). Our laboratory has developed expertise in this well-validated approach and care is taken to ensure there was no visible blood contamination of the water during the FST. Indeed, after a small incision at the end of a rat's tail, blood clots quickly and water is replaced after each animal test. Nevertheless, we cannot completely exclude the possibility of microscopic blood contamination of the water, the odour of which could have impacted the animal's habituation in the FST.

Animals exposed to MS exhibited a blunted corticosterone response when exposed to an acute stress which is in contrast to many findings reported in the literature (O'Mahony et al., 2009, Roque et al., 2014). One possible factor contributing to the distinct outcomes regarding the effects of MS manipulation on stress responsiveness may be sex-related, since most of the studies reporting an enhanced neuroendocrine stress response were conducted in male rats (Aoki et al., 2010, Dalla et al., 2011, Simpson and Kelly, 2012). This is certainly the case in previous studies from our laboratory where we have reported such alterations (O'Mahony et al., 2009). Indeed, the focus on female animals in this study is an important and understudied aspect of maternal separation which is in line with NIH policy which seeks to reduce the overreliance on male animals in preclinical studies. Moreover, another potential contributing factor to the discrepant results may be related to the amount of maternal care, specifically maternal licking and/or grooming towards the pup, following separation. Evidence suggests that under normal conditions there is variation in maternal care both among dams as well as

within the same litter, with some pups receiving more care than others (Champagne et al., 2003). It has been proposed that maternal care might function as a buffer against the enhanced HPA axis reactivity of MS adult in response to stress (Macri et al., 2008). This represents an interesting consideration that we will pursue in future studies.

HPA axis hypoactivity has been previously reported both in models of post-traumatic stress disorder (PTSD) (Yehuda et al., 2005a, Cohen et al., 2006) and of atypical depression (Bowens et al., 2012). In many ways the current MS paradigm reflects atypical rather than melancholic depression when viewed through the lens of HPA axis activity. It is conceivable that early-life stress induces increased release of proinflammatory cytokines which in turn activate the HPA axis. Persistent activation of this axis may eventually lead to end organ burnout. Future studies need to examine these changes in a detailed longitudinal manner.

Hippocampal GRs together with mineralcorticoid receptors (MRs) are considered the cornerstone of the adaptive HPA axis functionality (Lupien et al., 2009). We showed that EPA/DHA 1g/kg/day increased the abundance as well as the migration of GRs into the nucleus of the hippocampus. It is tempting to speculate a possible GR-binding site interaction with EPA/DHA 1g/kg/day which could shed light on a novel n-3 PUFAs induced steroid-independent GR translocation. According to the "nucleocytoplasmic traffic" model, the GR, in its inactivated form, resides in the cytosol complexed with a variety of proteins including heat shock protein 90 (hsp90), the heat shock protein 70 (hsp70) and the protein FKBP52 (FK506-binding protein 52) which form a multimeric complex (Pratt et al., 2006). After activation, due to ligand binding, GRs dissociates from the multimeric complex and undergoes a conformational change. This allows GR translocation from the cytoplasm to the nucleus, where it binds to glucocorticoid response elements (GREs) in order to regulate selective gene transcription in a cell-specific manner (Hayashi et al., 2004). Thus, in this

study, one possibility is that EPA/DHA 1g/kg/day may have a direct effect on one or more hsps facilitating the dissociation of GRs from the multimeric complex and its translocation into the nucleus. Moreover, the observed EPA/DHA 1g/kg/day-induced GRs activation in the hippocampus of female rats may suggest phosphorylation of GRs. Specifically, phosphorylation of GRs induced by adenylate cyclase and protein kinase A activators have been suggested to have a relevant role in the regulation of GR function, for example increasing GR-mediated gene transcription (Rangarajan et al., 1992). That EPA/DHA 1g/kg/day activates specific enzymes involved in the regulation of GR function, such as adenylate cyclase and protein kinase A, may represent another plausible explanation of EPA/DHA 1g/kg/day-induced GRs activation in the hippocampus of NS female rats. Future studies are necessary to determine whether n-3 PUFAs exert similar effects. Interestingly, the cited mechanisms could explain the reduced anxiety- and depressive-like behaviour together with increased cognition observed in NS rats treated with EPA/DHA 1g/kg/day. Indeed, GR activation has been shown to be part of the mechanisms exerted by antidepressants (Anacker et al., 2011). However, in our study this effect disappeared in the hippocampus of MS animals. In contrast to other studies (Ferreira et al., 2013) both BDNF expression levels and body weight were not affected either by maternal separation or by EPA/DHA supplementation. This may be due to strain- and sex-effects, as well as differences in the study design.

Altered HPA axis function can be associated with dysregulation in noradrenergic (NA) and serotonergic (5-HT) systems (Ressler and Nemeroff, 2000). The present study revealed that NA content was markedly decreased in the hippocampus of MS animals. Of importance, changes to monoamines are considered to be of relevance in the regulatory developmental ontogeny of anxiety at critical time windows (Ressler and Nemeroff, 2000). Interestingly, increased levels of 5-HT and reduced serotonin turnover in the brain stem, hippocampus and

prefrontal cortex of MS female animals were found, which contrasts with our previous studies which found that MS produces an increase in serotonin turnover (O'Mahony et al., 2008) together with an increase in corticosterone levels in male rats (O'Mahony et al., 2009). The discrepancy between our previous and current findings may be explained by the fact that either underactivity or overactivity of the 5-HT system causes anxiety and mood disorders or by sex-specific effects as noted above. This 'inverted-U' function has been described (Calabrese and Baldwin, 2001). Moreover, Olivier et al showed that knockout of the serotonin transporter (SERT) which increases 5-HT in the brain (Frazer and Benmansour, 2002), may exert anxiety- and depressive-like behaviours in mice (Olivier et al., 2008). Concerning the impact of n-3 PUFAs in the monoaminegic system, no influences exerted by EPA/DHA mixture have been found in our study. This is in marked contrast to previous positive findings (Kodas et al., 2004). Our findings suggest that GRs but not the monoaminergic system are involved in the resilience to behavioural despair and to anxiety-like behaviour exerted by n-3 PUFAs in NS female rats.

In conclusion, clinical studies have shown that adverse early life experiences render individuals vulnerable to psychopathology later in life. Using an animal model we provided further evidence for such arguments, showing that MS may lead to subsequent changes in monoaminergic and immune systems as well as altered neuroendocrine response to stressors. Furthermore, this study enlarges our knowledge on the beneficial role of n-3 PUFAs on healthy subjects with limitations in clinical conditions.

Chapter 3

The omega-3 polyunsaturated fatty acid docosahexaenoic acid reverses corticosterone-induced changes in cortical neurons

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3.1. Abstract

Background: Chronic exposure to the glucocorticoid hormone corticosterone (CORT) exerts cellular stress-induced toxic effects which have been associated with neurodegenerative and psychiatric disorders. Docosahexaenoic acid (DHA) is a polyunsaturated fatty acid that has been shown to be of benefit in stress related disorders, putatively through protective action in neurons.

Methods: We investigated the protective effect of DHA against CORT-induced cellular changes in cortical cell cultures containing both astrocytes and neurons.

Results: We found that CORT (100, 150, 200uM) at different time points (48, 72 hours), induced a dose- and time-dependent reduction in cellular viability as assessed by methyl thiazolyl tetrazolium (MTT). Moreover, CORT (200uM - 72 hours) decreased the percentage composition of neurons whilst increasing the percentage of astrocytes as assessed by βIII-tubulin and GFAP immunostaining, respectively. In contrast, DHA (6uM but not 3uM) attenuated CORT (200uM)-induced cell death (72 hours). This translates into a capacity for DHA to prevent neuronal death as well as astrocyte overgrowth following chronic exposure to CORT. Furthermore, DHA (6uM) reversed CORT-induced neuronal apoptosis as assessed by TUNEL, and attenuated CORT-induced reductions in BDNF and CREB mRNA expression in these cultures. Finally, DHA inhibited CORT-induced down-regulation of GR expression on βIII- tubulin-positive neurons.

Conclusions: This work supports the view that DHA may be beneficial in ameliorating stress-related cellular changes in the brain and may be of value in psychiatric disorders.

3.2. Introduction

Docosahexaenoic acid (DHA, 22:6n-3) represents the most abundant omega-3 polyunsaturated fatty acid (PUFA) in the mammalian brain and as such it has been implicated in the regulation of brain structure, signalling and plasticity (Salem et al., 2001, Sharma et al., 2012, Crupi et al., 2013). Growing evidence points to a decreased dietary PUFA intake associated with an increased prevalence of mental illness, including stress-related diseases (McNamara et al., 2007, Levant et al., 2008, Mathieu et al., 2011). Post-mortem orbitofrontal cortex DHA levels are 22% lower in major depressive disorder (MDD) patients relative to normal controls (McNamara et al., 2007). Clinical observations have shown PUFA supplementation to be beneficial in reducing certain anxiety behaviours as well as in treating MDD (Yehuda et al., 2005b, Grosso et al., 2014b). Consistent with these findings, chronic PUFA supplementation promotes beneficial effects on anxiety, and depressive-like behaviours in stress-induced animals (Ferraz et al., 2011).

Glucocorticoid stress hormone levels are known to be associated with neuropsychiatric disorders including anxiety and depression (Murray et al., 2008). Although acute treatment with glucocorticoids has been shown to be neuroprotective in rats (Jeanneteau et al., 2008), their chronic effects are deleterious to the structural and functional plasticity of adult rat brain (Sapolsky and Pulsinelli, 1985, Zhu et al., 2013). Chetty et al have recently shown that elevated glucocorticoid levels can alter the cellular composition and white matter structure of rodent hippocampus (Chetty et al., 2014). Moreover, chronic administration of glucocorticoids induces a number of behavioural abnormalities similar to depression and anxiety in mice (Ardayfio and Kim, 2006, Murray et al., 2008).

The prefrontal cortex (PFC) is one of the most sensitive brain areas to the detrimental effects of stress exposure (Arnsten, 2009). It is also a target region for glucocorticoid effects, as it has a rich population of glucocorticoid receptors (GRs) (Chao et al., 1989, Barsegyan et al., 2010). High doses of glucocorticoids have been shown to impair PFC-dependent working memory in humans (Lupien et al., 1999). Chronic corticosterone (CORT) treatment has also been shown to produce neuronal impairment in the PFC, such as reduced distal dendritic spines of neurons and neuronal loss in rats (Wellman, 2001, Seib and Wellman, 2003, Cerqueira et al., 2005). Moreover, an inverse relationship between the levels of glucocorticoids and brain derived neurotrophic factor (BDNF), a neurotrophin involved in cellular plasticity and neuronal survival (Lipsky and Marini, 2007), has been described both in humans and rodents (Issa et al., 2010). Interestingly, Rao et al have shown that DHA, mostly concentrated in the frontal cortex and hippocampus of rat (Xiao et al., 2005), regulates frontal cortex BDNF levels (Rao et al., 2007b).

In view of the above, we hypothesized that chronic treatment with CORT would affect the composition and the viability of rat cortical cells. Moreover, we investigated whether DHA could abrogate CORT-induced toxic effects in primary cortical cell cultures that contained both astrocytes and neurons. Furthermore, we predicted that these effects would be related to molecular changes at BDNF and GR levels.

3.3. Methods

Primary cultures of postnatal day (PND) 1/2 rat cortex

All scientific procedures were carried out in line with Directive 2010/63/EU and were approved by the Animal Experimentation Ethics Committee of University College Cork #2012/036. Mixed cortical cell cultures, consisting of both neurons and astrocytes, were prepared from PND 1/2 Sprague Dawley rats (Biological Services Unit, University College Cork) as previously described (Long-Smith et al., 2010). Rats were decapitated, the cerebral cortices dissected out, and the meninges removed. Tissue was mechanically dissociated, incubated in trypsin (0.25 μg/ml, Sigma-Aldrich) for 15 min at 37 °C, with 5% CO_2. This was replaced with pre-warmed dissociation medium (DMEM-F12 supplemented with foetal calf serum (10%, Sigma-Aldrich) and DNase (0.1 mg/ml, Sigma-Aldrich)). The suspension was inverted gently for 1 min, triturated with a flame-polished Pasteur pipette and passed through a sterile mesh filter (40 μm). The suspension was centrifuged at 200 × *g* for 5 min at 20 °C, and the pellet was re-suspended in warm culture media (DMEM-F12 supplemented with foetal calf serum (1%, Sigma-Aldrich), B-27 (2%, Gibco), penicillin/streptomycin (1%), d-glutamine (1%, Sigma-Aldrich) and d-glucose (55mM, Sigma-Aldrich)). Suspended cells were plated at a density of 4.5×10^4 cells on circular 13-mm diameter coverslips, coated with poly-D-lysine (0.1 mg/ml, Sigma-Aldrich) in a 24 well plate and incubated in a humidified atmosphere containing 5% CO_2: 95% O_2 at 37 °C for 2 hours prior to being flooded with pre-warmed culture media. The day after, all cells were grown in culture medium containing ∝-tocopherol (40 μM), BSA (0.01%) and ethanol (0.3%). Thereafter, one-half of the media was replaced every 2/3 days for the duration of the experiment.

Treatment

Depending on experimental design, cells were treated with/without DHA after 1 DIV, CORT after 7, 8 or 9 DIV, and RU486 or spironolactone (SPIRO) after 7 DIV. Cells were also pre-treated with DHA 6 days before treatment with CORT, or cells were co-treated with CORT and RU486 (after 7 DIV), or with CORT and SPIRO (after 7 DIV) as shown in Figures 1D and 2B. To avoid DHA oxidation, one-half of the media was replaced every 2/3 days. DHA, CORT, RU486, SPIRO, α-tocopherol and BSA-fatty acids free were purchased from Sigma-Aldrich. All treatments were freshly prepared. Corticosterone was dissolved in ethanol.

Methyl thiazolyl tetrazolium (MTT) assay

Cell viability was assessed by measuring the reduction of methyl thiazolyl tetrazolium (MTT) by mitochondrial dehydrogenase of viable cells as previously described (Godinho et al., 2014). Medium was removed and replaced with 300 µL of fresh culture medium containing MTT reagent (1mg/ml) for 3 h at 37 ºC. The formazan crystals produced were dissolved in 100 µL of dimethyl sulfoxide (DMSO). Absorbance was measured at 590 nm using a UV plate reader. Four wells per treatment were analysed from three independent experiments. Results were expressed as % dehydrogenase activity compared to untreated controls.

Immunocytochemistry

Culture media was removed and cells were washed with pre-warmed Hank's Balanced Salt Solution (HBSS). Cells were fixed with ice-cold methanol for 10 min at − 20 °C and subsequently washed and permeabilised in phosphate buffered solution (PBS) containing Tween 20 (0.02%) (PBS-T) three times for 5 min. Cells were incubated in 5% normal horse serum in PBS-T overnight at 4 °C to attenuate non-specific antibody binding. Cells were

incubated overnight at 4 °C in antibodies that target βIII-tubulin-positive neurons (1:300; mouse monoclonal, Promega), glial fibrillary acidic protein (GFAP)-positive astrocytes (1:300; rabbit polyclonal, Dako), or glucocorticoid receptors (GR) (1:100; rabbit monoclonal, Cell signaling). Cells were subsequently incubated in the appropriate secondary antibody: Alexa Fluor 488 donkey anti-rabbit IgG or Alexa Fluor 594 donkey anti-mouse IgG (all 1:2000; Molecular Probes). Cells were counterstained with bisbenzimide (1:2500; Sigma-Aldrich) to identify the nuclei and mounted on to glass coverslips with an aqueous mounting medium (Dako). For each antibody, the cells from one coverslip were incubated in blocking solution and secondary antibody, without primary antibody (to account for nonspecific binding of the secondary antibody), and showed a complete absence of immunoflouresencent staining (data not shown).

Cell counts and densitometry

An upright microscope (AX70, Olympus, Hamburg, Germany) was used to analyse the immuno-positive cell composition of the primary culture as previously described (Green and Nolan, 2012). Immuno-positive cells were counted in five randomly chosen fields of view from each of the four coverslips, and divided by the total number of cells per five fields of view to give an average percentage for each coverslip. Only βIII-tubulin or GFAP-positive cells with a differentiated phenotype were counted. Four wells per treatment were analysed from three independent experiments. The densitometry of forty GR-positive neurons, selected from five randomly chosen fields of view from each of the three coverslips per treatment, was carried out using Image J software (Version 1.38X, NIH, Bethesda, MD, USA). For each photomicrograph, background measurements were subtracted from each GR-positive cell value to obtain a corrected fluorescence measurement. Each experiment was independently repeated three times.

