Nourishing the brain from cradle to grave

The role of nutrients in neural development and neurodegeneration

Carola Janssen

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Carola IF Janssen, 2015; Nourishing the brain from cradle to grave: The role of nutrients in neural development and neurodegeneration.
PhD Thesis, Radboud university medical center

The research described in chapter 3 and 4 of this thesis was sponsored by Mead Johnson Pediatric Nutrition Institute.

The publication of this thesis was financially supported by the Department of Anatomy, Donders Institute for Brain, Cognition, and Behaviour, Radboud university medical center, Mead Johnson Pediatric Nutrition Institute and Alzheimer Nederland.

Cover design and lay-out
CIF Janssen
abstract | Shutterstock

Printing
Gildeprint, Enschede

ISBN
978-94-6284-033-1

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The role of nutrients in neural development and neurodegeneration

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. Th.L.M. Engelen,
volgens besluit van het college van decanen
in het openbaar te verdedigen op dinsdag 17 november 2015
om 10.30 uur precies

door

Carola Ingrid Frederika Janssen

geboren op 10 juni 1985
te Venlo

Promotoren
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Contents

Chapter 1

General Introduction

Nutrition plays an important role in developing and maintaining a healthy brain. In recent years, it has been shown that nutrients such as lipids and polyphenols influence processes supporting neuronal health (Cherniack, 2012; Gomez-Pinilla and Nguyen, 2012; Hooijmans *et al*., 2007; Muskiet, 2010; Vauzour, 2013). Current diets are associated with large cultural differences in health. For example, the typical Western diet is characterized by a high intake of saturated and trans-fatty acids and simple carbohydrates, increasing the risk of obesity and cognitive impairment (Davidson *et al*., 2005; Kanoski and Davidson, 2011). Adversely, it has been shown that the Mediterranean diet based on fish, olive oil, but also fruits, nuts, vegetables and whole grain leads to a lower risk of cardiovascular disease and contributes to a healthy brain (Cherniack, 2012; Estruch *et al*., 2013; Feart *et al*., 2009; Gardener *et al*., 2011; Guasch-Ferre *et al*., 2013; Hoevenaar-Blom *et al*., 2012; Jansen *et al*., 2013b; Nilsson *et al*., 2012; Renaud *et al*., 1995). In the 1970s, it was already demonstrated that the traditional Greenland Inuit diet was based on whale, seal, fish and wildfowl, which resulted in lower risk for ischemic heart disease (Bang *et al*., 1971; Bang *et al*., 1980; Muskiet, 2010). The common factor between these diets is that they are high in long-chain polyunsaturated fatty acids and low in saturated fats and refined grains. Furthermore, flavonoids, a type of polyphenols, originating from green tea have been used in Chinese medicine for thousands of years already (Gomez-Pinilla and Nguyen, 2012). It has been shown that epigallocatechin gallate, the main flavonoid in green tea alleviates stress and depression (Gomez-Pinilla and Nguyen, 2012). Likewise, flavonoids derived from cocoa and berries have demonstrated neuroprotective effects in ageing by supporting neurogenesis and cerebral blood flow (Cherniack, 2012; Francis *et al*., 2006; Vauzour, 2013). These studies suggest that nutrients supporting healthy cardiovasculature are also responsible for a healthy brain. Conversely, nutrients related to atherosclerosis, such as cholesterol, have also been associated with neurodegeneration and cognitive impairment (Hooijmans *et al*., 2007; Hooijmans *et al*., 2009; Jansen *et al*., 2012). This implies that the presence of genetic risk factors for Alzheimer's disease (AD), like ApoE4, combined with a high dietary intake of saturated fatty acids or cholesterol may result in a synergistic effect and dramatically increase the risk of AD.

In this thesis, we will focus not only on the influence of n-3 PUFA and flavonoids on neural development in (healthy) wild type mice, but also on the influence of cholesterol on neurodegeneration in ApoE4 and ApoE knockout mice.

LCPUFA

Long-chain polyunsaturated fatty acids (LCPUFA) are lipids that are mainly derived from diet and important in maintaining human health. The dietary intake in western countries has drastically changed during the industrial revolution from an omega-3 polyunsaturated fatty acids (n-3 PUFA) rich diet to an almost n-3 PUFA deficient diet accompanied by a sedentary lifestyle (Muskiet, 2010). Concerning fatty acid intake, Western diets have led to an increased intake of omega-6 polyunsaturated fatty acids (n-6 PUFA), saturated fatty acids (SFA) and trans fatty acids, and a decrease in n-3 PUFA (Muskiet, 2010). Human breast milk also reflects this shift in fatty acid composition (Ailhaud *et al*., 2006; Garcia *et al*., 2011; Makrides *et al*., 1996; Storck Lindholm *et al*., 2013). LCPUFA play an important role in brain development, especially during the growth spurt in the last trimester of pregnancy and the early postnatal period up to 2 years of age (Brenna, 2011; Gibson *et al*., 2011; Innis, 2009). The most abundant LCPUFA in the brain are docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) (Innis, 2009). The fetus obtains fatty acids through the placenta from the mother and is therefore depending on the maternal supply. After birth, breast milk is the only source of essential fatty acids for breast-fed infants.

Deficiency studies in rodents have demonstrated the importance of n-3 PUFA such as DHA, eicosapentaenoic acid (EPA, 20:5n-3), and their precursor α-linolenic acid (ALA, 18:3n-3) for brain development and cognitive functioning (Fedorova *et al*., 2009b; Fedorova and Salem, 2006; McNamara *et al*., 2009; Moriguchi and Salem, 2003). Research suggests that a n-3 PUFA

deficiency may lead to a variety of neuronal and psychological abnormalities, such as attention-deficit hyperactivity disorder (ADHD), depression, schizophrenia, autism, and anxiety (Chalon, 2006; Fedorova *et al*., 2009b; Fedorova and Salem, 2006; Gustafsson *et al*., 2010; Lyall *et al*., 2013; Muskiet, 2010; Perera *et al*., 2012; Yui *et al*., 2012). N-3 PUFA deficiency in rodents can result in hyperactivity, a characteristic underlying these neuropsychiatric disorders (Levant *et al*., 2004; Moriguchi and Salem, 2003; Umezawa *et al*., 1995). It has been proposed that n-3 PUFA deficiency may affect neurotransmission, especially within the dopaminergic and serotonergic systems, as a consequence of altered membrane fluidity and related receptor functions (Chalon, 2006; Muskiet, 2010). Furthermore, n-3 PUFA are important for the regulation of synaptic plasticity, and learning and memory via their involvement in regulating gene expression and retinoid signaling pathways (Cao *et al*., 2009a; Dyall and Michael-Titus, 2008; Dyall *et al*., 2010; Kitajka *et al*., 2004; Sidhu *et al*., 2011).

Due to the fact that both ALA and linoleic acid (LA, 18:2n-6; the precursor of n-6 PUFA) compete for the same conversion enzymes, delta-5-desaturase and delta-6-desaturase respectively (Schmitz and Ecker, 2008; Simopoulos, 2008a), a sufficient dietary intake of n-3 PUFA is necessary to maintain a healthy LCPUFA status. A high LA intake interferes with the desaturation and elongation of ALA and as well with the conversion of ALA via EPA to DHA (Simopoulos, 2008a). This imbalance in the n-6/n-3 ratio also results in the production of the n-6 ARA, leading to the formation of more pro-inflammatory eicosanoids, while the n-3 EPA is the precursor of anti-inflammatory eicosanoids (Schmitz and Ecker, 2008; Simopoulos, 2011; Tassoni *et al*., 2008). DHA can also interfere with neuroinflammation, as it is the precursor for resolvins and neuroprotectins, such as neuroprotectin D1 (NPD1) (Tapiero *et al*., 2002; Tassoni *et al*., 2008). Resolvins and neuroprotectins are produced in the brain to counteract and resolve neuroinflammation, thereby preventing excessive damage from the inflammatory response (Tassoni *et al*., 2008). NPD1 induces signaling for homeostatic maintenance of cellular integrity and can inactivate pro-apoptotic and pro-inflammatory signaling (Schmitz and Ecker, 2008). Therefore, it has been proposed that a balanced n-6/n-3 ratio is critical for maintaining a healthy brain and immune status.

Clinical studies have shown that (perinatal) LCPUFA supplementation may be beneficial for healthy neural development in both preterm and full term infants (Birch *et al*., 2010; Dunstan *et al*., 2008; Fang *et al*., 2005; Fewtrell *et al*., 2004; Henriksen *et al*., 2008; Isaacs *et al*., 2011; Janssen and Kiliaan, 2014; Makrides *et al*., 2010; Smithers *et al*., 2011; van Goor *et al*., 2011b; Westerberg *et al*., 2011; Willatts *et al*., 2013). Studies in full term infants indicate that pre- and postnatal supplementation are able to improve cognition which correspond with the found importance of starting supplementation in the last trimester of gestation in preterm infants (Birch *et al*., 2010; Birch *et al*., 2007; Birch *et al*., 2000; Dunstan *et al*., 2008; Helland *et al*., 2003b; Hoffman *et al*., 2003; Innis, 2008; Jensen *et al*., 2005; Judge *et al*., 2007a, b; Willatts *et al*., 2013).

Flavanols

Polyphenols are fruit and plant constituents that are well-known for their antioxidant properties. Flavonoids are a class of polyphenols that has been associated with improved cognition (Corcoran *et al*.; Ghosh and Scheepens, 2009; Hollenberg *et al*., 2009; Rendeiro *et al*., 2009; Spencer, 2009). Studies indicate that flavonoid consumption can beneficially affect normal cognitive function and cerebral blood flow in adults (Francis *et al*., 2006; Sorond *et al*., 2008; Vauzour, 2012).

In our study (chapter 4), we focused on flavanols, a group of flavonoids that are present in apple and grape seed extract. Flavanols are the most abundant flavonoids in the human diet and are mostly provided by fruits (such as apple and grapes), cocoa, tea, wine, nuts, and beans (Arola-Arnal *et al*., 2013). They have the ability to improve health by exerting cardioprotective, anti-inflammatory, and antioxidant effects (Arola-Arnal *et al*., 2013; Vauzour, 2012). Flavanols are best known for their antioxidant effects. Due to donation of a hydrogen atom from hydroxyl

groups to reactive oxygen species (ROS), phenoxyl radicals arise which can be stabilized by reactions with other radicals and stabilized oxygen species (Gomez-Pinilla and Nguyen, 2012). After ingestion, the largest portion of dietary flavonoids are metabolized in the small and large intestine, in the liver, and in the blood (Rendeiro *et al*., 2012). They form substrates for phase I and II enzymes and are de-glucosylated and further metabolized into glucuronides, sulphates, and O-methylated derivatives (Rendeiro *et al*., 2012). Flavonoids or their metabolites must pass the blood-brain barrier (BBB) in order to be able to influence brain health directly. The ability of structures to cross the BBB depends on their systemic availability and lipophilicity (Faria *et al*., 2014; Rendeiro *et al*., 2012). As a result, theoretically the O-methylated metabolites would be more potent to pass the BBB than sulphates and glucuronides, which has been shown in *in vitro* studies (Rendeiro *et al*., 2012; Vauzour, 2012; Youdim *et al*., 2003).

While the effects of flavonoid supplementation have been studied in normal aging and neurodegenerative models, little is known about their influence during development. In neurodegeneration, flavonoids have shown to exert neuroprotective properties such as the modulation of neuroinflammation and improvement of cognition (Vauzour, 2012). It has been shown that flavonoids modulate neuronal signaling pathways involved in synaptic plasticity and neurogenesis (Spencer, 2010; Vauzour, 2012). *In vivo*, flavonoids are able to affect these pathways by activation of cyclicAMP-response element binding (CREB) receptors and brain derived neurotrophic factor (BDNF), mechanisms that are involved in memory acquisition and consolidation (Spencer, 2010). Flavonoids possibly affect neural development, but this has not been studied yet.

Cholesterol & ApoE

A sedentary lifestyle and a high dietary intake of saturated and trans-fatty acids form risk factors for a spectrum of diseases, such as cardiovascular disease and neurodegenerative disorders (like AD). Risk factors for cardiovascular disease, such as hypertension and atherosclerosis, are also associated with cerebrovascular disease. Both hypertension and atherosclerosis cause impaired blood flow and blood brain barrier function, hypoperfusion and blood vessel wall pathology which may initiate the underlying neurodegenerative processes leading to cognitive impairment and ultimately AD (de la Torre, 2004, 2012; Grammas *et al*., 2002; Kalaria *et al*., 2012; Muller *et al*., 2007; Skoog and Gustafson, 2006). Hypertension can predict both vascular dementia and AD already 20 years before onset (Haan and Wallace, 2004; Launer and Hofman, 2000; Ruitenberg *et al*., 2001; Skoog and Gustafson, 2006). A genetic risk factor that has been associated with both cardiovascular disease and AD is Apolipoprotein E4 (ApoE4) (Tanzi and Bertram, 2001). Apolipoprotein E is the most common apolipoprotein in the brain and is involved in endocytosis, transport and clearance of lipids, such as cholesterol, playing an essential role in lipid metabolism in the brain (Kim *et al*., 2009). There are 3 alleles known for the APOE gene: ε2, ε3, and ε4. While APOEε4 seems to accelerate AD pathogenesis and vasculature, APOEε2 appears to have a protective effect (Kim *et al*., 2009). The most frequent allele is APOEε3, which regulates cholesterol transport and clearance in a normal manner (Knouff *et al*., 1999; Stojakovic *et al*., 2004). The APOEε4 allele does not only show a decrease in cholesterol clearance, but *in vitro* studies have also demonstrated a reduction in cholesterol synthesis by astrocytes and neurons (Jansen *et al*., 2009).

For a long time, two main hypotheses for the development of AD have been supported in the scientific community, with on one hand the amyloid hypothesis and on the other hand the vascular hypothesis (de la Torre, 2004; de la Torre and Stefano, 2000; Joseph *et al*., 2001). Recently, it has become clear that these hypotheses cannot be as clearly distinguished as previously suggested: AD is a multifactorial disease, caused by a combination of risk factors. For instance, the presence of APOEε4 (a risk factor for cardiovascular disease and AD) and advanced aging may lead to cerebral hypoperfusion. This will result in a neuroglial energy crisis and

subsequently white matter lesions causing neurodegeneration and AD. Cerebral hypoperfusion also leads to abnormal cleavage of β-amyloid (Aβ) (Kim *et al*., 2009). This causes Aβ depositioning and cerebral amyloid angiopathy (CAA) causing neurodegeneration and AD as well (Klann and Dever, 2004). CAA itself again leads to cerebral hypoperfusion which in its turn accelerates the process of neurodegeneration.