TUNEL - Apoptosis

Fragmented DNA of apoptotic cells was measured using the terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) staining provided by the DeadEnd™ Fluorometric TUNEL System according to manufacturer's instructions (Promega). Cells were counterstained with bisbenzimide (1:2500; Sigma-Aldrich) to label nuclei and double stained with BIII-tubulin (1:300; mouse monoclonal, Promega) to detect apoptotic neurons. Apoptotic-positive cells and apoptotic-positive neurons were counted in five randomly chosen fields of view from each of the four coverslips, and divided by the total number of cells or the total number of neurons, respectively, per five fields of view to give an average percentage for each coverslip. Four wells per treatment were analysed from three independent experiments.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from mixed cortical primary culture using the ReliaPrep™ RNA Cell Miniprep system as per manufacturer's instructions (Promega). Briefly, cells were harvested using a denaturing lysis solution. The lysate was then transferred to a microcentrifuge tube in presence of isopropanol and centrifuged. The filter was then washed and finally the RNA was eluted using Nuclease-Free Water. Isolated RNA was stored at −80 °C until further processing. RNA concentration was quantified using the ND-1000 spectrophotometer (NanoDrop®) and RNA quality was assessed using the Agilent 2100 Bioanalyzer.

Analysis of RNA expression levels was carried as previously described (O' Connor et al., 2013). Briefly, equal amounts of RNA were first reverse transcribed to cDNA using High

Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, CA). Real-time PCR was performed using TaqMan Universal Master Mix II, no UNG, and TaqMan Gene Expression Assays designed by Applied Biosystems for rat genes on the ABI7300 Real Time PCR machine (Applied Biosystems, Warrington, UK). Each sample was analysed in triplicate, both for target gene and for endogenous control using the 7300 System SDS Software (Applied Biosystems, Life Technologies). All assays were controlled for the absence of genomic DNA amplification. β-actin (Actb, VIC/MGB Probe, Primer Limited, Applied Biosystems) was used as an endogenous control. Cycle threshold (Ct) values were recorded. The Ct value for the target gene in each sample was normalized to its endogenous control transformed to relative gene expression value using the $2^{-\Delta Ct}$ method (Simen et al., 2006). Four wells per treatment were analysed from three independent experiments.

BDNF protein detection levels by ELISA

Supernatant was collected from cortical cell cultures. Cortical cells where lysed using T-PER buffer (Thermo scientific) containing protease and phosphatase inhibitor cocktail (Roche). Cell extracts were obtained after centrifugation at 14 000 g for 10 min. Protein concentrations from each sample was then measured using a Pierce BCA protein assay kit (Thermo scientific) and normalized. Thus, BDNF levels were measured in cellular media and cortical cellular extracts using an ELISA kit according to the manufacturer's instructions (BDNF Emax Immunoassay (Promega Corp, Madison, WI, USA). Briefly, the monoclonal antibody (mAb) was added to each well of a 96-well plate and incubated overnight at 4 °C. The following were added to the wells sequentially: samples and BDNF standards in duplicate (incubated 2 h at room temperature); anti-human BDNF pAb (incubated 2 h at room temperature); anti-IgY HRP (incubated 1 h at room temperature); and, TMB solution

(incubated 20 min at room temperature). The plate was washed with Tris-buffered saline 0.05% Tween 20 (TBST) buffer. Finally, a stop solution was added and absorbance was read at 450 nm using a microplate reader (Bio-Rad Laboratories) within 30 min of the addition of the stop solution. BDNF concentration was calculated based on a standard curve. Values are expressed in pg/ml protein.

Statistics

All data are presented as mean ± SEM. Data were analysed using a one or two-way ANOVA as appropriate, followed by Fisher's LSD post-hoc test, using the statistical software package SPSS 21.0 (IBM). A *p*-value of 0.05 was selected as the threshold of statistical significance.

3.4. Results

MTT – Cell viability

We first analysed the effect of a range of CORT concentration (75, 100, 150, 200 uM) on dehydrogenase activity in rat mixed cortical cultures at different time points (24, 48, 72 hours) using the MTT assay (Figure 1A, B and C). Administration of CORT for a period of 24 hours did not induce changes in dehydrogenase activity at any of the concentrations used (Figure 1A). However, after both 48 and 72 hours CORT treatment (200 uM) significantly reduced dehydrogenase activity ($p<0.05$; $p<0.001$) whilst also reducing its activity after treatment with 150uM for 72 hours ($p<0.01$) (Figure 1B and C). In order to determine if GR or mineralcorticoid receptors (MR) mediated the effect, we co-treated cells with CORT (200 uM) and with the GR antagonist RU486 (10 uM), or the MR antagonist spironolactone (SPIRO, 50 uM) for 72 hours. RU486, but not SPIRO significantly attenuated the CORT-induced decrease in dehydrogenase activity ($p<0.01$) indicating that this effect is mediated by the GR (Figure 1E). Secondly, we analysed the effect of DHA on dehydrogenase activity and its potential protective effect against CORT (200uM, 72 hours). DHA (6 uM), induced an increase in dehydrogenase activity compared to untreated control cultures ($p<0.01$, Figure 2A). Moreover, this concentration of DHA reversed CORT-induced decrease in dehydrogenase activity in rat mixed cortical cultures ($p<0.05$) as shown in Figure 2C. DHA concentration higher than 6 uM showed cellular toxicity assessed by MTT (data not shown).

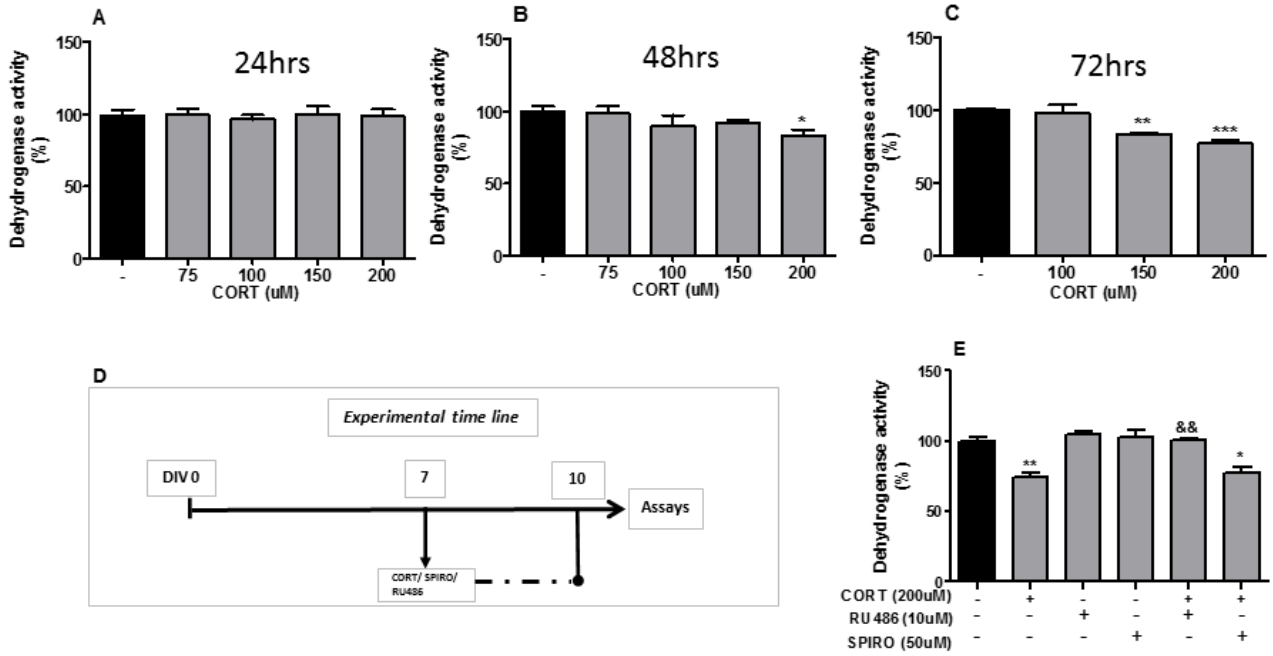


Figure 1. Corticosterone (CORT)-induced reduction in dehydrogenase activity in rat mixed cortical cultures is prevented by glucocorticoid receptor (GR) antagonist RU486. (**A**, **B** and **C**) CORT dose-dependent reduction in dehydrogenase activity at 24, 48 and 72 hours, respectively. (**D**) Schematic representing CORT, glucocorticoid receptor (GR) antagonist RU486, and mineralcorticoid receptor (MR) antagonist spironolactone (SPIRO) treatments (**E**) RU486 (10 uM), but not SPIRO (50 uM), blocks the decrease in dehydrogenase activity upon treatment with CORT. Intergroup comparisons: *, ** and ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively compared to control (-); &&: $P < 0.01$ compared to CORT (200 uM). Data are presented as mean ± S.E.M. ($n = 3$).

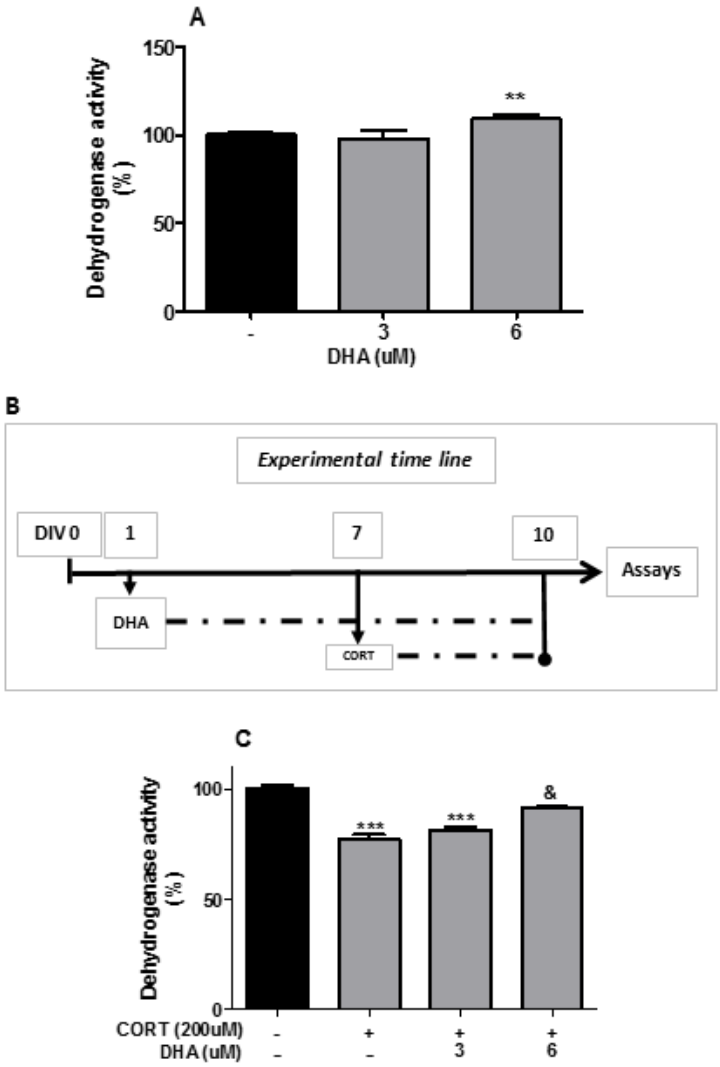


Figure 2. DHA prevented corticosterone (CORT)-induced reduction in dehydrogenase activity in rat mixed cortical cultures. (**A**) DHA dose-dependent increase in dehydrogenase activity. (**B**) Schematic representing CORT and DHA treatments. (**C**) DHA prevention of CORT-induced reduction in dehydrogenase activity. Intergroup comparisons: ** and ***: $P < 0.01$ and $P < 0.001$ respectively compared to control (-); &: $P < 0.05$ compared to CORT (200 uM). Data are presented as mean ± S.E.M. ($n = 3$).

Immunostaining – Quantification of cell phenotypes

In order to examine the effects of both CORT and DHA on the cellular composition of rat mixed cortical cultures, cells were treated with CORT (200 uM, 72 hours), DHA (6uM, 9 DIV) or pre-treated with DHA for 6 days before treatment with CORT, as described above. Immunocytochemistry for bisbenzimide, βIII-tubulin and GFAP was used to visualize cellular nuclei, post mitotic neurons and astrocytes, respectively (Figure 3A). CORT decreased the number of total cells ($p<0.05$) as shown in Figure 3B. Moreover, CORT exerted a bimodal effect on cellular composition. Specifically, CORT decreased the percentage composition of neurons ($p<0.001$) whilst increasing the percentage of astrocytes in culture ($p<0.01$) (see Figure 3C and D). DHA attenuated the CORT-dependent decrease in number of total cells stained with bisbenzimide. Furthermore, DHA reversed both the CORT-induced decrease in the percentage composition of neurons ($p<0.01$) and the increase in the percentage composition of astrocytes ($p<0.05$) in these cultures (Figure 3C and D).

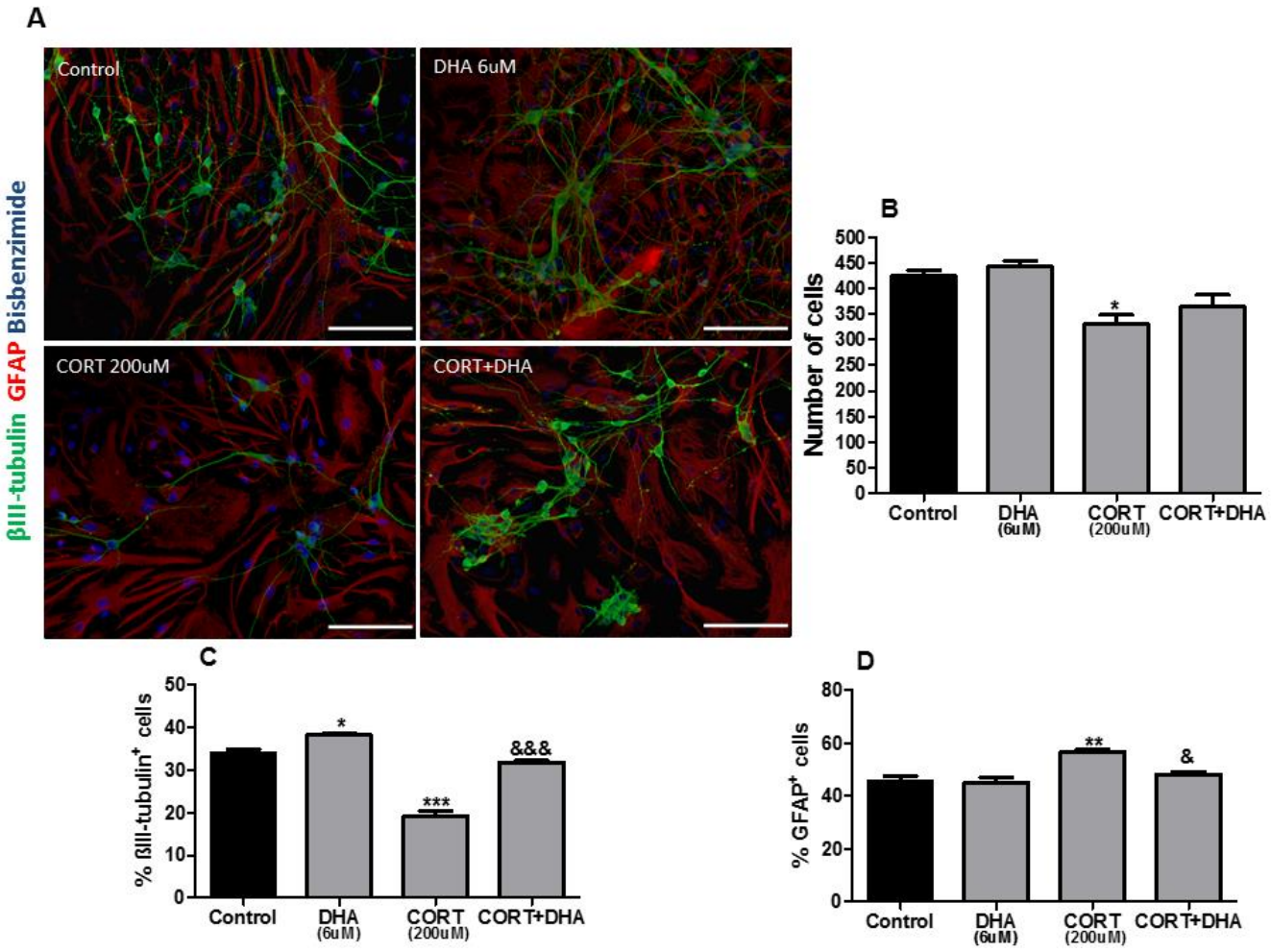


Figure 3. DHA prevented corticosterone (CORT)-induced changes in the cellular composition of rat mixed cortical cultures. (**A**) Representative photomicrographs of cells immunocytochemically stained for βIII-tubulin (green), GFAP (red) and bisbenzimide (blue). (**B**) Quantification of total cells stained with bisbenzimide. (**C**) Percentage composition of βIII-tubulin$^+$cells (**D**) and GFAP$^+$cells. Scale bar=50 μm. Intergroup comparisons: *and **: $P < 0.05$ and $P < 0.01$ respectively compared to control; & and &&&: $P < 0.05$ and $P < 0.01$ respectively compared to CORT (200 uM). Data are presented as mean ± S.E.M. ($n = 3$).