Carriers of the APOEε4 allele show high serum cholesterol levels. Cholesterol is absorbed from food or synthesized in the liver, intestine or brain. It is required for the formation of bile acids, steroid hormones and membrane synthesis, but excessive levels lead to hypercholesterolemia. Cholesterol circulating in the blood is not able to cross the blood brain barrier (BBB). Yet, the brain is the organ in the human body that contains the most cholesterol (Jenner *et al*., 2010). In the brain, it is mainly synthesized by astrocytes and especially present in the myelin sheaths and membranes of neurons and astrocytes (Donahue and Johanson, 2008). In general, increased serum levels of cholesterol (hypercholesterolemia) lead to increased blood levels of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) (Libby *et al*., 2013). LDL and VLDL invade the endothelium and start accumulating, initiating a state commonly known as atherosclerosis (Libby *et al*., 2013). The damage to the endothelium initiates an inflammatory response and monocytes and macrophages start accumulating, while platelets adhere to the affected area (Libby *et al*., 2013). Eventually, this will evolve into a chronic inflammatory state if the cholesterol intake is not changed. This situation increases (cerebral) hypoperfusion and ultimately increase the risk of cardiovascular disease. Birdsill *et al*. have demonstrated that decreased cerebral blood flow in patients with metabolic syndrome, a collection of cardiovascular risk factors, is associated with impaired cognitive functions (Birdsill *et al*., 2013). Metabolic syndrome is characterized by high triglyceride and low HDL cholesterol levels. Furthermore, it has been shown that ApoE4 and ApoE knockout mouse models display reduced cerebral blood flow (Zerbi *et al*., 2014b).

Mouse models expressing human ApoE4 (C57BL/6J background strain with a knock-in of the human APOEε4 allele) have shown cognitive impairment and vascular changes (Knouff *et al*., 1999). This homozygous knock-in model has more difficulty clearing serum cholesterol from blood vessel membranes and therefore shows impaired vasculature more often (Knouff *et al*., 1999). Furthermore, this model displays an increased risk of developing atherosclerosis. It serves as a model for vascular pathology in AD. It was demonstrated that a DHA enriched diet can prevent the pathological phenotype in ApoE4 targeted replacement mice (Kariv-Inbal *et al*., 2012). APOE deficient mice are used as a model for atherosclerosis (Piedrahita *et al*., 1992). This model will display accumulation of cholesterol in the body, because of its impairment to clear cholesterol. Supplementation with a n-3 fatty acid enriched diet indicated a reduction of the increased levels of reactive oxygen species in ApoE-/- mice (Suchy *et al*., 2009).

Outline thesis

This thesis aims to determine the influence of several nutrients on neural development and neurodegeneration. Little is known about the effect of both LCPUFA and flavanols on neural development. Therefore, we studied the effect of supplementation starting during gestation and lasting throughout life in C57Bl/6J mice. Furthermore, we also studied the effect of a cholesterol enhanced diet on neurodegeneration and cognitive impairment in mouse models at risk for atherosclerosis and Alzheimer's disease.

A range of methodologies were applied throughout this thesis to gain a better understanding of the role of nutrients on neural development and neurodegeneration. Animals underwent cognitive and behavioral tests to study locomotion, sensory motor integration, and spatial learning and memory. We used magnetic resonance imaging and spectroscopy (MRI and MRS) at 7 and 11.7 Tesla to determine cerebral blood flow (CBF), white and grey matter integrity, and brain metabolite levels. Neurohistology was assessed using immunohistochemical stainings for synaptic plasticity, neurogenesis, neuroinflammation, and vascular density. Quantitative real-time PCR (qRT-PCR) was used to study gene expression of inflammatory markers, angiogenesis, and synaptic plasticity.

Chapter 2

In this review, we will focus on the involvement of LCPUFA from genesis to senescence. We will cover the stages of neural development, normal aging, and neurodegeneration. In all these stages, the role of LCPUFA in the brain will be discussed with emphasis on synaptic plasticity, neurogenesis, cognition, and vascular health.

Chapter 3

The aim of this study was to obtain detailed insights into the mechanisms underlying the long-term beneficial effects of n-3 PUFA availability during gestation and throughout life in mice. A broad combination of parameters was determined to assess effects on behavior, brain structure and function. With behavioral and cognitive tests, changes in cortical and hippocampal functionality were studied. Cerebral metabolite status was measured with phosphorus magnetic resonance spectroscopy (^{31}P MRS) to assess energy metabolism and neuronal membrane turnover, immunohistochemistry evaluated neurogenesis and synaptic plasticity, and qRT-PCR was used to study gene expression of inflammatory markers.

Chapter 4

The impact of flavanol availability during gestation and the postnatal period was assessed in mice to understand the mechanisms underlying potential long-term beneficial effects throughout life. A broad spectrum of parameters was investigated in order to evaluate effects on brain structure and function. Changes in cortical and hippocampal functionality were studied with behavioral and cognitive tests. Proton magnetic resonance spectroscopy (^{1}H MRS) was used to assess brain metabolism, while CBF was measured with arterial spin labeling (ASL), and diffusion tensor imaging (DTI) was performed to study brain integrity. Neurogenesis, synaptic plasticity and capillary density were evaluated immunohistochemically, and qRT-PCR was used to study gene expression of inflammatory markers, synaptic plasticity and angiogenesis.

Chapter 5

In this study, we hypothesize that ApoE4 and ApoE deficient mice are vulnerable to high-fat diet induced neurodegeneration and cognitive impairment. Cognitive tests were performed to study (spatial) learning and memory, while neuropathology was evaluated with immunohistochemical stainings for neurogenesis, synaptic plasticity, neuroinflammation, and vascular density.

Chapter 6

All study findings presented in this thesis are summarized and discussed in this chapter, and we propose suggestions for future experiments to gain a better understanding of the role of nutrients on neural development and neurodegeneration.

Chapter 2

Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: the influence of LCPUFA on neural development, aging, and neurodegeneration

Carola IF Janssen, Amanda J Kiliaan

Department of Anatomy, Radboud university medical center, Donders Institute for Brain, Cognition, and Behaviour

Prog Lipid Res 53 (2014) 1-17

Abstract

Many clinical and animal studies demonstrate the importance of long-chain polyunsaturated fatty acids (LCPUFA) in neural development and neurodegeneration. This review will focus on involvement of LCPUFA from genesis to senescence. The LCPUFA docosahexaenoic acid and arachidonic acid are important components of neuronal membranes, while eicosapentaenoic acid, docosahexaenoic acid, and arachidonic acid also affect cardiovascular health and inflammation. In neural development, LCPUFA deficiency can lead to severe disorders like schizophrenia and attention deficit hyperactivity disorder. Perinatal LCPUFA supplementation demonstrated beneficial effects in neural development in humans and rodents resulting in improved cognition and sensorimotor integration. In normal aging, the effect of LCPUFA on prevention of cognitive impairment will be discussed. LCPUFA are important for neuronal membrane integrity and function, and also contribute in prevention of brain hypoperfusion. Cerebral perfusion can be compromised as result of obesity, cerebrovascular disease, hypertension, or diabetes mellitus type 2. Last, we will focus on the role of LCPUFA in most common neurodegenerative diseases like Alzheimer's disease and Parkinson's Disease. These disorders are characterized by impaired cognition and connectivity and both clinical and animal supplementation studies have shown the potential of LCPUFA to decrease neurodegeneration and inflammation. This review shows that LCPUFA are essential throughout life.

Introduction

Long-chain polyunsaturated fatty acids (LCPUFA) are lipids which are mainly derived from diet and important in maintaining human health. With the industrial revolution, the Western dietary intake has drastically changed from an omega-3 polyunsaturated fatty acids (n-3 PUFA) rich diet to an almost n-3 PUFA deficient diet accompanied by a sedentary lifestyle (Muskiet, 2010). Regarding fatty acid content, Western dietary intake has shifted to an increase in omega-6 polyunsaturated fatty acids (n-6 PUFA), saturated fatty acids (SFA) and trans fatty acids, and a decrease in n-3 PUFA (Muskiet, 2010). The current diets are also associated with large cultural differences in health. For example, the traditional Greenland Inuit diet was based on whale, seal, fish and wildfowl, which was demonstrated to result in lower risk for ischemic heart disease (Bang *et al*., 1971; Bang *et al*., 1980; Muskiet, 2010). The Mediterranean diet is not only based on fish, but also fruits, vegetables and whole grain and it has been shown that it leads to a lower risk of cardiovascular disease and contributes to a healthy brain (Estruch *et al*., 2013; Feart *et al*., 2009; Guasch-Ferre *et al*., 2013; Hoevenaar-Blom *et al*., 2012; Jansen *et al*., 2013b; Nilsson *et al*., 2012; Renaud *et al*., 1995). The common factor between these diets is that they are low in saturated fats and refined grains.

Over the years, the importance of LCPUFA in neural development, aging, and neurodegeneration has been shown in both clinical and animal studies (Agostoni, 2008; Belkind-Gerson *et al*., 2008; Calon and Cole, 2007; Das, 2008; Freund-Levi *et al*., 2008; Hooijmans *et al*., 2007; Innis, 2008; van Goor *et al*., 2010). Supplementation with LCPUFA has shown to be beneficial in the development of both children and (young) rodents. Several animal studies and studies in children revealed an improvement in cognition and motor skills after LCPUFA supplementation (Coluccia *et al*., 2009; Fedorova *et al*., 2009b; van Goor *et al*., 2010). On the other hand, LCPUFA deficiency can lead to neurodevelopmental disorders such as schizophrenia, ADHD or mood disorders (Fedorova *et al*., 2009a; Mathieu *et al*., 2011; McNamara *et al*., 2013a; Richardson, 2006; Sethom *et al*., 2010). LCPUFA have also been shown to be advantageous in neurodegenerative disorders. For example, dietary LCPUFA supplementation showed an attenuation of cognitive impairment and decreased anxiety in both human and animal studies (Kamphuis *et al*., 2011; Scheltens *et al*., 2010; Shah, 2011).

In this review, we will focus on the involvement of LCPUFA from genesis to senescence. We will cover the stages of neural development, normal aging, and neurodegeneration. In all these stages, the role of LCPUFA in the brain will be discussed with emphasis on synaptic plasticity, neurogenesis, cognition, and vascular health.

We searched the PubMed database for original articles published in English from 1995 until August 31, 2013. The main search topics concerned LCPUFA, influence of LCPUFA in neural development of preterm and full term infants, influence of LCPUFA in disorders such as autism, attention deficit hyperactivity disorder (ADHD), mild cognitive impairment (MCI), cerebrovascular disease, Alzheimer's disease (AD) and Parkinson's disease (PD). The search strategy was based on the following search terms: LCPUFA, neural development, cognition, autism, ADHD, healthy aging, MCI, cerebrovascular disease, AD, PD, filter: clinical trials. Moreover, to identify potentially relevant new papers we filtered our total list of relevant papers by hand. Based on the title and abstract, we selected the studies. If these two components were not sufficient for selection, we evaluated the total publication.

LCPUFA in neural development

During the embryonic phase in humans (until 7 weeks) the structure of the brain is defined, while growth during the fetal phase (start at 8 weeks) is characterized by functional development (Fitzgerald, 2002; Larsen *et al*., 2011). At birth, the brain is fully developed but only 25% of its definitive volume; postnatally, the brain expands by an increase in glial cells, outgrowth of axons and dendrites, and myelination of nerve fibers. This human brain growth spurt starts prenatally

in the third trimester of pregnancy (Mostofsky, 2001). At this time, the infant brain starts accumulating docosahexaenoic acid (DHA, 22:6n-3) in utero and this continues up to the first 24 months of neonatal brain growth, although the postnatal DHA accumulation occurs at a slower rate (Dagai *et al*., 2009; Mostofsky, 2001). In this period, neural development is most dependent on an adequate supply of LCPUFA.

LCPUFA are essential nutrients in the development and functioning of brain and visual system (Belkind-Gerson *et al*., 2008; Innis, 2008; Koletzko *et al*., 2008). The most abundant LCPUFA in the brain are DHA which is mainly derived from fish, and arachidonic acid (ARA, 20:4n-6) from animal sources like meat and eggs. Linoleic acid (LA, 18:2n-6) is the precursor molecule of ARA which is derived from LA by desaturation and elongation of the carbon chain. DHA is derived from α-linolenic acid (ALA, 18:3n-3), forming eicosapentaenoic acid (EPA, 20:5n-3) in the process. The placental fatty acid composition is dependent on the supply from maternal plasma fatty acids. After birth, breast-fed infants are subsequently supplied with n-3 and n-6 fatty acids from breast milk, which support the rapid growth and development of the infant brain (Hadders-Algra, 2005; Helland *et al*., 2003b; Hoffman *et al*., 2009; Innis, 2008).The most important LCPUFA responsible for the growth of the brain are DHA and ARA. Aside from inflammation and cardiovascular health, LCPUFA are important building blocks of neuronal membranes. The lipid bilayer of neuronal membranes consists of phospholipids, with DHA, ARA, and EPA as their main components. Three compounds are important for the membrane formation as shown in the Kennedy cycle (Figure 1): a uridine source, a fatty acids source, and a choline source (Kennedy and Weiss, 1956; Wurtman *et al*., 2009). Other phospholipids are also synthesized via the Kennedy cycle and incorporate LCPUFA, such as phosphatidylethanolamine (PE) that uses ethanolamine instead of choline (Sastry, 1985). Phosphatidylserine (PS) exchanges a serine molecule for choline in phosphatidylcholine (PC) or ethanolamine

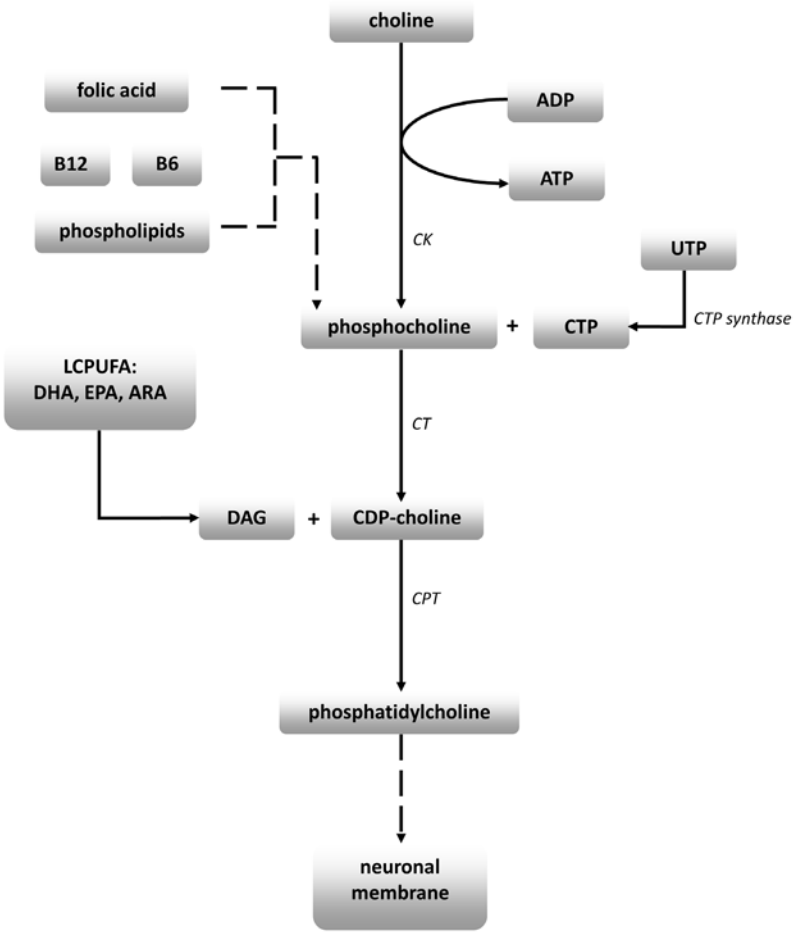


Figure 1: Formation of neuronal membranes. Schematic overview of the formation of neuronal membranes from LCPUFA, also known as the Kennedy pathway (Cansev, 2006; Kennedy and Weiss, 1956; Wurtman *et al*., 2009; Wurtman *et al*., 2000).

in PE (Sastry, 1985).