TUNEL – Apoptosis

In order to establish whether the CORT-induced reduction of the mitochondrial dehydrogenase activity is due to apoptosis, we analysed the percentage of total apoptotic cells as well as of apoptotic neurons in mixed cortical cultures (Figure 4A). Furthermore, we investigated a potential reversal effect exerted by DHA. CORT treatment (200 uM, 72 hours) induced an increase in the percentage of apoptotic cells (p = 0.079) as well as significantly increasing the percentage composition of apoptotic neurons (p<0.01). In contrast, pre-treatment with DHA (6 uM, 6 DIV) reversed the CORT-induced increase in total apoptotic cells (p<0.01) and in apoptotic neurons (p<0.01) (Figure 4B and C).

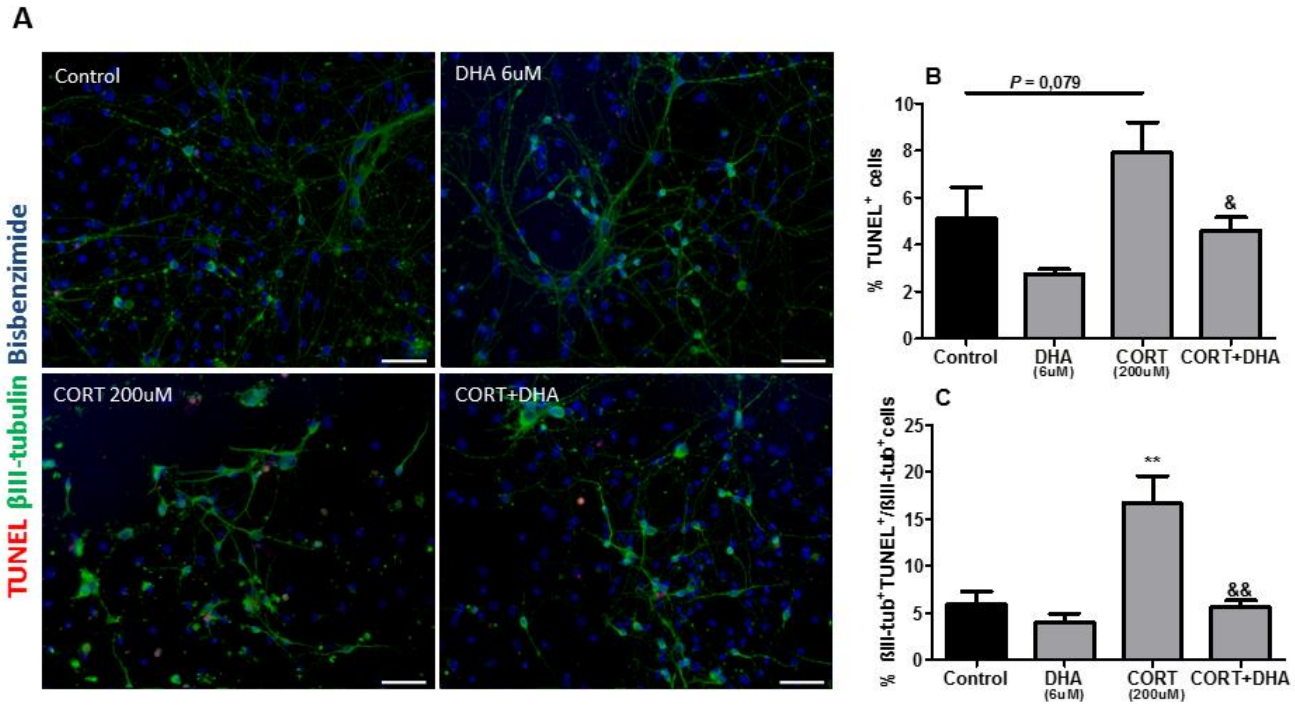


Figure 4. DHA prevented corticosterone (CORT)-induced apoptosis in rat mixed cortical cultures. (**A**) Representative photomicrographs of cells immunocytochemically stained for TUNEL (red), βIII-tubulin (green) and bisbenzimide (blue). (**B**) Percentage composition of TUNEL$^+$cells (**C**) and βIII-tub$^+$TUNEL$^+$/ βIII-tub$^+$cells. Scale bar=100 µm. Intergroup comparisons: **: $P < 0.01$ compared to control; & and &&: $P < 0.05$ and $P < 0.01$ respectively compared to CORT (200 uM). Data are presented as mean ± S.E.M. (n = 3).

Glucocorticoid receptor (GR) expression

Having established that DHA ameliorated CORT-related cellular changes in rat mixed cortical cultures, we wanted to establish the molecular mechanisms implicated in these effects. We focused specifically on the GR, as treatment with the GR antagonist RU486 prevented CORT-induced cell death. Immunocytochemical analyses revealed that CORT decreased the expression levels of GR in mature neurons ($p<0.01$). Interestingly, pre-treatment with DHA inhibited the CORT-induced down-regulation of GR expression on βIII-tubulin-positive neurons (Figure 5A, B).

BDNF and CREB1 expression

Subsequently, we wished to establish whether CORT-induced GR down-regulation in neurons is associated with reductions in the neurotrophin BDNF and the transcriptional factor cAMP response element-binding protein (CREB) mRNA levels and whether such effects are reversed by DHA intervention. In CORT-treated (200 uM, 72 hours) rat mixed cortical cultures we found a significant reduction in BDNF ($p<0.05$) as well as a negative trend in CREB expression levels ($p = 0.055$). DHA reversed the CORT-induced decrease in BDNF mRNA expression levels, as shown in Figure 5C and D.

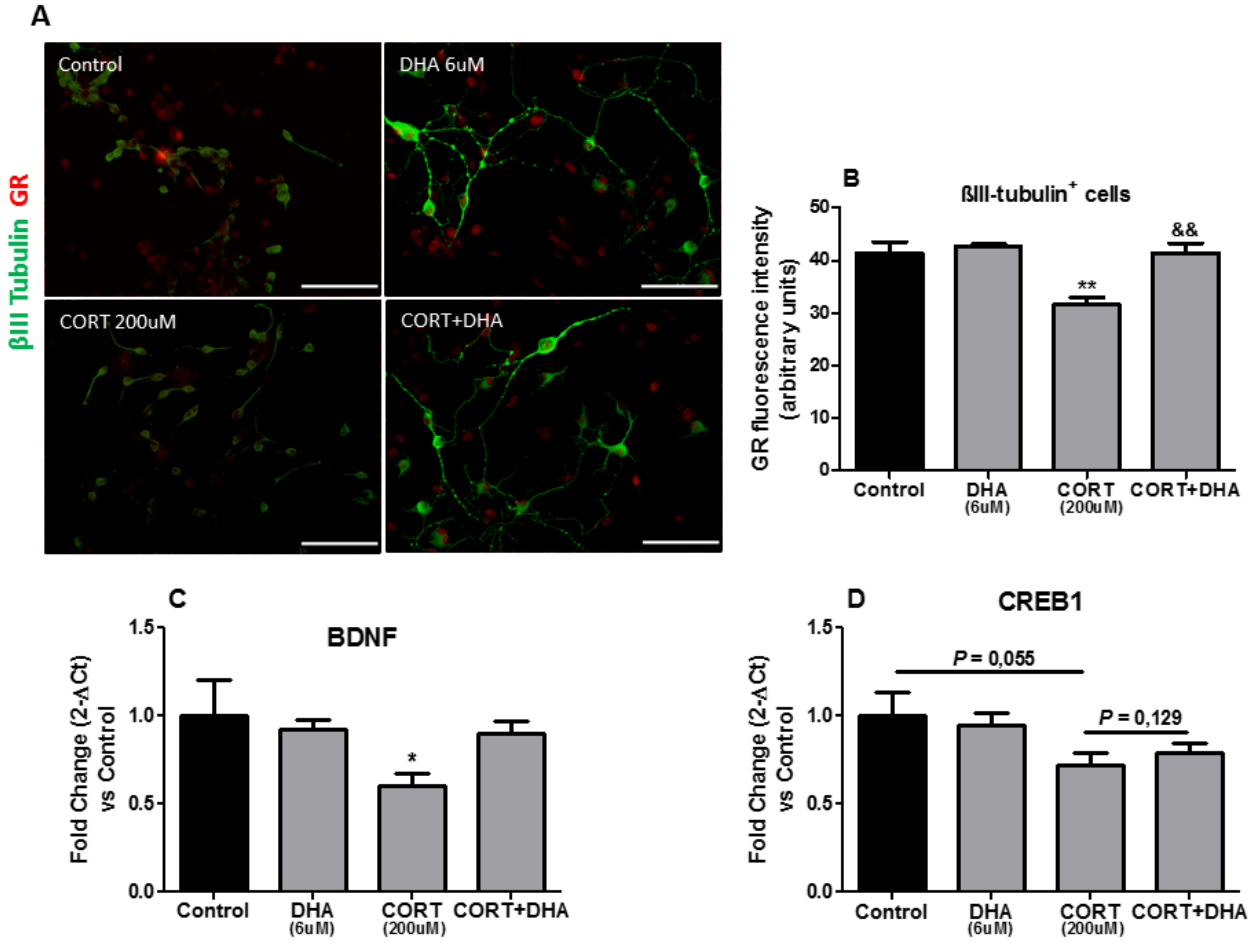


Figure 5. DHA prevented corticosterone (CORT)-induced changes in the glucocorticoid receptor (GR) and in the gene expressions of rat mixed cortical cultures. (**A**) Representative photomicrographs of cells immunocytochemically stained for βIII-tubulin (green) and GR (red). (**B**) Mean densitometry analysis of GR protein in βIII-tubulin^{+} cells. Scale bar=100 μm. (**C-D**) Relative BDNF and CREB1, gene expression from RNA extracted by rat mixed cortical cultures. Intergroup comparisons: **: $P < 0.01$ compared to control; &&: $P < 0.01$ compared to CORT (200 uM). Data are presented as mean ± S.E.M. ($n = 3$).

3.5. Discussion

The present study demonstrates a key role for DHA in reversing CORT-induced cellular changes in cerebral cortex. Specifically, we show that CORT-induced cellular toxicity exerts a bimodal effect on the neuronal and glial cell composition which effect is abolished by DHA treatment in vitro. We demonstrate that DHA reverses CORT-induced neuronal apoptosis, and attenuates CORT-induced reductions in BDNF expression levels in these cultures. We show that DHA inhibits CORT-induced down-regulation of GR expression on neurons.

We first demonstrated that CORT, in a dose- and time-dependent manner, reduces rat cortical cell viability. Blockade of this CORT-induced change by co-treatment of the cell culture with RU486, a GR antagonist but not with spironolactone, an MR antagonist, suggests that CORT exerts its effect on cell viability through the activation of the GR only. Moreover, our data show that chronic CORT treatment induces cortical neuronal cell death through apoptosis. These data are in agreement with several studies which report a CORT-dependent decreased cellular viability in different rat brain areas such as cortex (Liu et al., 2010a, b), hippocampus (Zhou et al., 2000) and hypothalamus (Zhang et al., 2012). Moreover, previous studies have suggested a differential effect of MR and GR on cell death in rodents (Behl et al., 1997, Almeida et al., 2000). For instance, it has been shown that GR-mediated apoptosis is counteracted by MR agonist aldosterone in hippocampal neurons, which apoptosis would lead to impaired regulation of the hypothalamic–pituitary–adrenal (HPA) axis and stress response (Crochemore et al., 2005). Moreover, $GR^{+/-}$ mice (expressing a 50% gene dose), exhibited reduced hippocampal neurogenesis and stress hypersensitivity (Ridder et al., 2005, Kronenberg et al., 2009). In our study, chronic CORT treatment down-regulated GR expression levels in cortical neurons. Interestingly, in addition to the hippocampus, a number of findings have implicated reduced forebrain GR levels in the dysregulation of the HPA axis and the development of affective disorders both in humans and mice (Holsboer, 2000, Boyle

et al., 2005, Boyle et al., 2006). Accordingly, disruption of forebrain GR has been associated with alterations in stress induced HPA axis activation leading to despair- and anxiety-like behaviours in GR conditional knock-out mice (Boyle et al., 2006). This is in agreement with clinical studies reporting abnormal GR isoform expression levels in the PFC of patients with psychiatric disorders like schizophrenia, bipolar and major depressive disorders (Sinclair et al., 2011, Sinclair et al., 2012, Qi et al., 2013).

Interestingly, our study showed differential CORT effects on the cellular composition of the rat mixed cortical primary culture. Indeed, the percentage composition of neuronal cells was reduced by CORT, whereas the percentage of astrocytes was increased. That CORT decreases the number of neurons is in line with earlier in vitro and in vivo investigations at hippocampal, cortical and hypothalamic levels (Sapolsky et al., 1985, Crochemore et al., 2005, Liu et al., 2010a, b, Zhu et al., 2013). However, evidence of the impact of glucocorticoids on glial cells is limited. Anacker et al have shown a cortisol-induced shift of progenitor cell fate from neuronal- to astrocyte-differentiation using immortalized, multipotent human foetal hippocampal progenitor cell line HPC03A/07 (Anacker et al., 2013). Moreover, the authors suggested the activation of Notch- and Hairy/Enhancer of Split (Hes)-signaling as pathway potentially involved in the observed cortisol-induced shift from neurogenesis to astrogliogenesis. Indeed, the Notch-Hes pathway has been reported to be crucially involved in changing neuronal- to astroglial cell fate (Kageyama and Ohtsuka, 1999). Bridges et al, have shown that chronic CORT administration, over a period of 21 days, increases astrocyte numbers in the CA1 hippocampal region of both male and female rats (Bridges et al., 2008). Accordingly, it has been shown that stress increases the hippocampal glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes by 30% compared to control (Lambert et al., 2000). However, the meaning of astrocytes overgrowth subsequent to stress or CORT treatment remains unknown. This phenomenon could be considered as part of a

greater neuronal damage by impeding the neuron-glia interaction. Indeed, astrogliosis has been shown to impede the movement of substances through the extracellular space (ECS), altering the extra-synaptic communication between glia and glia, and glia and neurons (Sykova, 2001, Sykova et al., 2002, Sykova, 2004, 2005). This may also provide further understanding in regards to impairments in cognition and emotional state observed as consequence of chronic stress (Lupien et al., 2009). Noteworthy, alteration in neuronal and glial cell loss, cell atrophy or increased number of cells have been reported post mortem in psychiatric disorders (Rajkowska, 2000).

Moreover, we demonstrated that chronic CORT treatment decreases BDNF and CREB cortical expression levels, both of which are involved in neuronal survival and cell plasticity (Lipsky and Marini, 2007). This is in agreement with several studies showing decreased BDNF levels subsequent to chronic CORT treatment both in the rat frontal cortex (Dwivedi et al., 2006) and hippocampus (Schaaf et al., 1998, Schaaf et al., 2000, Dwivedi et al., 2006). Of note, pharmacological inhibition of GR deactivates the CamKII-BDNF-CREB pathway which mediates long-term memory formation in hippocampal adult rats (Chen et al., 2012).

Regarding the reversal effect exerted by DHA, we found that a specific DHA dose (6uM) prevented CORT cellular death and neuronal apoptosis. These data are in agreement with other studies which showed increased neuronal survival as well as prevention of cortical neuronal apoptosis in primary culture, after DHA treatment (Cao et al., 2005, Florent et al., 2006). Accordingly, increased DHA content in the brain has been found to protect against several neurotoxic insults such as ischemia (Belayev et al., 2009), glutamate excitotoxicity (Blondeau et al., 2002, Ozyurt et al., 2007), and traumatic head injury (Wu et al., 2004).

Moreover, DHA attenuated CORT-induced reduction in BDNF expression level which may be a mechanism by which DHA reverses CORT-induced cellular toxicity. Indeed, DHA has been shown to increase CREB and BDNF levels both *in vitro* (Rao et al., 2007b) and *in vivo* (Lipsky and Marini, 2007, Rao et al., 2007b, Wu et al., 2008) suggesting potential neurotrophic and neuroprotective properties. Of note, other neuroprotective agents, such as curcumin, have already shown reversal in CORT-induced cortical and hippocampal BDNF levels as well as antidepressant-like behavioural effect in rats (Huang et al., 2011b). Moreover, treatment with DHA impeded GR down-regulation induced by CORT, which receptor has been shown to be involved in the regulation of BDNF levels in the rat hippocampus (Chen et al., 2012).