Humans, like all mammals, can synthesize saturated and monounsaturated fatty acids, but they are not able to synthesize the n-3 fatty acid ALA and the n-6 fatty acid LA due to lack of the conversion enzyme n-3-desaturase, making ALA and LA essential fatty acids (Simopoulos, 2008b). Humans are able to convert EPA to DHA, and ARA to all-cis-4,7,10,13,16-docosapentaenoic acid (osbond acid), but the conversion rate by the responsible delta-5- and delta-6-desaturase is very slow (Rzehak *et al*., 2009; Xie and Innis, 2008). These n-3 and n-6 PUFA are obtained by dietary intake or endogenous conversion of the parent precursors. LA and ALA require the same conversion enzymes, which means that there is competitive inhibition between these 2 substrates. Especially delta-6-desaturase favors the conversion of n-3 fatty acids to that of n-6 fatty acids (Schmitz and Ecker, 2008; Simopoulos, 2008b). Despite the preference for conversion of n-3 PUFA, a high LA intake may shift the balance towards conversion of n-6 PUFA and can interfere with the desaturation and elongation of ALA (Gibson *et al*., 2011). This imbalance can also lead to inhibition of the conversion of ALA to DHA, by slowing down the conversion rate of ALA into EPA and of EPA into DHA by delta-6-desaturase. The fatty acid desaturase (FADS) 1 and FADS2 genes are responsible for the expression of the conversion enzymes delta 5 desaturase and delta 6 desaturase making them a rate limiting factor in the LCPUFA conversion (Rzehak *et al*., 2009; Schaeffer *et al*., 2006; Xie and Innis, 2008). Thus, polymorphisms in these genes are able to influence the conversion of LCPUFA. Some studies suggest that these polymorphisms may increase the sensitivity of LCPUFA intervention, which recommend investigation of the genetic variation when performing a supplementation study (Schaeffer *et al*., 2006; Simopoulos, 2010; Xie and Innis, 2008).

EPA and ARA are also important during this period for the production of eicosanoids (prostaglandins, thromboxanes, leukotrienes) and their involvement in inflammation (Figure 2). Eicosanoids are lipid mediators that are involved in a wide array of physiological functions, some of which are vasoreactivity, platelet aggregation, and inflammation (Schmitz and Ecker, 2008; Simopoulos, 2011). The eicosanoids derived from LCPUFA exert effects that are involved in inflammation, for example the regulation of arrhythmia, platelet activation, vasoreactivity, and inflammation, resulting in either enhanced or compromised immunity (Muskiet, 2010; Schmitz and Ecker, 2008; Simopoulos, 2011). Prostaglandins, leukotrienes and thromboxanes are metabolites that regulate inflammatory mediation and they are metabolized by cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) (Tapiero *et al*., 2002; Tassoni *et al*., 2008). ARA is the precursor for the 2-series of prostaglandins and thromboxanes, and the 4-series of leukotrienes. EPA is a precursor for the 3-series of prostaglandins and thromboxanes, and the 5-series of leukotrienes and these eicosanoids are less pro-inflammatory (Schmitz and Ecker, 2008). The prostaglandin 2-series therefore exert pro-arrhythmic effects, while the 3-series shows anti-arrhythmic effects and the thromboxane 2-series act as platelet activator and vasoconstrictor, while the 3-series performs as a platelet inhibitor and vasodilator. Furthermore, leukotrienes of the 4-series have pro-inflammatory effects, while the 5-series exert anti-inflammatory effects. As a result, ARA shows typical pro-inflammatory properties opposed to EPA that overall exerts anti-inflammatory effects (Schmitz and Ecker, 2008). Furthermore, 5-LOX is responsible for the generation of anti-inflammatory eicosanoids that are derived from DHA and EPA, the resolvins and neuroprotectins (Tapiero *et al*., 2002; Tassoni *et al*., 2008). Neuroprotectin D1 (NPD1) induces signaling for homeostatic maintenance of cellular integrity, and also has the ability to inactivate pro-apoptotic and pro-inflammatory signaling (Schmitz and Ecker, 2008). Besides regulation of inflammation, these physiological properties of LCPUFA contribute to cardiovascular health. EPA and DHA have the potential to prevent arrhythmia, lower blood pressure, reduce plasma triglycerides, improve vasoreactivity, increase plaque stability, reduce platelet aggregation, and decrease inflammation (Breslow, 2006; Kris-Etherton *et al*., 2003; Mozaffarian and Wu, 2012). This will be discussed in more detail in the Section on 'LCPUFA in normal aging'.

Several studies in humans and rodents have shown that an n-3 PUFA deficiency may lead to a variety of neuronal abnormalities, such as ADHD, depression, schizophrenia, autism spectrum disorders, and anxiety (Bourre *et al*., 1984; Chalon, 2006; Fedorova *et al*., 2009a; Fedorova *et al*., 2009b; Levant *et al*., 2004; Moriguchi and Salem, 2003; Umezawa *et al*., 1995). It is proposed that an n-3 PUFA deficiency reduces neurotransmission processes, especially the dopaminergic and serotonergic system, by affecting membrane fluidity and related receptor functions and thereby ultimately affecting brain structure and function (Chalon, 2006; Muskiet, 2010). Studies in n-3 PUFA deficient rodents indicate that this deficiency may result in hyperactivity which underlies disorders such as ADHD and schizophrenia (Chalon, 2006; Levant *et al*., 2004; Moriguchi and Salem, 2003; Umezawa *et al*., 1995). ADHD is the most common developmental disorder occurring during childhood and has been shown to persist into adulthood (Kooij *et al*., 2005; Richardson, 2006). ADHD often shows comorbidity with behavioral and learning disorders in childhood, while it is linked to mood disorders during adulthood (Chalon, 2009; Muskiet, 2010; Richardson, 2006). A genetic association has been found between the occurrence of ADHD and disorders such as dyslexia, antisocial behavior, mood disorders, and schizophrenia. Studies have also shown that environment, in particular nutrition, strongly influences genetic expression (Luchtman and Song, 2013; Richardson, 2006). It has been demonstrated that LCPUFA and their metabolites are involved in regulating gene expression such as cAMP response element binding protein (CREB), which plays an important role in long term potentiation (Cao *et al*., 2009b; Sidhu *et al*., 2011). But also in the retinoid signaling pathways regulating synaptic plasticity, learning, and memory involving the retinoic acid receptor (RAR), retinoid X receptor (RXR), and peroxisome proliferator-activated receptor (PPAR) (Dyall and Michael-Titus, 2008; Dyall *et al*., 2010; Kitajka *et al*., 2004).

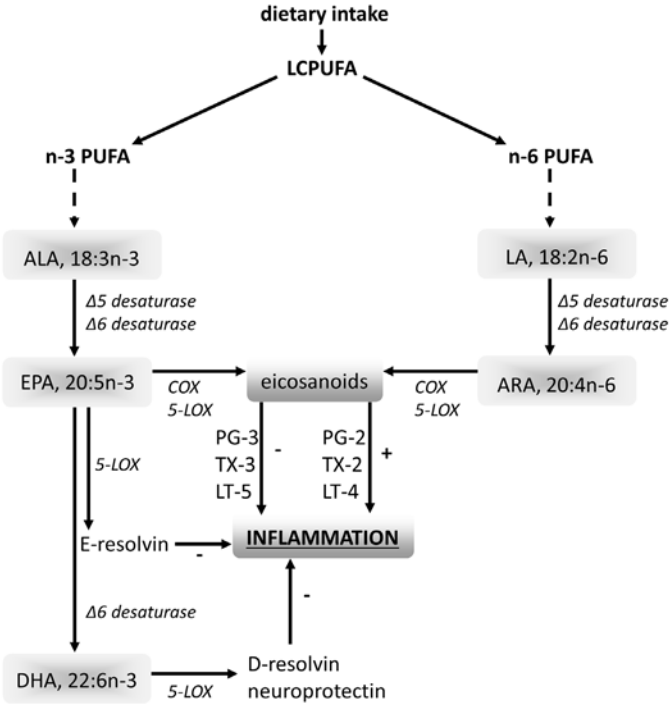


Figure 2: Involvement of LCPUFA in inflammation. Schematic overview of the involvement of EPA, ARA, and DHA in inflammation.

In short, LCPUFA have the ability to influence human health in many different ways. DHA, EPA and ARA are important components of neuronal membranes, while DHA and EPA also contribute to cardiovascular health, and finally ARA and EPA act as precursors in the production of eicosanoids. As mentioned before, ARA (n-6) tends to lead to more pro-inflammatory eicosanoids, while EPA (n-3) leads to mostly anti-inflammatory eicosanoids. For this reason, the balance between n-3 and n-6 PUFA is critical in maintaining a healthy LCPUFA status and it has been suggested that a n-6/n-3 ratio of 1/1 or 2/1 would be the optimal ratio (Simopoulos, 2011). A balanced ratio is very important for many bodily and brain functions like in inflammation, endocrine and cardiovascular system and (lipid) metabolism (Hjorth and Freund-Levi, 2012; Innis, 2008; Simopoulos, 2008b; van Goor *et al*., 2010; Wall *et al*., 2010).

Clinical studies

Table 1A, B and C show an overview of LCPUFA supplementation studies during pregnancy and/or lactation in pre- and full term infants, and postnatal supplementation in patients that are diagnosed with either autism spectrum disorders or ADHD (Table 1A, B and C).

The influence of LCPUFA in the neural development of preterm infants is being studied more and more over the last decade (Table 1A). Only one study investigated prenatal supplementation, while most other studies focused on postnatal supplementation (Fang *et al*., 2005; Fewtrell *et al*., 2004; Fewtrell *et al*., 2002; Henriksen *et al*., 2008; Isaacs *et al*., 2011; Makrides *et al*., 2010; O'Connor *et al*., 2001; Smithers *et al*., 2010; Smithers *et al*., 2008; Westerberg *et al*., 2011). The results of these studies have been ambiguous, with some studies indicating slight beneficial effects (Fang *et al*., 2005; Henriksen *et al*., 2008; O'Connor *et al*., 2001; Smithers *et al*., 2008; Westerberg *et al*., 2011) and others demonstrating no neurodevelopmental effects (Fewtrell *et al*., 2004; Fewtrell *et al*., 2002; Isaacs *et al*., 2011; Makrides *et al*., 2010; Smithers *et al*., 2010) and none showing major adverse effects. Overall, the preterm supplementation studies show beneficial effects of the supplementation when the n-6/n-3 ratio is at an optimal balance of 1/1 to 2/1.

The problem with studies on preterm infants is that these infants cannot fully benefit from the accumulation of LCPUFA which start in the last trimester of gestation, because of their preterm birth. Thus, the nutritional LCPUFA status of preterm infants needs to be optimal in order to complement the LCPUFA accumulation. Makrides *et al*. demonstrated that prenatal supplementation did not affect neural development in preterm infants (Table 1A) (Makrides *et al*., 2010). They supplemented preterm infants starting before 21 weeks of gestation until birth (Makrides *et al*., 2010). However, these infants are born in the last trimester of gestation, missing the supplementation at a crucial time point. Another issue is that the preterm infants in this study were supplemented with n-3 PUFA only (Makrides *et al*., 2010). It is important in preterm infants that they are supplemented with both n-3 and n-6 PUFA. It is also advisable to add the n-6 ARA to n-3 PUFA supplements, because n-3 PUFA are known to decrease the plasma concentrations of ARA due to the competition for conversion by delta-6 desaturase (Aggett *et al*., 1991; Carlson, 1996; Carlson *et al*., 1991; Carlson *et al*., 1987; Clandinin *et al*., 1992; Foreman-van Drongelen *et al*., 1995; Koletzko *et al*., 1989).

The postnatal supplementation studies in preterm infants show more beneficial effects of LCPUFA than in the prenatal phase (Table 1A). These studies started supplementation at birth or directly after birth, a time point that would originally mark the last trimester of gestation when DHA accumulation begins. Table 1A shows that in only one cohort the supplementation was aborted at the estimated due date (Smithers *et al*., 2010; Smithers *et al*., 2008). Smithers *et al*. reported no difference in visual acuity at 2 months, but they did find an improvement at 4 months of age (Smithers *et al*., 2008). In the same cohort no differences in language development were shown at 26 months, and there was no difference in behavior between 3 and 5 years of age (Smithers *et al*., 2010). In this study only n-3 PUFA was supplemented. As shown in Table 1A, the other postnatal supplementation studies continued supplementation ranging from 3 weeks up until 12 months (corrected age) with both n-3 and n-6 PUFA (Fang *et al*., 2005; Fewtrell *et al*., 2004; Fewtrell *et al*., 2002; Henriksen *et al*., 2008; Isaacs *et al*., 2011; O'Connor *et al*., 2001; Westerberg *et al*., 2011). These studies stress the importance of an optimal n-6/n-3 ratio (1/1 to 2/1). A 9 months supplementation with a LCPUFA ratio of 1/15 did not affect cognition at 9 months, 18 months, or 10 years of age (Fewtrell *et al*., 2004; Isaacs *et al*., 2011). Studies using a ratio of 1/1 or 2/1 did show long term improvement of cognitive functions starting already with 9 weeks of supplementation (Fang *et al*., 2005; Henriksen *et al*., 2008; O'Connor *et al*., 2001; Westerberg *et al*., 2011). However, Fewtrell *et al*. used a "healthy" n-6/n-3 ratio of 1.5/1 but did not find positive effects on cognition at 9 or 18 months of age. In this study the duration of supplementation was 33 days on average which appears to be too short to establish an effect on cognition. These

studies indicate that apart from a balanced n-6/n-3 ratio, the duration of supplementation is another key factor contributing to beneficial effects of LCPUFA supplementation.