In conclusion, clinical studies have shown that high levels of glucocorticoids induce vulnerability to psychopathology. Using an in vitro model, we provided further evidence for such arguments, showing that CORT may lead to subsequent changes in cortical neuron and astrocyte composition, BDNF and CREB expression levels as well as GR down-regulation in mixed cortical cultures. Furthermore, this study contributes to our knowledge on the beneficial role of PUFAs that may be considered as future treatment strategies to counteract glucocorticoids-induced impairments in clinical settings.

Chapter 4

n-3 Polyunsaturated Fatty Acids (PUFAs) Reverse the Impact of Early-Life Stress on the Gut Microbiota

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4.1. Abstract

Background

Early life stress is a risk factor for many psychiatric disorders ranging from depression to anxiety. Stress, especially during early life, can induce dysbiosis in the gut microbiota, the key modulators of the bidirectional signalling pathways in the gut-brain axis that underline several neurodevelopmental and psychiatric disorders. Despite their critical role in the development and function of the central nervous system, the effect of n-3 polyunsaturated fatty acids (n-3 PUFAs) on the regulation of gut-microbiota in early-life stress has not been explored.

Methods and Results

Here, we show that long-term supplementation of eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) (80% EPA, 20% DHA) n-3 PUFAs mixture could restore the disturbed gut-microbiota composition of maternally separated (MS) female rats. Sprague-Dawley female rats were subjected to an early-life stress, maternal separation procedure from postnatal days 2 to 12. Non-separated (NS) and MS rats were administered saline, EPA/DHA 0.4 g/kg/day or EPA/DHA 1 g/kg/day, respectively. Analysis of the gut microbiota in adult rats revealed that EPA/DHA changes composition in the MS, and to a lesser extent the NS rats, and was associated with attenuation of the corticosterone response to acute stress.

Conclusions

In conclusion, EPA/DHA intervention alters the gut microbiota composition of both neurodevelopmentally normal and early-life stressed animals. This study offers insights into the interaction between n-3 PUFAs and gut microbes, which may play an important role in advancing our understanding of disorders of mood and cognitive functioning, such as anxiety and depression.

4.2. Introduction

Stress, especially in early life has been identified as a cause of the disruption of this developmental pattern leading to a variety of disorders ranging from gastrointestinal disorders (Mayer et al., 2014b), to anxiety and depression (Borre et al., 2014). In rodents, the maternal separation (MS) model is a well-known paradigm that induces brain-gut axis dysfunction (O'Mahony et al., 2011). The separated phenotype alters many components of the brain-gut axis throughout the body including the hypothalamic–pituitary adrenal (HPA) axis (O'Mahony et al., 2011), the immune and neuroendocrine systems (O'Mahony et al., 2009). Growing evidence considers these abnormalities comorbid with changes in the gut microbiota (Diaz Heijtz et al., 2011, Clarke et al., 2014, El Aidy et al., 2015) as well as crucial risk factors for the development of mental illnesses such as anxiety and depression (Dinan and Scott, 2005, Bakunina et al., 2015).

There is increasing evidence suggesting a bi-directional communication between the central nervous system (CNS) and the gut-microbiota which is recognized as the microbiome–gut–brain axis (Cryan and Dinan, 2012, Bercik and Collins, 2014, Mayer et al., 2014a). This communication is believed to influence the parallel development of both CNS and gut microbiota which can remarkably influence health and disease (Moloney et al., 2014). Emerging evidence has shown the involvement of the gut microbiota in maternal stress and maternal separation in brain and associated behaviour (Bailey and Coe, 1999). Prenatal stress has been shown to change the composition of the microbiome in adult rat (Golubeva et al., 2015) neonatal mice (Jasarevic et al., 2015) and infant humans (Zijlmans et al., 2015). Changes in the gut microbiota composition were reported in monkeys subjected to maternal separation between six and nine months of age with shedding of lactobacilli three days following separation, followed by the return of normal lactobacilli levels seven days later. Moreover, we have previously shown, albeit using somewhat crude Denaturing Gradient Gel

Electrophoresis- based analysis, that adult rats that underwent maternal separation showed altered faecal microbial composition compared with normally reared control animals (O'Mahony et al., 2009).

It is well recognized that eating habits are of relevance to (mental) health (Logan and Jacka, 2014). Moreover, there is a growing appreciation for the impact of dietary fatty acids on the intestinal microbiota composition of the host (Patterson et al., 2014, Yu et al., 2014, Marques et al., 2015). Being critical in the development and function of the CNS, n-3 polyunsaturated fatty acids (n-3 PUFAs) have been under the spotlight for decades (Grosso et al., 2014a). The possible underlying mechanisms by which n-3 PUFAs exert their beneficial effects on health are diverse, involving for instance, HPA axis, neuroendocrine and immune regulations (Song, 2013, Dyall, 2015).

In light of these observations, we reported recently the beneficial effects of n-3 PUFAs on reduction of anxiety-like, depressive-like behaviours and improved cognition in female rats (Pusceddu et al., 2015). Here we hypothesize that these beneficial effects of long-term intake of n-3 PUFAs would have an impact intestinal microbiota populations, which in turn, would contribute to the reverse of gut-brain axis dysfunction associated with maternal separation. To the best of our knowledge, this is the first study to describe the impact of n-3 PUFAs on the gut-microbiota of female rats exposed to early-life stress.

4.3. Methods

Maternal separation

Maternal separation was performed as previously described by our group (O'Mahony et al., 2008, Pusceddu et al., 2015). Briefly, male and female rats were obtained from Harlan Laboratories UK (250 – 300 g) and mated in the local animal unit. Food and water was available *ad libitum* and animals were maintained on a 12:12-h dark–light cycle with temperature at 20 ± 1°C. MS animals were separated from their mothers from postnatal day (PND) 2 to 12, for three hours a day. Separations were conducted between 0900h and 1200h a.m. in plastic cages placed on top of heater pads (30 - 33 °C) in a separate room to the main holding room. Non-separated (NS) animals were left undisturbed in their home cages with their respective dams and were returned to the holding room. After postnatal day 12, rats were left undisturbed except for routine cage cleaning every two days and a weekly body weight measurement until they were 5 weeks old. Animals were group-housed 5 per cage in plastic cages with sawdust bedding in an enriched environment with shredded paper and a cardboard roll. All experiments were in full accordance with the European Community Council Directive (86/609/EEC).

Treatments

Oral administration of an eicosapentaenoic acid (EPA)/docosaexaenoic acid (DHA) (80 % EPA, 20 % DHA) n-3 PUFAs mixture was administered by gavage when animals reached 5 weeks of age. In order to avoid any confounding litter effects, individual groups consisted of rats from multiple litters. Treatments consisted of 1) saline water; 2) EPA/DHA 0.4 g/kg/day or Low Dose (LD); 3) EPA/DHA 1 g/kg/day or High Dose (HD). The chosen EPA/DHA concentrations were based on the Food and Agriculture Organization of the United Nations (FAO) recommendations. FAO recommends a minimum DHA intake of 10-12 mg/kg per body weight for children 6-24 months old and EPA/DHA 100-250 mg/day for children aged

2-10 years. In our study, the maximum EPA/DHA intake was 100 and 250 mg for the low dose and the high dose per body rat, respectively. Moreover, previous studies have used the same concentrations proposed in this study (Calviello et al., 2004, Yilmaz et al., 2004). Treatments were prepared freshly every day and administered between 0900h and 1100h a.m. The experimental time line is shown in Figure 1A.

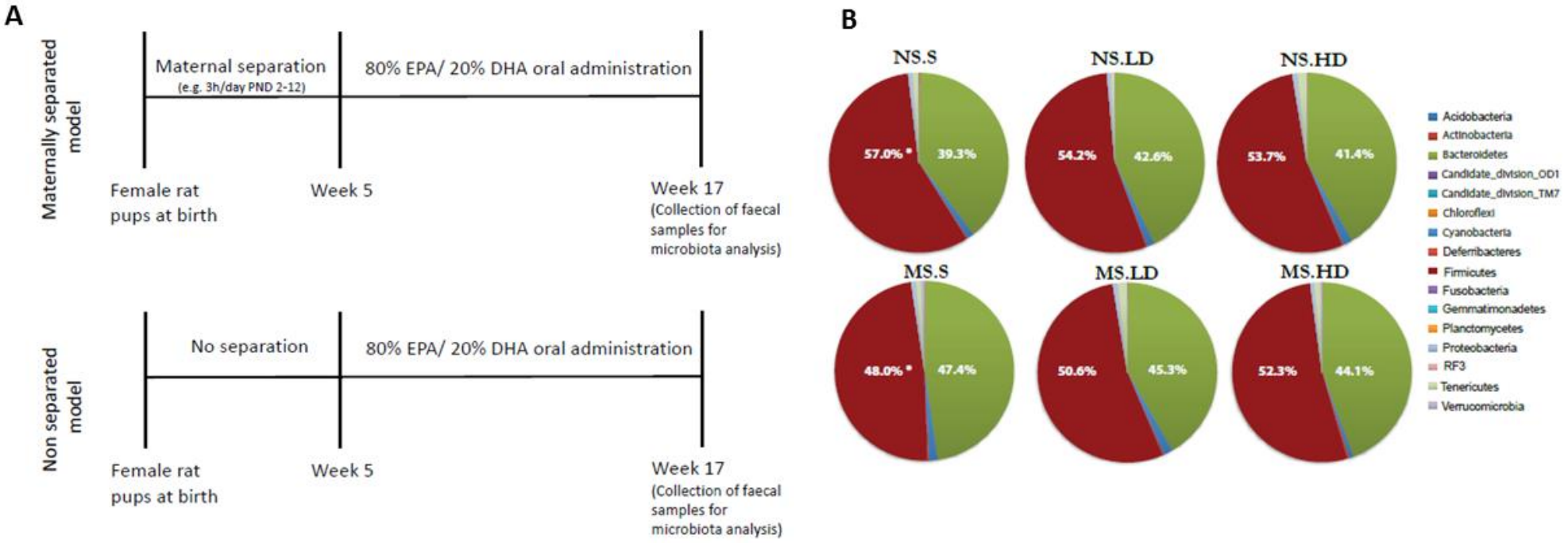


Figure 1. (A) Schematic representation of the time course of the maternal separation procedure and EPA/DHA treatment. (B) Global average microbial composition of faecal 17 weeks old rats samples (n = 10 per group) at phylum-level. * Indicate bacterial group significantly different between MS and NS groups.

Sample collection

Faecal pellets were collected from 17 weeks old female rats. All samples were, then, frozen at −80°C for microbiota analysis.

Microbiota analysis

DNA was extracted using the DNA Fast Stool DNA extraction kit (Qiagen) using the protocol for Gram positive bacteria and including an additional bead beating step at the beginning of the procedure. DNA was quantified using the Qubit High Sensitivity Kit (Life Technologies), standardised and then used as a template for PCR. PCR primers and conditions are essentially as outlined in the Illumina 16S Metagenomic Sequencing Library preparation guide (Illumina) with the following exceptions: For the initial 16S PCR, the PCR was performed in duplicate 50 ul reaction volumes, and 40 cycles were used in the PCR. Products were then pooled, cleaned with an appropriate volume of Ampure beads and eluted in 30ul/sample. This was then used as the template for the index PCRs as outlined in the protocol (Illumina). Library quantification, normalisation, and pooling were as outlined in the protocol. After pooling, the sample was requantified using the Qubit High Sensitivity Kit (Life Technologies) and run on an agilent high sensitivity chip (Agilent). Library denaturation and MiSeq sample loading were then performed as described in the protocol. The final concentration of the library was 4pM and PhiX was spiked in as a control at 5% v/v. A 2 x 300bp MiSeq reagent was used for sequencing. Diversity analyses were performed in QIIME and correlations used the websites Calypso at http://bioinfo.qimr.edu.au/calypso.

4.4. Results

Long-term supplementation of EPA/DHA restores the microbiota composition in the MS rats

In this study, the microbial composition of the faecal samples collected from MS and NS EPA/DHA treated and saline rats revealed significant changes in the relative abundance of the main dominant phyla; Bacteroidetes and Firmicutes between the MS-saline and NS-saline (Figure 1B). These data suggest a state of microbial dysbiosis in MS-saline group. The results coincide with our recent findings that early life stress was associated with induced inflammatory cytokines in plasma (Pusceddu et al., 2015). Indeed, a recent study using bacterial tag encoded FLX amplicon pyrosequencing demonstrated that repeated social stress, associated with elevated levels of inflammatory cytokines decreased the relative abundance in cecal bacteria of the genus Bacteroidetes, while increasing the relative abundance of bacteria in the genus Firmicutes (Bailey et al., 2011). Moreover, reduced Bacteroidetes: Firmicutes ratio in human stool specimen has also been shown in depressed individuals as well as in irritable bowel syndrome (IBS) patients, which is often accompanied by depressive symptoms (Jeffery et al., 2012, Jiang et al., 2015). Interestingly, long-term administration of EPA/DHA reversed the early-life stress-induced Bacteroidetes: Firmicutes shift in MS adult rats (Figure 1B). Presumably, this shift suggests an anti-inflammatory effect (Troy and Kasper, 2010) of EPA/DHA supplementation, as we previously reported (Pusceddu et al., 2015).

Long-term EPA/DHA supplementation shifts the microbiota composition in MS rats towards a profile similar to that in NS rats regardless of the dose

The difference of the global microbiota composition from the 16S rDNA data of the six groups was assessed by ordination (Figure 2A). Statistics based on random permutations of

the redundancy analysis (RDA) showed that the MS-saline group can significantly be separated at genus level ($p<0.001$) from the MS EPA/DHA treated groups and the NS-saline and EPA/DHA treated groups. The centroids of the MS-saline and NS groups were clearly separated; whereas the MS EPA/DHA treated groups were in an intermediate position between the MS-saline and NS groups. Long-term supplementation of low dose of EPA/DHA in NS rats appears to have a different impact on the microbiota composition when compared to the NS-saline and NS-HD groups. Together, the results point to possible interactions between EPA/DHA and members of the gut microbiota, which may eventually influence their biological roles. In fact, *in vitro* interactions of PUFAs with some probiotics have been shown to affect the growth and adhesion of different Lactobacillus strains (Kankaanpaa et al., 2001).

Statistical analyses of the differences of microbiota composition among the six groups at genus level identified several taxa differentially present between the MS, NS EPA/DHA treated and saline rats (Table 1). The significance of the taxa *Akkermansia, Rikenella, Prevotella,* and *Flexibacter* are among the main discriminants between MS-saline, MS EPA/DHA treated and NS groups (Figure 2B). The relative abundance of *Akkermansia* was induced by maternal separation suggesting elevated inflammatory response, as we recently reported (Pusceddu et al., 2015). In an inflammatory milieu, *Akkermansia*, the mucus degrader, facilitates the microbial translocation to come in direct contact with the intestinal epithelium, therefore exacerbating gut inflammation (Stecher et al., 2007, Ganesh et al., 2013). The reduced abundance of Akkermansia in EPA/DHA treated groups coincide with the findings reporting that high concentrations of PUFAs inhibited growth and adhesion to mucus of several bacterial strains (Kankaanpaa et al., 2001). Similarly, *Flexibacter*, a member of Bacteroidetes, was elevated in the microbiota isolated from tissue specimens of a subset of patient with Crohn's disease and ulcerative colitis (Frank et al., 2007). *Prevotella*

was shown to increase the sensitivity to chemically induced colitis in experimental mice (Scher et al., 2013). Collectively, the data reveals the abundance of members of the gut microbiota previously reported to be associated with a state of inflammation, in MS rats. Long-term n-3 PUFAs supplementation appears to restore the microbial balance to a state similar to that in NS rats. Therefore, it is tempting to speculate a possible EPA/DHA anti-inflammatory effect through the regulation of the gut microbiota composition.