Numerous studies have been performed investigating the impact of LCPUFA supplementation on general and neurological development of full term infants (Table 1B) (Auestad *et al*., 2001; Auestad *et al*., 2003; Birch *et al*., 2010; Birch *et al*., 2007; Birch *et al*., 2000; Bouwstra *et al*., 2006b; de Jong *et al*., 2010; Dunstan *et al*., 2008; Firmansyah *et al*., 2011; Helland *et al*., 2003b; Hoffman *et al*., 2003; Horby Jorgensen *et al*., 1998; Innis and Friesen, 2008; Jensen *et al*., 2005; Judge *et al*., 2007a, b; Lauritzen *et al*., 2005; Lucas *et al*., 1999; Malcolm *et al*., 2003; Pivik *et al*., 2009; Scott *et al*., 1998; Smithers *et al*., 2011; van Goor *et al*., 2011a; Willatts *et al*., 2013). However, there are only few studies exceeding the age of 18 months (Auestad *et al*., 2003; Birch *et al*., 2007; Bouwstra *et al*., 2006b; de Jong *et al*., 2010; Dunstan *et al*., 2008; Firmansyah *et al*., 2011; Helland *et al*., 2003b; Jensen *et al*., 2005; Willatts *et al*., 2013). Often, clinical supplementation studies show little to no effect of LCPUFA supplementation (Auestad *et al*., 2001; Auestad *et al*., 2003; Bouwstra *et al*., 2006b; de Jong *et al*., 2010; Firmansyah *et al*., 2011; Horby Jorgensen *et al*., 1998; Lucas *et al*., 1999; Malcolm *et al*., 2003; Pivik *et al*., 2009; Smithers *et al*., 2011; van Goor *et al*., 2011a) and the few effects found showed just slight improvements in cognition, visual function, or motor skills (Birch *et al*., 2010; Birch *et al*., 2007; Birch *et al*., 2000; Dunstan *et al*., 2008; Helland *et al*., 2003b; Hoffman *et al*., 2003; Innis and Friesen, 2008; Jensen *et al*., 2005; Judge *et al*., 2007a, b; Willatts *et al*., 2013). Two studies reported decreased language skills at 12 and 14 months of age after supplementation with only n-3 PUFA after 4 and 12 months of supplementation respectively (Lauritzen *et al*., 2005; Scott *et al*., 1998). Table 1B demonstrates the overall finding that both pre- and postnatal supplementation are potent in achieving effects on LCPUFA supplementation of full term infants. This is in line with the finding in preterm infants that it is important to start supplementation in the last trimester of gestation. However, in contrast to preterm supplementation where the n-6/n-3 ratio was of importance, full term supplementation studies remain inconclusive whether only n-3 PUFA or both n-3 and n-6 PUFA supplementation are more suitable. Overall, n-3 PUFA show beneficial effects prenatally, while both n-3 and n-6 PUFA demonstrate to be favorable during postnatal supplementation (Table 1B).

Several studies and reviews have made recommendations for a healthy LCPUFA intake in preterm and full term infants based on the available data from randomized controlled trials (Hadders-Algra, 2011; Koletzko *et al*., 2001; Lapillonne *et al*., 2013; Meldrum *et al*., 2011; Simmer *et al*., 2011). In these studies a DHA intake of 0.35-1% in preterm infants and 0.2-0.32% for full term infants is suggested. The advised concentration of ARA is 0.4-0.8% for preterm infants and 0.35% for full term infants. It should be noted that these concentrations are quite conservative as they reflect the average concentrations in maternal milk from Western countries.

A key factor that one has to keep in mind while interpreting supplementation studies on full term infants is that it is plausible that the effect of LCPUFA supplementation is limited in a healthy (LCPUFA sufficient) full term cohort. Prenatal supplementation studies have shown to be most likely to show beneficial effects, because supplementation already starts at the critical time point which is the start of the last trimester of gestation.

It is difficult to study underlying mechanisms in humans due to limited parameters. In infants, the outcomes are limited to non-invasive parameters. It is possible that the tests used (mainly Bayley scales of infant development and visual acuity tests) are not sensitive enough to measure all effects caused by LCPUFA supplementation (Eilander *et al*., 2007; Meldrum *et al*., 2011). Animal studies have the advantage that they enable invasive techniques and to perform histological and biochemical techniques on for example brain tissue, unlike studies on infants. Furthermore, animal supplementation studies are less time consuming and long term effects can be studied in a broader lifespan. When studying the effect of perinatal supplementation in later childhood and beyond in humans, there is a large timeframe that makes the study vulnerable to external influences. However, animal studies indicate that it is important to take into account

Table 1A: Overview of supplementation studies in neural development, perinatal supplementation in preterm infants.

Author	Year	N	Prenatal versus postnatal	Start supplementation	Duration supplementation	Daily dose					Primary outcome
						DHA	EPA	ARA	ALA	LA	
Preterm infants											
Isaacs *et al.*	2011	107	postnatal	birth	9 months	0.5g/100g	0.1 g/100g	0.04 g/100g	-	-	No overall change in cognitive measures at 10 years
Westerberg *et al.*	2011	92	postnatal	birth	9 weeks	32mg	-	31mg	-	-	Increased attention at 20 months
Makrides *et al.*	2010	694	prenatal	<21 weeks of gestation	birth	800mg	100mg	-	-	-	No difference BSID at 18 months
Smithers *et al.*	2010	125	postnatal	birth – 5 days commencing enteral feeds	estimated due date	1%	-	-	-	-	No difference language development at 26 months; no difference in behavior between 3 and 5 years
Henriksen *et al.*	2008	105	postnatal	birth	9 weeks	32mg/100ml	-	31mg/100ml	11mg/100ml	88mg/100ml	Increased problem-solving skills and discrimination of familiar/ unfamiliar objects at 6 months
Smithers *et al.*	2008	143	postnatal	birth – 5 days commencing enteral feeds	estimated due date	1%	-	-	-	-	No difference VEP acuity at 2 months, improvement at 4 months
Fang *et al.*	2005	27	postnatal	birth	6 months	0.05%	-	0.10%	-	-	Improved PDI and MDI between 6 and 12 months. No difference visual acuity
Fewtrell *et al.*	2004	298	postnatal	birth	9 months	0.5g/100g	0.1g/100g	0.04g/100g	-	-	No difference BSID at 9 and 18 months
Fewtrell *et al.*	2002	174	postnatal	≤10 days p.p.	≥3 weeks until discharge neonatal unit	0.17g/100g	0.04g/100g	0.31g/100g	-	-	No difference BSID at 9 and 18 months
O'Connor *et al.*	2001	463	postnatal	≤28 days p.p.	12 months (corrected age)	1) 0.16-0.27g/100g 2) 0.15-0.24g/100g	1) 0.08g/100g 2) -	1) 0.43g/100g 2) 0.41g/100g	-	-	Increased BSID at 12 months in group 1.

Table 1B: Overview of supplementation studies in neural development, perinatal supplementation in healthy full term subjects.