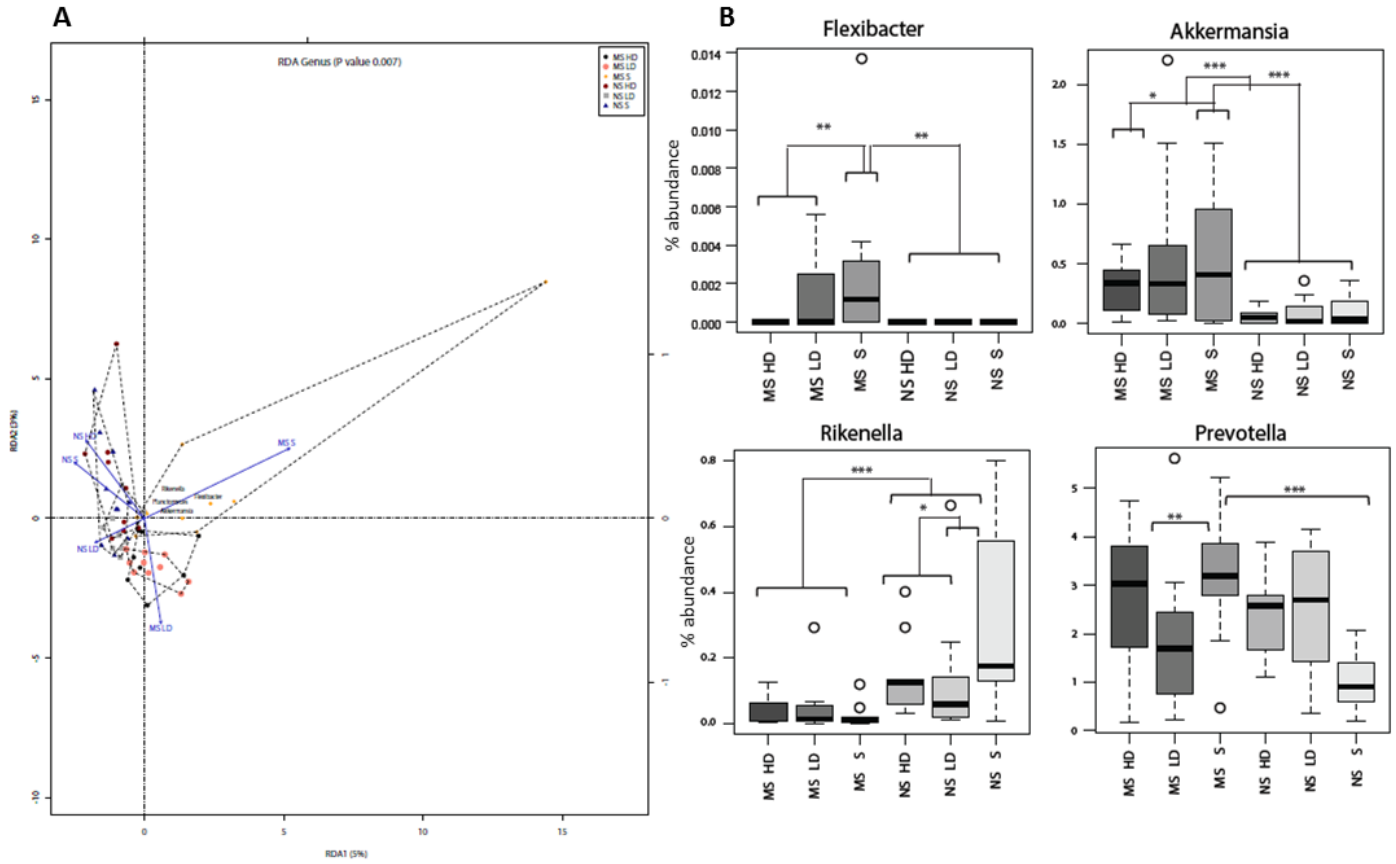


Figure 2. (A) Redundancy analysis (RDA) based on the genus level showing a significant separation between the EPA/DHA treated and saline MS groups (P=0.007). The hulls identify the centroids of each dataset. (B) Relative abundance of selected genera in the MS and NS saline and EPA/DHA treated groups.

Table 1. Significant differentially abundant taxa between MS and NS EPA/DHA treated and saline groups as calculated by Wilcoxon rank test at genus level, indicated by the p-value. Values for the six groups are medians of the relative abundance of the indicated genus (% of all sequences). The FDR q-values are adjusted p-values that correct for multiple testing at a defined false discovery rate (Benjamini et al., 1995).

Taxa	P	FDR qValue	Median NS S	Median NS LD	Median NS HD	Median MS S	Median MS LD	Median MS HD
Planctomycetes-Phycisphaerae-WD2101	0.00045	0.0319	0	0	0	0.00484	0.00325	0.011374
Rikenellaceae-Rikenella	0.00052	0.0319	0.175714	0.058656	0.124997	0.012741	0.014224	0.032709
Acidobacteria-Acidobacteria_bacterium	0.00066	0.0319	0	0	0	0.002806	0.002105	0.001315
Aerococcaceae-Aerococcus	0.0013	0.047125	0	0	0	0	0	0.001569
Cytophagaceae-Flexibacte	0.0018	0.0522	0	0	0	0.001165	0	0
Burkholderiaceae-Cupriavidus	0.0025	0.059813	0	0	0	0.002325	0	0
Oxalobacteraceae-Massilia	0.0029	0.059813	0	0	0	0.000695	0	0
Xanthomonadaceae-Rhodanobacter	0.0033	0.059813	0	0	0	0	0	0.00215
Planctomycetaceae-Planctomyces	0.0053	0.076962	0	0	0	0.001545	0	0.00052
Chitinophagaceae-uncultured	0.0057	0.076962	0	0	0	0	0	0.00131
Acidobacteria-DA023-uncultured_bacterium	0.0059	0.076962	0	0	0	0.001602	0	0.000259
Acidobacteria-DA023-uncultured_bacterium	0.0067	0.076962	0	0	0.000315	0.001149	0	0.00271
Verrucomicrobiaceae-Akkermansia	0.0069	0.076962	0.034586	0.017324	0.048543	0.41013	0.330376	0.336236
Mollicutes-RF9-uncultured	0.0082	0.080233	0.257038	0.334292	0.392858	0.249543	0.21356	0.114447
Acidobacteriaceae-Candidatus	0.0083	0.080233	0	0	0	0.00291	0	0
Prevotellaceae-Prevotella	0.0092	0.083375	0.9102	2.68956	2.576339	3.190481	1.688466	3.033729
Spartobacteria-Chthoniobacterales	0.025	0.1885	0	0	0	0.00914	0.006015	0.00289
Caldicoprobacteraceae- Caldicoprobacter	0.026	0.1885	0.019758	0.010302	0.012136	0.00734	0.00214	0.002545
Rikenellaceae-Alistipes	0.037	0.2262	0	0	0	0	0	0.000525
Clostridiaceae-Clostridium	0.039	0.2262	0.167448	0.154021	0.194113	0.13282	0.086339	0.103405
Ruminococcaceae-uncultured_bacterium	0.039	0.2262	0.001072	0.00103	0.000944	0	0	0
Bacillaceae-Bacillus	0.043	0.239808	0	0	0	0.000396	0	0

Low corticosterone levels in MS rats is correlated with Akkermansia and Rikenella

Recently, we showed that stress-induced corticosterone levels were reduced in MS group compared to the respective controls when exposed to acute stress (Pusceddu et al., 2015). In order to investigate a possible correlation between the altered microbiota in the gut of MS-saline group and the HPA axis, regression analysis was performed between corticosterone levels and the gut microbiota of MS and NS groups. Interestingly, a negative correlation with *Akkermansia* (R=0.4097, P=0.0019) and positive correlation with *Rikenella* (R= 0.4481, P=6e-04) was observed (Figure 3). While *Rikenella* was reported to be associated with reduced risk of colitis (Couturier-Maillard et al., 2013)(Couturier-Maillard, J Clin Invest. 2013 Feb 1; 123(2): 700–711), *Akkermansia* has been previously shown to exacerbate gut inflammation in mice (Stecher et al., 2007, Ganesh et al., 2013). Thus, we consider that the observed high *Akkermansia* abundance in MS rats may contribute to elevated levels of inflammation,, which in turn may activate the HPA axis as recently shown (Pusceddu et al., 2015). In view of that, persistent activation of HPA axis may eventually lead to end organ burnout and consequent lower CORT release. Accordingly, HPA axis hypoactivity has been previously reported in other animal models of stress (Yehuda et al., 2005a, Cohen et al., 2006, Bowens et al., 2012).

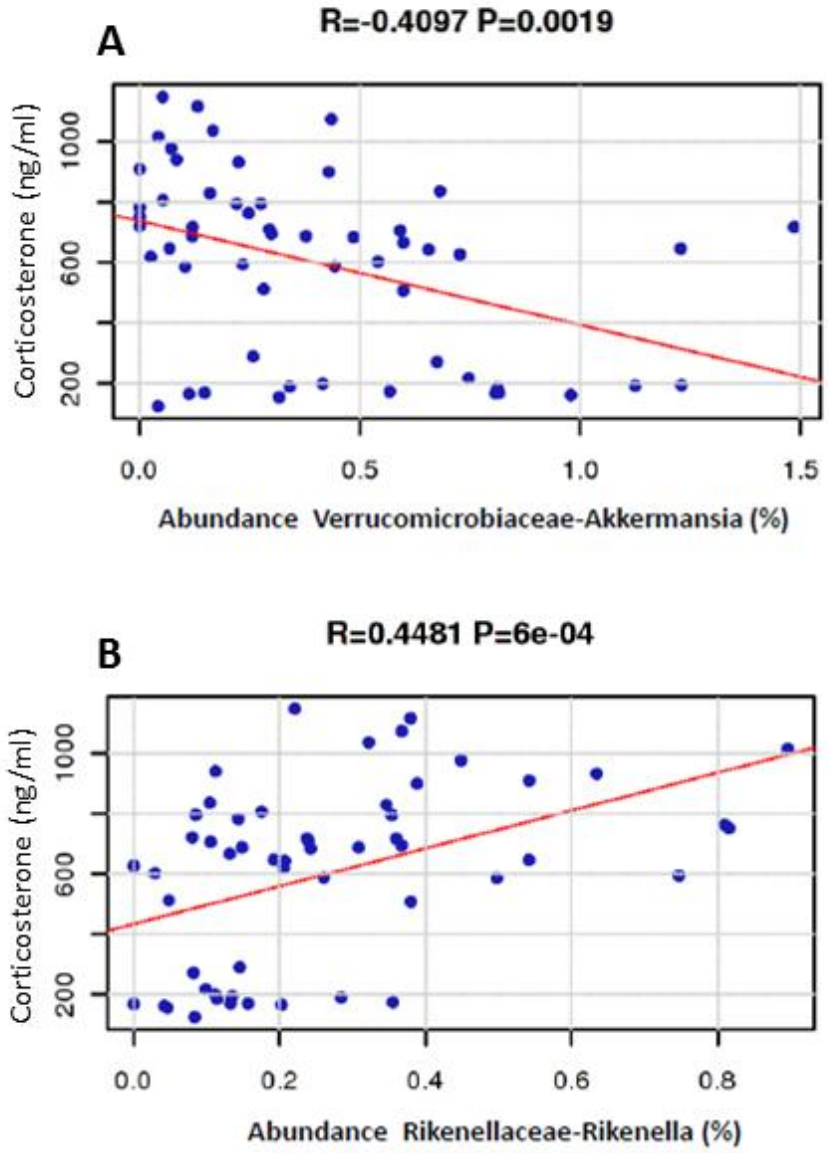


Figure 3. Correlation between percentage abundance of *Akkermansia* and *Rikenella* and corticosterone (CORT) plasma levels. Regression analysis revealed (A) negative correlation between low CORT levels in MS-saline group and low abundance of *Akkermansia* and (B) positive correlation between low CORT levels in MS-saline group and low abundance of *Rikenella.*

Effects of long-term EPA/DHA supplementation on the composition of the gut microbiota is more pronounced in MS rats

Long-term EPA/DHA supplementation has a strong impact on the composition of the gut microbiota in MS rats (Figure 4A). In particular, high dose administration of EPA/DHA was associated with higher levels of the butyrate producing bacteria; Butyrivibrio. Moreover, high dose supplementation of EPA/DHA elevated the levels of several members Actinobacteria (such as *Aerococcus*), with a concomitant reduction of the abundance of members of Proteobacteria (such as *Undibacterium*). The results are in agreement with a recent study on maternal prenatal stress, which reported lower quantities of lactic acid bacteria such as Lactobacillus, Lactococcus, Aerococcus and Bifidobacteria and significantly higher relative abundance of Proteobacterial members (Zijlmans et al., 2015). Altogether, this pattern of altered gut microbiota in MS rats support the existence of a potentially increased level of inflammation, which could be reversed by long-term supplementation of high dose of n-3 PUFAs. In fact, diets rich in PUFAs have been shown to positively influence immune function (Russo et al., 1995). The possible underlying mechanisms by which PUFAs exert their beneficial effects on health were shown to involve the formation of pro-inflammatory cytokine production (tumor necrosis factor alpha and interleukin-1), modulation of the hypothalamic-pituitary-adrenal anti-inflammatory responses, and induction of the release of acetylcholine (Das, 2000).

Long-term EPA/DHA supplementation in NS rats had less significant impact on their gut microbiota in comparison to their effect observed in MS rats (Figure 4B). In NS rats, long-term administration of EPA/DHA was associated with changes in the abundance of three taxa; *Enterorhabdus*, *Sutterella,* and *Prevotella* (Figure 3B). *Sutterella* was shown to be associated with some gastrointestinal infections in humans (Molitoris et al., 1997). Moreover, *Sutterella* was shown to be of significantly higher prevalence in biopsies taken from the gut

of autistic children with gastrointestinal disturbance compared to controls with GI disturbance (Finegold et al., 2010). Together, our data supports the expected beneficial effects of n-3 PUFAs particularly in a state of microbial dysbiosis, which is associated with inflammation.

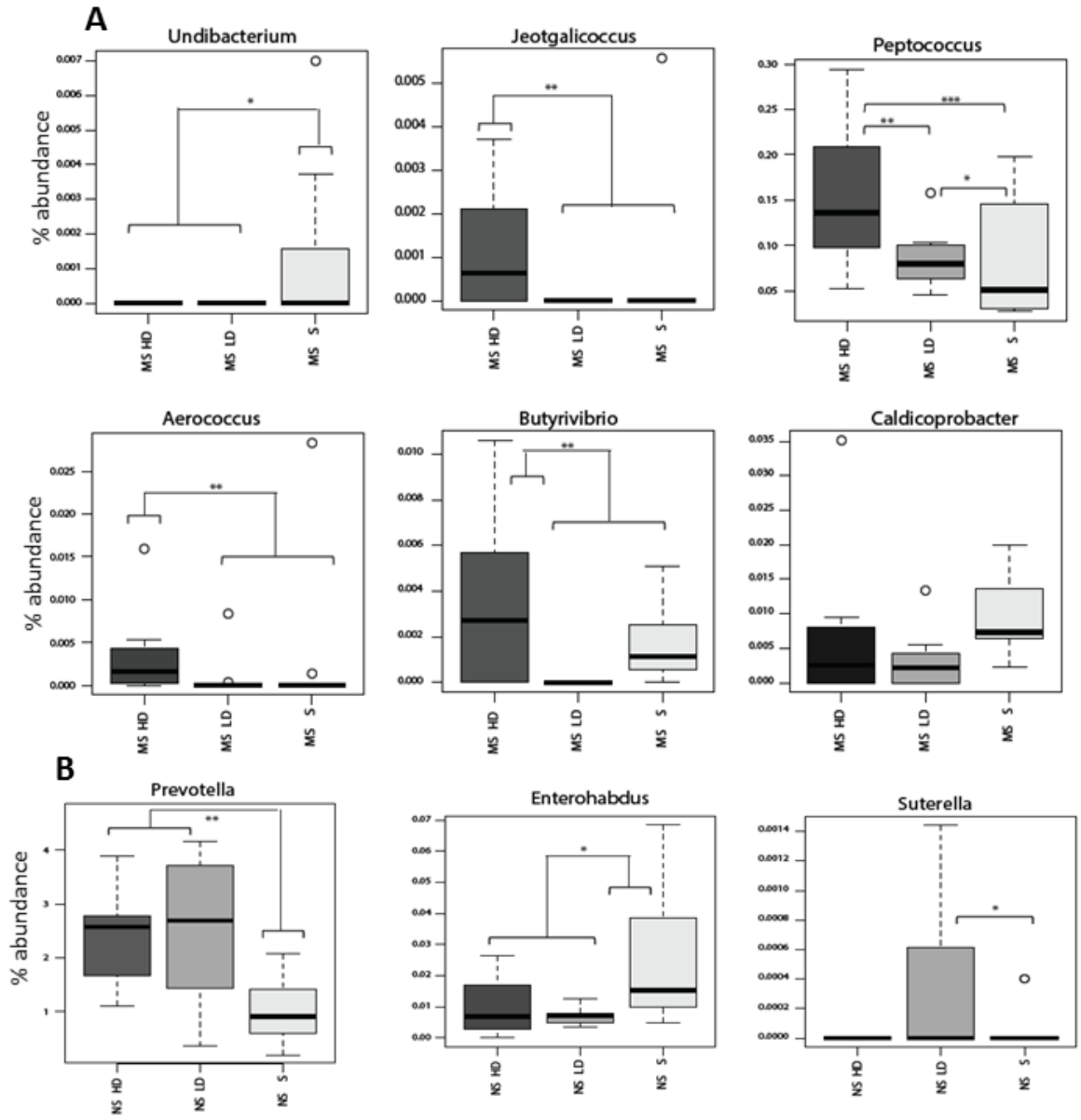


Figure 4. Effects of maternal separation (MS) and EPA/DHA administration on rat gut microbiota. Relative abundance of significantly altered microbial genera in the MS-saline and EPA/DHA treated groups (A) and NS-saline and EPA/DHA treated groups (B). Median with interquartile ranges is depicted. Significant difference indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.5. Discussion

We demonstrate what is to our knowledge the first time that EPA/DHA treatment normalized early-life stress-induced disruption of female rat gut microbes. Analysis of the gut microbiota of MS rats showed altered microbial composition with abundance of members previously shown to be associated with inflammation. These results indicate that transient stress-induced alteration during a crucial developmental time-window for neonatal rats has long-lasting effects on the gut microbiota composition in adulthood. Supplementation with EPA/DHA restored the composition of the gut microbiota in MS rats. Presumably, the EPA/DHA effect on the gut microbiota is related to PUFAs anti-inflammatory activity. Recently, it has been shown that omega-3 and omega-6 dramatically reduce the endotoximic and inflammatory status in metabolic endotoxemia (Kaliannan, 2015). Intriguingly, the observed effects involved changes in the gut microbiota, through the impact of omega-6, -3 on intestinal production and secretion of intestinal alikaline phosphatase. The induced alterations in gut microbiota composition resulted in reduction in levels of lipopolysachharides and gut permeability, which in turn reduces the onset of inflammation.

Overall, the healthy benefits at CNS level have ascertained the contribution made by n-3 PUFAs to stress-related disorders and put them under the spotlight for decades (Peet, 2008, Hibbeln and Gow, 2014, McNamara et al., 2015). The current study offers insight into a potential role of n-3 PUFAs through the modification of the gut microbiota in an animal model of stress. Our data supports the previous reports showing that the absence as well as the exacerbation of certain bacterial taxa in the gut of early-life stressed rats may represent risk factors for the development of anxiety, depression and inflammatory diseases such as IBS. We postulate that EPA/DHA administration is beneficial for restoring members of the

microbiota with immunoregulatory functions in order to prevent an overly robust stress-induced inflammatory response which may contribute to the onset of mental illnesses.

Chapter 5

The role of n-3 PUFAs and phospholipids in inflammation: Effects of lipopolysaccharide

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5.1. Abstract

Background: Neuroinflammation has been shown to contribute to the development of stress-related disorders such as depression and anxiety due to the inappropriate release of pro-inflammatory cytokines from glial cells. Both phospholipids and n-3 polyunsaturated fatty acids (n-3 PUFAs) have been shown to be essential for the development of the central nervous system as well as of benefit in stress related disorders.

Methods: We investigated the protective effect of both the phospholipid phosphatidylserine (PS) and the n-3 PUFAs docosahexaenoic acid (DHA) against lipopolysaccharide (LPS)-induced cellular changes in astrocytes cortical cell cultures.

Results: We found that LPS (5, 1, 0.5, 0.25, 0.1 ug/ml) increased the mRNA expression levels of tumour neuronal factor (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) pro-inflammatory cytokines after 24 hours of treatment. Neither DHA nor PS were able to attenuate LPS-induced inflammation.

Conclusions: This work suggests that the reversal of pro-inflammatory cytokines may not represent one of the mechanisms by which phospholipids and n-3 PUFAs may be of value in psychiatric disorders.

5.2. Introduction

Chronic inflammation is now considered to be central to the pathogenesis of stress-related disorders. In the brain, pro-inflammatory cytokines, interleukin-1 beta (IL-1β), tumour necrosis factor (TNF-α), interleukin-6 (IL-6), are released primarily from glial cells after inflammatory stimulation. Elevated levels of these cytokines have been linked with acute and chronic neurological disorders, as well as with psychiatric disorders (Leonard, 2007). High pro-inflammatory cytokines levels have been found to promote neuronal death (Long-Smith et al., 2010), to cause depressive-like behaviour in rodents and have an anti-neurogenic effect on rat neural precursor cells (Yirmiya and Goshen, 2011, Green et al., 2012). Moreover, patients with major depression have raised pro-inflammatory cytokines in the plasma or serum (Maes, 1999).

Phospholipids constitute the major component of all cell membranes. As such, they confer on membrane property of fluidity, and thus, determine and influence the behaviour of membrane-bound enzymes and receptors. Within the phospholipids, phosphatidylserine (PS) has received most attention from researchers. PS is mostly enriched in docosahexaenoic acid (DHA) which is the most abundant n-3 PUFAs in the brain. Both, PS and DHA have been shown to be involved in the development and functionality of the central nervous system (CNS) (Newton and Keranen, 1994, Salem et al., 2001, Huang et al., 2011a, Sharma et al., 2012).

For instance, PS has been shown to improve cognition both in rats (Park et al., 2013) and humans (Delwaide et al., 1986). Furthermore, chronic DHA supplementation promoted beneficial effects on anxiety, and depressive-like behaviours in stress-induced animals (Ferraz et al., 2011). Consistent with these findings, clinical observations have shown n-3 PUFAs supplementation to be beneficial in reducing certain anxiety behaviours as well as in treating MDD (Yehuda et al., 2005b, Grosso et al., 2014b).

Nonetheless, there is a paucity of information regarding the direct effects of PS and n-3 PUFA in neuroinflammation. In view of the above, we hypothesized that chronic treatment with LPS would induce the production of pro-inflammatory cytokines in astrocytes cortical cultures. We thus, investigated whether PS or DHA could abrogate LPS-induced inflammation in astrocytes cortical cell cultures.

5.3. Methods

Cortical astrocytes primary cultures

P1/P2 Sprague–Dawley rats were anaesthetised by hypothermia on ice and were killed by decapitation. Brains were removed and transferred to sterile Petri dishes containing ice-cold HBSS. The cerebral hemispheres were separated and the cortex was dissected out. The meninges were carefully removed and cortical tissue was placed in ice-cold sterile HBSS. The dissected cortical tissue was incubated in 0.1% trypsin solution in sterile HBSS for 15 min at 37 °C with 5% CO2. This was replaced with pre-warmed dissociation medium (10% heat-inactivated (50 °C, 30 min), normal horse serum (NHS) (Sigma) and DNase (100 µg/ml) in DMEM F12). The suspension was inverted gently for 1 min, triturated with a flame-polished Pasteur pipette and passed through a 40 µm cell strainer to remove cellular debris. The suspension was centrifuged for 5 min at 1000 rpm at room temperature, the supernatant was removed and the pellet was resuspended in 1 ml of medium (10% HS and 100× penicillin/streptomycin (1%; 100 U/ml penicillin, 100 µg/ml streptomycin) in DMEM-F12). Cells from each pup were cultured in a t-75 flask (Sarstedt) at 37 °C with 5% CO2. After 10 days in vitro (DIV), mixed glial-enriched cultures underwent a 16 h ‘shake off’ on an orbital shaker after which detached cells in the supernatant were discarded, and the remaining adherent astroglial cells were separately further cultured in 6 well plates at a density of 1 x 10^6 cells/well. After a total of 11 DIV, astroglial-enriched cultures were treated for 24 h

with LPS (5, 1, 0.5, 0.25 or 0.1 ug/ml from Escherichia coli serotype O111:B4; Sigma). Cells were also pre-treated with PS or DHA, one hour before LPS treatment.

Immunocytochemistry

In order to assess the percentage of astrocytes contained in the cultures, cells were grown in 24 well plates for immuno-staining. In brief, culture media was removed and cells were washed with pre-warmed Hank's Balanced Salt Solution (HBSS). Cells were fixed with ice-cold methanol for 10 min at − 20 °C and subsequently washed and permeabilised in phosphate buffered solution (PBS) containing Tween 20 (0.02%) (PBS-T) three times for 5 min. Cells were incubated in 5% normal horse serum in PBS-T overnight at 4 °C to attenuate non-specific antibody binding. Cells were incubated overnight at 4 °C in antibodies that target βIII-tubulin-positive neurons (1:300; mouse monoclonal, Promega), glial fibrillary acidic protein (GFAP)-positive astrocytes (1:300; rabbit polyclonal, Dako). Cells were subsequently incubated in the appropriate secondary antibody: Alexa Fluor 488 donkey anti-rabbit IgG or Alexa Fluor 594 donkey anti-mouse IgG (all 1:2000; Molecular Probes). Cells were counterstained with bisbenzimide (1:2500; Sigma-Aldrich) to identify the nuclei. For each antibody, the cells from one coverslip were incubated in blocking solution and secondary antibody, without primary antibody (to account for nonspecific binding of the secondary antibody), and showed a complete absence of immunoflouresencent staining (data not shown).

Cell counts by high content analysis

High content analysis (HCA) is a high throughput technique that allows for screening of multiple cellular features based on automated cell imaging analysis (Godinho et al., 2014). In this study, Cytiva™ Cell Integrity HCA Assay was used to investigate the percentage composition of astrocytes in the cultures. Five images per well were

acquired using the IN Cell Analyser 1000 (GE Healthcare, UK) with a 20× objective. After acquisition, data were analysed using In Cell® 1000 Workstation software (GE Healthcare, UK). Immuno-positive cells were counted in five randomly chosen fields of view from each of the four coverslips, and divided by the total number of cells per five fields of view to give an average percentage for each coverslip. Only βIII-tubulin or GFAP-positive cells with a differentiated phenotype were counted. Four wells per treatment were analysed from three independent experiments.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from cortical astrocytes primary cultures using the ReliaPrep™ RNA Cell Miniprep system as per manufacturer's instructions (Promega). Briefly, cells were harvested using a denaturing lysis solution. The lysate was then transferred to a microcentrifuge tube in presence of isopropanol and centrifuged. The filter was then washed and finally the RNA was eluted using Nuclease-Free Water. Isolated RNA was stored at −80 °C until further processing. RNA concentration was quantified using the ND-1000 spectrophotometer (NanoDrop®) and RNA quality was assessed using the Agilent 2100 Bioanalyzer.

Analysis of RNA expression levels was carried as previously described (O' Connor et al., 2013). Briefly, equal amounts of RNA were first reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, CA). Real-time PCR was performed using TaqMan Universal Master Mix II, no UNG, and TaqMan Gene Expression Assays designed by Applied Biosystems for rat genes on the ABI7300 Real Time PCR machine (Applied Biosystems, Warrington, UK). Each sample was analysed in triplicate, both for target gene and for endogenous control using the 7300 System SDS Software (Applied Biosystems, Life Technologies). All assays were

controlled for the absence of genomic DNA amplification. β-actin (Actb, VIC/MGB Probe, Primer Limited, Applied Biosystems) was used as an endogenous control. Cycle threshold (Ct) values were recorded. The Ct value for the target gene in each sample was normalized to its endogenous control transformed to relative gene expression value using the $2^{-\Delta Ct}$ method (Simen et al., 2006). Four wells per treatment were analysed from three independent experiments.

Statistics

All data are presented as mean ± SEM. Data were analysed using a one or two-way ANOVA as appropriate, followed by Fisher's LSD post-hoc test, using the statistical software package SPSS 21.0 (IBM). A *p*-value of 0.05 was selected as the threshold of statistical significance.

5.4. Results

Cell identification

We identified the specific cells which populate the cultures used for this investigation. Data showed that the percentage composition of astrocytes was 98%, as shown in Figure 1.

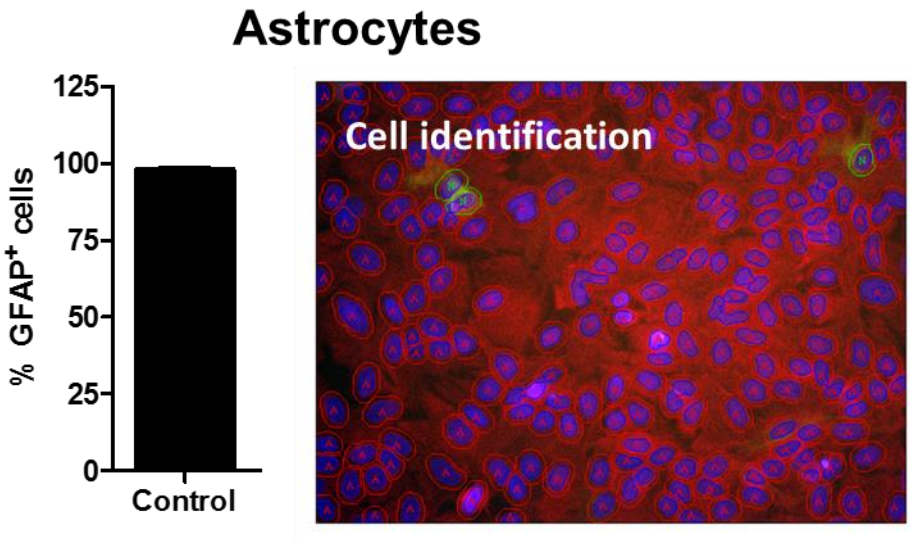


Figure 1. Representative photomicrographs of cells immunocytochemically stained for GFAP (red), βIII-tubulin (green), and bisbenzimide (blue).

TNF-α, IL-1β, and IL-6 expression

We first established whether LPS-induced pro-inflammatory cytokines is associated with increased mRNA levels of TNF-α, IL-1β, and IL-6 mRNA levels in rat cortical astrocytes primary culture and whether such effects are reversed by either PS or DHA intervention. In LPS-treated (5, 1, 0.5, 0.25 or 0.1 ug/ml, 24 hours) rat cortical astrocytes cultures we found a significant increase in TNF-α, IL-1β, and IL-6 expression levels , respectively, as shown in Figure 2A, B, and C.

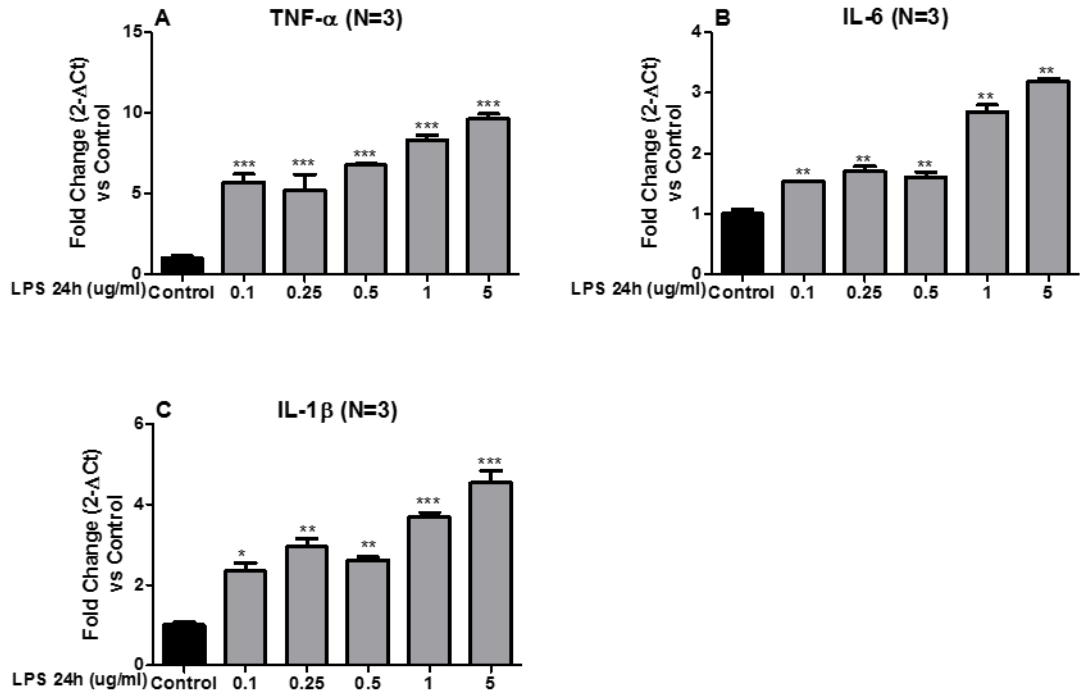


Figure 2. (**A**, **B** and **C**) LPS dose-dependent increase in TNF-α, IL-1β, and IL-6 mRNA expression levels. Intergroup comparisons: *, ** and ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively compared to control. Data are presented as mean ± S.E.M. ($n = 3$).

PS was not able to reverse LPS-induced increase in TNF-α, IL-1β, and IL-6 mRNA expression levels, as shown in Figure 3A, B, and C. While higher PS doses (5, 10, 20 uM) showed cell toxicity (data not shown).

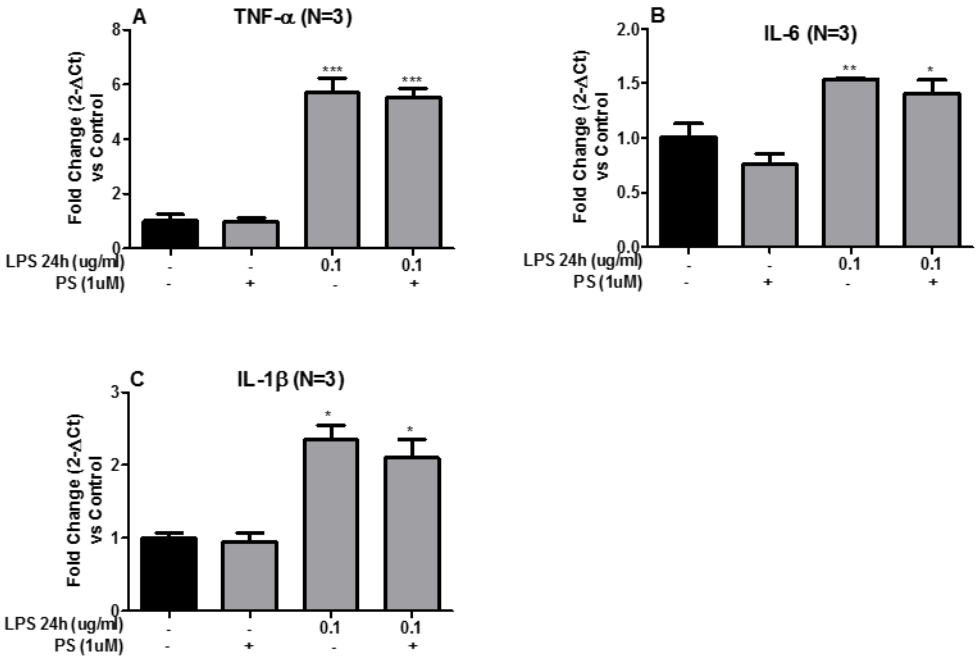


Figure 3. (**A**, **B** and **C**) PS was not able to reverse LPS-induced TNF-α, IL-1β, and IL-6 mRNA expression levels. Intergroup comparisons: *, ** and ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively compared to control. Data are presented as mean ± S.E.M. ($n = 3$).

DHA was not able to reverse LPS-induced increase in TNF-α, IL-1β, and IL-6 mRNA expression levels, as shown in Figure 4A, B, and C.

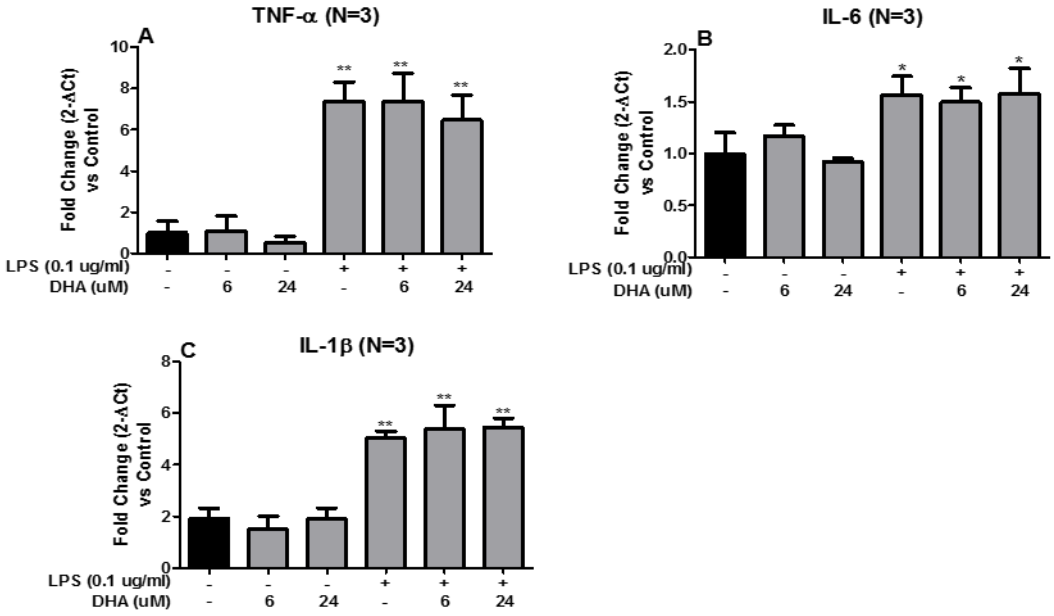


Figure 4. (**A**, **B** and **C**) DHA was not able to reverse LPS-induced TNF-α, IL-1β, and IL-6 mRNA expression levels. Intergroup comparisons: * and **: $P < 0.05$ and $P < 0.01$, respectively compared to control. Data are presented as mean ± S.E.M. ($n = 3$).

5.5. Discussion

The present study demonstrates that reversal of LPS-induced inflammation in cortical astrocytes primary cultures is not underlying the beneficial effects exerted by either PS or DHA in CNS. Specifically, we showed that LPS-induced inflammation is associated with increased mRNA levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in rat cortical astrocytes primary culture. We demonstrated that neither PS nor DHA reversed LPS-induced increased mRNA levels of TNF-α, IL-1β, and IL-6 in these cultures.

As expected, and in agreement with previous studies on glial cultures (Long-Smith et al., 2010, Wang et al., 2010), we showed that a range of doses of LPS induced an increase in TNF-α, IL-6, and IL-1β mRNA expression levels in cortical astrocytes cultures (Borysiewicz et al., 2009, Gong et al., 2014). Considering the increase in the levels of pro-inflammatory cytokines in a dose-dependent fashion after stimulation with LPS, we suggested that the lowest dose of LPS (0.1 ug/ml) was sufficient to induce an inflammatory response from the astrocytes present in these cultures.

In our culture system and at the concentration used, PS did not block the LPS (0.1 ug/ml)-induced increase in TNF-α, IL-6 or IL-1β mRNA levels and accordingly higher concentrations of LPS were not investigated, while higher doses of PS showed cell toxicity. There is an increasing body of scientific literature probing the role of individual phospholipids in CNS functioning and development (Delwaide et al., 1986, Lee et al., 2010) so it is being increasingly appreciated that dietary intake inter alia may have a role to play in the execution of appropriate clinical management strategies. Nonetheless, the biological mechanisms by which phospholipids function is not clear yet. To the best of our knowledge there has not been investigation on the effect of phospholipids in the inflammatory system, yet. Perhaps, the inability of PS on reversing LPS-induced pro-inflammatory cytokines may predict the exclusion of inflammation as an explicative target of the beneficial effects exerted

by phospholipids in CNS. However, further studies need to be envisaged in order to address to this question.

Being n-3 PUFAs of relevance for the function and development of the CNS, it would be plausible to consider the benefits exerted by phospholipids due to their high content in n-3 PUFAs. Dietary phospholipids are hydrolysed during digestion, so orally administered phospholipids most likely are not absorbed intact. Because of the partial or complete hydrolysis of phospholipids, the release of n-3 PUFAs maybe the cause of the beneficial effects of phospholipids rather than the intact phospholipids their self. Of note, phospholipids metabolism and consequent n-3 PUFAs release does not occur in cell cultures, hence this could give an explanation to the inefficacy of PS against LPS-induced pro-inflammatory cytokines. In regard of this hypothesis, we investigated the DHA potential reversal effect against LPS in cortical astrocytes cultures. Indeed, DHA has been shown to have anti-inflammatory properties (Raederstorff et al., 1996). However, DHA was not able to counteract the TNF-α, IL-6 or IL-1β increased expression levels induced by LPS stimulation. Perhaps different kind of n-3 PUFAs may constitute better candidates for this challenge. For instance, the eicosapentaenoic acid (EPA) n-3 PUFAs has been shown to have anti-inflammatory effects both in vitro (Kim et al., 2012) in vivo (Koto et al., 2007) and in human studies (Su et al., 2014).

In conclusion, recent evidence has shown that dysregulation of the inflammatory system may induce vulnerability to psychopathology. Using an in vitro model, we provided further evidence for such arguments, showing that LPS leads to increased pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β, mRNA expression levels in mixed cortical cultures. Future research in this area is necessary to determine whether either phospholipids or n-3 PUFA directly target brain inflammatory pathways as mechanisms for prevention from neurological disorders with a neuroinflammatory component.

Chapter 6
General Discussion

6.1. Overview and summary

In this thesis we have demonstrated that targeting n-3 PUFAs can be considered as a potential approach for the prevention of stress-related psychiatric disorders. Using a well-known model of early-life stress, the maternal separation (MS) paradigm, we have investigated the functional consequences of chronic dietary lipids intake in female rats. We have thus demonstrated that EPA/DHA supplementation decreased anxiety- and depressive-like behaviours as measured in the elevated plus maze (EPM) and forced swim test (FST), respectively, and increased cognitive behaviour assessed by the novel object recognition task (NOR) in non-separated (NS) animals. As a driver of these behavioural improvements, we proposed the activation of glucocorticoid receptors (GR) observed in the hippocampus of female rats upon EPA/DHA treatment. However, MS-induced changes in monoaminergic and immune systems as well as altered neuroendocrine response to stressors were not reversed by EPA/DHA intervention, highlighting limitations of n-3 PUFAs in pathological conditions.

We also investigated the impact of both EPA/DHA treatment and MS in the gut microbiota composition. We observed that MS-induced alteration during a crucial developmental time-window for neonatal rats had long-lasting effects on the gut microbiota composition in adulthood. Notably, a spectrum of microbes, believed to be involved in anti-inflammatory activity, were the most altered by MS. These microbes constituted a specific target for EPA/DHA intervention in animals who were exposed as pups to a maternal separation paradigm as well as in neurodevelopmentally normal rats.

Further, this research has shed light on some of the underlying neurobiological mechanisms that may mediate n-3 PUFAs reversal of corticosterone (CORT)-induced cellular changes in cortical cell cultures containing both astrocytes and neurons. We showed that CORT-induced cellular stress exerted down-regulation of GR expression on neurons, an effect which was

abolished by DHA treatment. Furthermore, we demonstrated that DHA reversed CORT-induced neuronal apoptosis, and attenuated CORT-induced reductions in BDNF expression levels in these cultures. We also observed that CORT-induced cellular stress exerted a bimodal effect on the neuronal and glial cell composition whose effect was abolished by DHA treatment in vitro. In light of this evidence, we also explored DHA as well as the phospholipid phosphatidylserine (PS), enriched in DHA, on reversing LPS-induced pro-inflammatory cytokines expression levels in astrocytes cortical cultures. However, both PS and DHA failed to induce a response.

Nonetheless, the studies outlined in this thesis advance our understanding of the role of n-3 PUFAs as future preventive strategies to counteract glucocorticoid-induced impairments in clinical settings. Furthermore, the current findings support the idea that dietary lipids intake could represent a beneficial long-term intervention to reduce the risk of development of stress related pathologies, especially anxiety and depression.

6.2. n-3 PUFAs: a preventive strategy for stress-related psychopathology

The impact of lifetime dietary habits and their role on health, especially mental health, represents an important topic for the discovery of alternative potential approaches for the prevention of stress-related psychiatric disorders (Jacka et al., 2014). Women in particular appear to have an increased risk of suffering from stressful life events (Dinan, 2005). However, in contrast to men, there is a distinct paucity of information describing stress responses in women (Taylor et al., 2000b). In chapter 2, we hypothesized that an eicosapentaenoic acid (EPA)/ docosahexaenoic acid (DHA) (80% EPA, 20% DHA) n-3 PUFAs mixture improved stress-related behavioural and neurobiological responses both in neurodevelopmentally normal female animals and in animals who were exposed as pups to a maternal separation paradigm. In rodents, the maternal separation (MS) model is a well-known paradigm used to investigate the biological consequences of early-life stress (O'Mahony et al., 2011). Several behavioural responses are altered by MS in adult rodents (O'Mahony et al., 2009) mimicking a phenotype which is comparable to depression in adult humans (Schmidt et al., 2011). Moreover, inadequate maternal care has been linked to developmental, emotional, and social deficits in human infants (Field, 1998) and in the rat (Caldji et al., 1998).

In chapter 2 we showed that a specific EPA/DHA dose (1g/kg/day) increased resilience to psychological stress as indicated by the elevated plus maze paradigm and antidepressant type effects in the forced swim test. Moreover, improvement in long term memory was shown by NS female rats treated with n-3 PUFAs during the novel object recognition task. In accordance with our observations, it has been already shown that rats deficient in n-3 PUFAs exhibit impaired performance on cognition and mood disorders as well as neurobiological effects associated with depression (Levant et al., 2008). Therefore, it is plausible to conclude

that EPA/DHA can exert positive outcomes on stress-related behavioural responses following normal neurodevelopment. That n-3 PUFAs had little impact on animals subjected to MS is perhaps not surprising. Separation from the dam represents one of the most potent stressors for pups inducing alterations at different levels: animal behaviour (Berman et al., 2014), the HPA axis (Liu et al., 1997), the immune system (O'Mahony et al., 2009) and the brain-gut axis (O'Mahony et al., 2011). Furthermore, these previous studies were conducted in male animals, but we used female animals as females are more prone to depression and anxiety disorders. Results in humans with depression indicate that n-3 PUFAs alone do not have antidepressant properties but may augment antidepressants (Su et al., 2003).

The responsivity of the HPA axis plays a pivotal role in behavioural abnormalities induced by stress (Lupien et al., 2009). In chapter 2, evaluation of the HPA axis response to a novel stressful environment was assessed by measuring corticosterone levels via tail incision during behavioural testing.

Animals exposed to MS exhibited a blunted corticosterone response when exposed to an acute stress which is in contrast to many findings reported in the literature (O'Mahony et al., 2009, Roque et al., 2014). One possible factor contributing to the distinct outcomes regarding the effects of MS manipulation on stress responsiveness may be sex-related, since most of the studies reporting an enhanced neuroendocrine stress response were conducted in male rats (Aoki et al., 2010; Dalla et al., 2011; Simpson and Kelly, 2012). This is certainly the case in previous studies from our laboratory where we have reported such alterations (O'Mahony et al., 2009). Indeed, the focus on female animals in this study is an important and understudied aspect of maternal separation which is in line with NIH policy which seeks to reduce the overreliance on male animals in preclinical studies. Moreover, another potential contributing factor to the discrepant results may be related to the amount of maternal care, specifically maternal licking and/or grooming towards the pup, following separation. Evidence suggests

that under normal conditions there is variation in maternal care both among dams as well as within the same litter, with some pups receiving more care than others (Champagne et al., 2003). It has been proposed that maternal care might function as a buffer against the enhanced HPA axis reactivity of MS adult in response to stress (Macri et al., 2008). This represents an interesting consideration that we will pursue in future studies.

HPA axis hypoactivity has been previously reported both in models of post-traumatic stress disorder (PTSD) (Cohen et al., 2006; Yehuda et al., 2005) and of atypical depression (Bowens et al., 2012). In many ways the current MS paradigm reflects atypical rather than melancholic depression when viewed through the lens of HPA axis activity. It is conceivable that early-life stress induces increased release of proinflammatory cytokines which in turn activate the HPA axis. Persistent activation of this axis may eventually lead to end organ burnout. Future studies need to examine these changes in a detailed longitudinal manner.

Hippocampal GRs together with mineralcorticoid receptors (MRs) are considered the cornerstone of the adaptive HPA axis functionality (Lupien et al., 2009). We showed that EPA/DHA 1g/kg/day increased the abundance as well as the migration of GRs into the nucleus of the hippocampus. This could explain the reduced anxiety- and depressive-like behaviour together with increased cognition observed in NS rats. Indeed, GR activation has been shown to be part of the mechanisms exerted by antidepressants (Anacker et al., 2011). However, in our study this effect disappeared in the hippocampus of MS animals. In contrast to other studies (Ferreira et al., 2013) both BDNF expression levels and body weight were not affected either by maternal separation or by EPA/DHA supplementation. This may be due to strain- and sex-effects, as well as differences in the study design.

Altered HPA axis function can be associated with dysregulation in noradrenergic (NA) and serotonergic (5-HT) systems (Ressler and Nemeroff, 2000). In chapter 2 we revealed that NA

content was markedly decreased in the hippocampus of MS animals. Of importance, changes to monoamines are considered to be of relevance in the regulatory developmental ontogeny of anxiety at critical time windows (Ressler and Nemeroff, 2000). Interestingly, increased levels of 5-HT and reduced serotonin turnover in the brain stem, hippocampus and prefrontal cortex of MS female animals were found, which contrasts with our previous studies which found that MS produces an increase in serotonin turnover (O'Mahony et al., 2008) together with an increase in corticosterone levels in male rats (O'Mahony et al., 2009). The discrepancy between our previous and current findings may be explained by the fact that either underactivity or overactivity of the 5-HT system causes anxiety and mood disorders or by sex-specific effects as noted above. This 'inverted-U' function has been described (Calabrese and Baldwin, 2001). Moreover, Olivier et al showed that knockout of the serotonin transporter (SERT), which increases 5-HT in the brain (Frazer and Benmansour, 2002), may exert anxiety- and depressive-like behaviours in mice (Olivier et al., 2008). Concerning the impact of n-3 PUFAs in the monoaminegic system, no influences exerted by the EPA/DHA mixture have been found in our study. This is in marked contrast to previous positive findings (Kodas et al., 2004). Our findings suggest that GRs but not the monoaminergic system are involved in the resilience to behavioural despair and to anxiety-like behaviour exerted by n-3 PUFAs in NS female rats.

Using cortical cell cultures, we further investigated the potential reversal effect of n-3 PUFAs against glucocorticoid-induced cellular changes *in vitro* (Chapter 3). The concept of targeting glucocorticoid-induced cellular changes through pharmacological means is to produce a therapeutically beneficial effect for the treatment of stress-related pathologies, such as depression and anxiety (Anacker et al., 2011, Lucassen et al., 2014). Chronic administration of glucocorticoids has been shown to be deleterious to the structural and functional plasticity of the adult rat brain (Sapolsky and Pulsinelli, 1985, Zhu et al., 2013) provoking a number of

behavioural abnormalities similar to depression and anxiety in mice (Ardayfio and Kim, 2006, Murray et al., 2008). One of the most sensitive brain areas to the detrimental effects of stress exposure is the prefrontal cortex (PFC) which has a rich population of GRs (Chao et al., 1989, Barsegyan et al., 2010). PFC is a target region for glucocorticoid effects. For instance, chronic corticosterone (CORT) treatment has been shown to produce neuronal impairment in the PFC, such as reduced distal dendritic spines of neurons and neuronal loss in rats (Wellman, 2001, Seib and Wellman, 2003, Cerqueira et al., 2005). In chapter 3 we supported the hypothesis that glucocorticoids mediate deleterious cellular changes in cortical cultures containing both astrocytes and neurons. Chronic CORT treatment reduced cellular viability and specifically induced cortical neuronal cell death through apoptosis. This is in agreement with previously generated data (Crochemore et al., 2005, Liu et al., 2010a, b). Many effects of stress on the brain are tightly regulated by the action of MRs and GRs (McEwen, 2007, de Kloet et al., 2008). Accordingly, we showed that CORT exerted its toxic effect directly through the activation of GR but not MR. Furthermore, chronic CORT treatment showed down-regulated GR expression levels in cortical neurons. This evidence is in line with clinical observations reporting abnormal GR isoform expression levels in the PFC of patients affected by stress-related pathologies, such as bipolar, depression disorder and schizophrenia (Sinclair et al., 2011, Sinclair et al., 2012, Qi et al., 2013). In agreement with previous findings at the cortical level in the rat (Dwivedi et al., 2006), we also found that CORT reduced BDNF expression levels in our cultures, highlighting loss in cellular plasticity and survival (Lipsky and Marini, 2007). Astrocyte overgrowth was another interesting effect observed upon CORT treatment in cortical cultures. This phenomenon could be considered as part of a greater CORT-induced neuronal damage by impeding the neuron-glia interaction, as already shown by previous findings (Sykova, 2001, Sykova et al., 2002, Sykova, 2004, 2005).

We sought to characterize the potential n-3 PUFAs protective effects against CORT-induced cellular changes in cortical cultures. For this study, we used the n-3 PUFAs docosahexaenoic acid (DHA) and found that a specific DHA concentration (6 uM) protected from CORT-induced changes in cortical neuron and astrocyte composition as well as cellular death and apoptosis. This evidence is in line with several other studies showing that DHA pharmacological intervention increased neuronal survival and prevented cortical neuronal apoptosis in primary culture (Cao et al., 2005, Florent et al., 2006). Interestingly we found DHA to be effective in impeding GR down-regulation induced by CORT, which may be a mechanism by which DHA reversed CORT-induced reduction in BDNF expression levels. Indeed, GR have been reported to recruit the CaMKIIα-BDNF-CREB-dependent neural plasticity pathways mediating long-term memory formation in rats (Chen et al., 2012). Moreover, DHA attenuated CORT-induced reduction in BDNF expression level may be a mechanism by which DHA reverses CORT-induced cellular toxicity and apoptosis. Of note, alteration in neural and astrocyte composition generated by CORT exposure were reversed upon DHA treatment.

In conclusion, the studies advance our understanding on the role of n-3 PUFAs as future preventive strategies to counteract CORT-induced cellular changes in the brain, and support the concept that n-3 PUFAs intake could represent a beneficial habit to reduce the risk of development of stress related pathologies.

6.3. Gut microbiota: an attractive target for potential n-3 PUFAs mechanisms against stress-related pathologies

There is increasing evidence suggesting a bi-directional communication between the central nervous system (CNS) and the gut-microbiota which is recognized as the microbiome–gut–brain axis (Cryan and Dinan, 2012). This communication is believed to influence the parallel development of both CNS and gut microbiota which can remarkably influence health and disease (Moloney et al., 2014). It is now evident that this gut-brain cross-talk may be involved in the aetiology of several metabolic and mental dysfunctions/disorders (Dinan et al., 2015; Montiel-Castro et al., 2013). Indeed, there is an expanding volume of evidence to support the view that cognitive and emotional processes can be altered by microbes acting through the brain-gut axis (Bravo et al., 2011; Dinan and Cryan, 2013).

Stress, especially in early life, has been identified as a cause of the disruption of this developmental pattern leading to a variety of disorders ranging from gastrointestinal disorders (Mayer et al., 2014), to anxiety and depression (Borre et al., 2014). In rodents, the maternal separation (MS) model is a well-known paradigm that induces brain-gut axis dysfunction (O'Mahony et al., 2011). The separated phenotype alters many components of the brain-gut axis throughout the body including the hypothalamic–pituitary adrenal (HPA) axis (O'Mahony et al., 2011), the immune and neuroendocrine systems (O'Mahony et al., 2009). Growing evidence considers these abnormalities comorbid with changes in the gut microbiota (Clarke et al., 2014; Diaz Heijtz et al., 2011; El Aidy et al., 2015) as well as crucial risk factors for the development of mental illnesses such as anxiety and depression (Bakunina et al., 2015; Dinan and Scott, 2005).

It is well recognized that eating habits are of relevance to health in general and mental health in particular (Logan and Jacka, 2014). Being critical in the development and function of the CNS, n-3 polyunsaturated fatty acids (n-3 PUFAs) have been under the spotlight for decades

(Grosso et al., 2014). The possible underlying mechanisms by which n-3 PUFAs exert their beneficial effects on health are diverse, involving, for instance, HPA axis, neuroendocrine and immune regulations (Dyall, 2015; Song, 2013). Moreover, there is a growing appreciation of the impact of dietary fatty acids on the intestinal microbiota composition of the host (Marques et al., 2015; Patterson et al., 2014; Yu et al., 2014). In chapter 4 we investigated the impact of EPA/DHA n-3 PUFAs mixture on the intestinal microbiota populations of female rats exposed to early-life stress.

In chapter 4 we demonstrated what is to our knowledge the first time that EPA/DHA treatment normalized early-life stress-induced disruption of female rat gut microbes. These results indicate that temporary stress-induced alteration during a crucial developmental time-window for neonatal rats has long-lasting effects on the gut microbiota composition in adulthood. Notably, a spectrum of microbes, believed to be involved in anti-inflammatory activity, were the most altered by MS. These microbes constituted a specific target for EPA/DHA intervention in animals who were exposed as pups to a maternal separation paradigm as well as in neurodevelopmentally normal rats.

In adults, reduced bacterial diversity and altered microbial composition seem to be correlated with both inflammatory diseases and psychiatric illnesses (Abrahamsson et al., 2014; Kennedy et al., 2014; Neufeld and Foster, 2009). Of interest, MS animals clearly showed a disrupted microbiota compared to their counterpart, as already observed in previous studies in our lab (O'Mahony et al., 2009). This microbial disruption seems to be mainly due to a shift in Bacteroidetes: Firmicutes, which dominate the adult microbiota, between MS and NS females. Reduced Bacteroidetes: Firmicutes ratio in human stool specimen has been revealed in depressed individuals as well as in irritable bowel syndrome (IBS) patients (Jeffery et al., 2012; Jiang et al., 2015); this syndrome is often accompanied by depressive symptoms (Bengtson et al., 2015). Interestingly, EPA/DHA chronic administration reversed the early-

life stress-induced Bacteroidetes: Firmicutes shift in MS adult rats. Moreover, EPA/DHA increased Bacteroidetes abundance in adult early-life stressed rats. This presumably refers to an anti-inflammatory effect (Troy and Kasper, 2010), especially with regards to the EPA/DHA high dose treatment on the MS model. Moreover, the early-life stress-induced alteration of the microbiota was further confirmed by the diverse distribution of microbial community within the groups. Such distribution revealed all the NS groups clustered together.

Regarding the bacterial composition of the intestinal microbiota, phylum Verrucmicrobia and Akkermansia muciniphila is the most interesting. The relative abundances of both phylum were decreased by maternal separation, suggesting the MS-saline group were more susceptible to inflammation. Indeed, Verrucmicrobia and Akkermansia muciniphila have been shown to exacerbate gut inflammation in mice (Ganesh et al., 2013; Stecher et al., 2007). Similarly, Coriobacteriaceae abundance, reductions in which were associated with increased anti-inflammatory markers in humans (Martinez et al., 2013), was similarly reduced by EPA/DHA treatment both in NS and MS female rats. This reveals a possible EPA/DHA anti-inflammatory effect through the regulation of the gut microbiota composition. Such an effect is further confirmed by EPA/DHA-induce decreased abundance of Porphyromonadaceae, a family of bacteria that is likely to be involved in anti-inflammation (Zackular et al., 2013).

In conclusion, health benefits at the CNS level have indicated the contribution made by n-3 PUFAs to stress-related disorders (Hibbeln and Gow, 2014; McNamara et al., 2015; Peet, 2008). The study outlined in chapter 4 offers insight into a novel n-3 PUFAs mechanism through the modification of the gut microbiota in an animal model of stress. Our data suggest that the absence as well as the exacerbation of certain bacterial classes in the gut of early-life stressed rats may represent risk factors for the development of anxiety, depression and

inflammatory diseases such as IBS. We postulate that EPA/DHA administration is essential for the maintenance of certain taxa which immunoregulatory functions are necessary to prevent an overly robust stress-induced inflammatory response which may contribute to the onset of mental illnesses. Moreover, these findings emphasize the microbiota as an attractive target for potential therapeutic strategies since altered host's gut microbiota may infer health benefits, including mental health, to the host.

6.4. Phospholipid inefficacy in stress-related pathologies

There is an increasing body of scientific literature probing the role of individual phospholipids (PL) in ameliorating clinical problems such as the progression of cognitive decline in the elderly (Delwaide et al., 1986, Lee et al., 2010) so that it is being increasingly appreciated that dietary intake inter alia may have a role to play in the execution of appropriate clinical management strategies. Furthermore, while a considerable body of scientific evidence exists on the role of n-3 PUFAs in relation to neuronal development and cell signalling, the biological mechanisms by which PL function is less clear. PL constitutes the major component of all cell membranes. As such, they confer on membrane property of fluidity, and thus, determine and influence the behaviour of membrane-bound enzymes and receptors. Interestingly, adequate levels of PL in the cellular membrane have been observed to be crucial for the activation of several key signalling pathways involved in CNS function (Newton and Keranen, 1994; Huang et al., 2011).

During the past two decades growing evidence has linked alterations in the inflammatory system to stress-related pathologies, such as depression, including the presence of elevated levels of pro-inflammatory cytokines, together with other mediators of inflammation (Cattaneo et al., 2015). Within the phospholipids, it is the phosphatidylserine (PS) which has received the most attention from researchers. PS is the most abundant phospholipid in the

brain, comprising 13–15% of the phospholipids in the human cerebral cortex (Svennerholm, 1968). Moreover, it is enriched in DHA which is the most abundant n-3 PUFAs in the brain. Given the potential benefit in cognition and cell signalling exerted by PS, we undertook to study the potential PS protective effect against lipopolysaccharide (LPS)-induced increased cytokines levels in cortical astrocytes cultures. Contrary to our hypothesis, PS failed to reverse the effects of LPS in cortical cultures.

The biological mechanisms by which PL function is not yet clear. For instance, incontrovertible experimental evidence indicating that orally or intravenously administered PL actually alter neuronal membrane properties is lacking. How the administered PL are transported in the plasma, how much enters the brain, whether they are taken up intact, and whether they are incorporated into neurons or glia are not known. Dietary PL are hydrolysed during digestion, so orally administered PS most likely are not absorbed intact. Because the administered PL probably undergo partial or complete hydrolysis, the beneficial effects of PL are produced by the n-3 PUFAs released from the PL rather than the intact PL themselves. Of note, the metabolism of PL does not occur in cell cultures, hence this could explain the inefficacy of PL in the *in vitro* studies we carried out. These issues will have to be investigated in order to obtain some mechanistic insight into how PL may regulate cellular signalling and cognitive functioning.

6.5. n-3 PUFAs: future directions

In view of the evidence highlighted by this thesis, n-3 PUFAs have shown to improve anxiety- and depressive-like behaviours as well as cognition in female rats. Thereby, this finding raises the possibility that n-3 PUFAs may represent a potential strategy to reduce the risk of development of psychopathologies. However, further preclinical studies are required to more thoroughly investigate the effect of n-3 PUFAs in emotional state and cognition in different animal models. Such studies will facilitate informed prediction of expected effects in humans.

Despite additional research is required, our results nevertheless highlight a possible interaction within n-3 PUFAs and GR functionality, which may be considered as an explanation to our behavioural findings (*Chapter 3*). Moreover, we have observed regulation of GR abundance upon DHA treatment against CORT-induced GR down-regulation (*Chapter 2*). Therefore, it is tempting to speculate a possible GR-binding site interaction with DHA which could shed light on a novel n-3 PUFAs mechanism. With the noted limitations in mind, GR abundance resulted of importance for the regulation of BDNF expression levels upon both DHA and CORT treatments (*Chapter 2*). Further studies are needed to evaluate which are the molecular pathways by which CORT and DHA compete on the regulation of BDNF expression levels in order to prioritise specific molecular targets for follow up clinical investigation. This findings may open to novel investigations towards the role of n-3 PUFAs in relation to stress-related pathologies, such as anxiety and depression. At present, despite two promising results from our preclinical studies (Chapter 2 and 3; Pusceddu et al., 2015), there is insufficient data available to conclusively state whether n-3 PUFAs improve mood and cognition through GR regulation.

Our research also offers insights into the interaction between n-3 PUFAs and gut microbes in non-separated and early-life stressed female rats (*Chapter 4*). EPA/DHA treatment increased the abundance of specific members involved in anti-inflammatory activity. Being inflammation recognized to be linked with the occurrence of psychopathologies (Horowitz et al., 2013), the EPA/DHA-induced alteration of gut microbes opens to the possibility of an adjunct strategy by which n-3 PUFAs may improve mood and cognition. Thus, considering the increased evidence pointing to an effect of gut microbes on the CNS (Cryan and Dinan, 2012), it is tempting to speculate whether the EPA/DHA-induced improvement at behavioural and biochemical levels (chapter 2; Pusceddu et al., 2015) can be found in vagotomised rats. Moreover, individuate the pathways which regulate the EPA/DHA-microbiota interaction may lead to the manufacture of adjunct pharmacotherapies pointing to specific molecular targets useful in stress-related pathologies.

6.6. Conclusions

In this thesis, we have conducted research which has furthered our understanding of the role played by n-3 PUFAs in the brain and gut-microbiota as a beneficial dietary habit capable to reduce the risk of development of stress-related pathologies. This thesis, to our knowledge showed for the first time the role of DHA in the modulation of CORT-induced cellular changes in cortical cultures which may open novel avenues to counteract glucocorticoids-induced impairments in clinical settings. Further, this thesis emphasizes the relevance of dietary n-3 PUFAs in the improvement of emotional states and cognition highlighting the modulation of GR as a novel target for n-3 PUFAs. Moreover, this thesis sheds light on the interaction between n-3 PUFAs and the gut-microbiota composition which may have potentialities in health and mental health improvements as well as disease prevention. To build on this work, further studies are now needed to address unanswered questions regarding the role of n-3 PUFAs in mental health. Indeed, both *in vitro* and *in vivo* studies with a particular focus on the mechanisms by which n-3 PUFAs improve mood and cognition are necessary. Specifically, an investigation on the interaction between n-3 PUFAs and GR functionality is required to provide more conclusive information in this regard.

In this thesis sequencing of faecal samples has associated dietary n-3 PUFAs with specific bacterial species which have anti-inflammatory properties. These initial findings can undoubtedly open up to further investigation for a complete elucidation of the molecular processes linking bacteria, metabolic enzymes, and metabolites with their corresponding biological functions. Indeed, integration of the gene sequence of the microbiome with metaproteomics, metatranscriptomics, and metabolomics will pave the way toward a better molecular and functional understanding of the interplay between gut microbiota and host and its modulation by dietary n-3 PUFAs. Taken together these findings indicate that n-3 PUFAs may represent a potential strategy to prevent the development of stress-related pathologies.

This thesis represents a well-characterised portfolio of preclinical studies representing an appropriate starting point for human clinical studies in follow-on research.

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