Author	Year	N	Prenatal versus postnatal	Start supplementation	Duration supplementation	Daily dose					Primary outcome
						DHA	EPA	ARA	ALA	LA	
Full term infants											
Willatts *et al.*	2013	147	post	≤7 days p.p.	4 months	0.21g/100g	-	0.35g/100g	-	-	No difference IQ at 6 years; improvement of information processing
van Goor *et al.*	2011	114	pre + post	gestational week 14-20	until 3 months p.p.	220mg	-	220mg	-	-	No difference BSID at 18 months
Firmansyah *et al.*	2011	290	post	12 months p.p.	until 24 months p.p.	23mg/100g	-	24mg/100g	0,48mg/100g	4,3mg/100g	No difference BSID at 24 months
Smithers *et al.*	2011	183	pre	18-20 weeks of gestation	birth	800mg	100mg	-	-	-	No difference VEP acuity and latency at 4 months
de Jong *et al.*	2010	341	post	birth	2 months p.p.	0.30%	-	0.45%	-	-	No difference neurological functioning at 9 years
Birch *et al.*	2010	244	post	≤9 days p.p.	12 months p.p.	1) 0.32% 2) 0.64% 3) 0.96%	- - -	1) 0.64% 2) 0.64% 3) 0.64%	- - -	- - -	Increased VEP acuity at 12 months
Pivik *et al.*	2009	28-44	post	birth	6 months p.p.	1) 0.15% 2) 0.32%	- -	1) 0.40% 2) 0.64%	- -	- -	No difference BSID at 3 and 6 months
Dunstan *et al.*	2008	83	pre	20 weeks of gestation	birth	2200mg	1100mg	-	-	-	Higher eye and hand coordination at 2.5 years
Innis *et al.*	2008	135	pre	16 weeks of gestation	birth	400mg	-	-	40mg	-	Higher visual acuity at 60 days
Birch *et al.*	2007	52	post	≤ 5 days p.p.	17 weeks	1) 0.35% 2) 0.36%	-	1) - 2) 0.72%	- -	- -	Improvement of visual acuity and verbal IQ at 4 years in group 2
Judge *et al.* (a)	2007	29	pre	<20 weeks of gestation	birth	214mg	-	-	-	-	Better problem solving at 9 months
Judge *et al.* (b)	2007	30	pre	<20 weeks of gestation	birth	294mg	-	-	-	-	Higher visual acuity at 6, but not 4 months
Bouwstra *et al.*	2006	270	post	neonatal age	2 months p.p.	0.30%	-	0.45%	-	-	No difference BSID at 18 months
Jensen *et al.*	2005	160	post	birth	4 months p.p.	200mg	-	-	-	-	Higher PDI at 30 months
Lauritzen *et al.*	2005	89	post	<14 days p.p.	4 months p.p.	900mg	-	-	-	-	No difference problem solving, lower early language acquisition at 1 year

Author	Year	N	Prenatal versus postnatal	Start supplementation	Duration supplementation	Daily dose					Primary outcome
						DHA	EPA	ARA	ALA	LA	
Full term infants											
Auestad *et al.*	2003	210	post	<7 days p.p.	12 months	1) 0.12g/100g 2) 0.23g/100g	- -	1) 0.43g/100g 2) -	- -	- -	No difference in cognition and visual acuity at 39 months
Helland *et al.*	2003	83	pre	18 weeks of gestation	3 months p.p.	1183mg	803mg	-	-	-	Improved cognition at 4 years
Hoffman *et al.*	2003	61	post	4-6 months p.p.	until 12 months p.p.	0.36%	-	0.72%	-	-	Higher VEP acuity at 1 year
Malcolm *et al.*	2003	55	pre	15 weeks of gestation	birth	40.4% (200mg)	4.1%	0.1%	0.8%	1.2%	No difference VEP at 6 months
Auestad *et al.*	2001	294	post	Birth - ≤ 11 days	12 months	1) 0.14g/100g 2) 0.13g/100g	1) - 2) ≤0.4g/100g	1) 0.45g/100g 2) 0.46g/100g	- -	- -	No difference BSID or visual acuity at 6 and 12 months
Birch *et al.*	2000	56	post	≤ 5 days p.p.	17 weeks	1) 0.35% 2) 0.36%	- -	1) - 2) 0.72%	- -	- -	Improvement MDI at 18 months in both groups
Lucas *et al.*	1999	354	post	1st week p.p.	6 months	0.32%	-	0.30%	-	-	No difference BSID at 18 months
Hørby-Jørgensen *et al.*	1998	37	post	<30 days p.p.	4 months	0.3%	0.4%	-	-	-	No difference VEP at 4 months
Scott *et al.*	1998		post	<7 days p.p.	12 months	1) 0.12g/100g 2) 0.23g/100g	- -	1) 0.43g/100g 2) -	- -	- -	No difference BSID at 14 months; lower vocabulary scores in group 2

Table 1C: Overview of supplementation studies in neural development, supplementation in autism spectrum disorders and ADHD.

Author	Year	N	Start supplementation	Duration supplementation	Daily dose					Primary outcome
					DHA	EPA	ARA	ALA	LA	
Autism spectrum disorders										
Yui *et al.*	2012	13	6-28 years	16 weeks	120-240mg	-	120-240mg	-	-	Improvement of social impairment
Bent *et al.*	2011	25	3-8 years	12 weeks	460mg	700mg	-	-	-	No difference in hyperactivity and core symptoms of autism
Politi *et al.*	2008	19	18-40 years	6 weeks	DHA + EPA: 930mg					No difference in behavior
Meguid *et al.*	2008	60	3-11 years	3 months	240mg	52mg	20mg	-	-	Improvement of concentration, eye contact, language development, and motor skills
Amminger *et al.*	2007	12	5-17 years	6 weeks	700mg	940mg	-	-	-	No difference in aberrant behavior
ADHD										
Perera *et al.*	2012	94	6-12 years		n-3 PUFA: 592.74mg n-6 PUFA: 361.5mg					Improvement of learning and behavior
Milte *et al.*	2012	67	7-12 years	4 months	1) 108mg 2) 1032mg 3) -	1) 1109mg 2) 264mg 3) -	- - -	- - -	1) - 2) - 3) 1467mg	No differences between groups. Increased erythrocyte DHA improves literacy and behavior within ADHD group
Gustafsson *et al.*	2010	74	7-12 years	15 weeks	2.7mg	500mg	-	-	-	Improvement of oppositional behavior, hyperactivity, and impulsivity
Bélanger *et al.*	2009	26	6-12 years	8 weeks rdbpc 8 weeks open label	200-400mg	500-1000mg	-	-	-	Improvement of ADHD core symptoms
Johnson *et al.*	2009	59	8-18 years	3 months rdbpc 3 months open label	174mg	558mg	-	-	-	No difference in ADHD core symptoms
Raz *et al.*	2009	63	7-13 years	7 weeks	-	-	-	120mg	480mg	No difference in ADHD core symptoms
Sorgi *et al.*	2007	9	8-16 years	4 weeks open label 4 weeks open label	5400mg 2700-4000mg	10800mg 5400-8100mg	- -	- -	- -	Improvement of ADHD core symptoms
Hirayama *et al.*	2004	40	6-12 years	2 months	3600mg per week	700mg per week	-	-	-	No difference in ADHD core symptoms
Voigt *et al.*	2001	63	6-12 years	4 months	345mg	-	-	-	-	No difference in ADHD core symptoms

multigenerational influences. In order to reach a model for n-3 PUFA depletion, several studies use a multigenerational animal model (Aid *et al*., 2003; Fedorova *et al*., 2009b; Moriguchi *et al*., 2000). Moriguchi *et al*. point out stronger effects of n-3 PUFA deficiency when comparing second generation to first generation rats (Moriguchi *et al*., 2000).

Multiple studies have investigated the effect of n-3 PUFA supplementation in neurodevelopmental disorders (Table 1C). As mentioned before, it is thought that n-3 PUFA deficiency impairs neurotransmission processes, by affecting membrane fluidity and related receptor functions (Chalon, 2006; Muskiet, 2010). This suggests that ADHD patients and autism spectrum disorders may benefit from LCPUFA supplementation as well. Studies on n-3 PUFA supplementation in autism spectrum disorders are scarce and do not show significant results due to small sample sizes (Amminger *et al*., 2007; Bent *et al*., 2011; Meguid *et al*., 2008; Politi *et al*., 2008; Yui *et al*., 2012). However, there are indications that supplementation may be able to decrease hyperactivity symptoms (Gillies *et al*., 2012; James *et al*., 2011). Table 1C shows that studies on n-3 PUFA supplementation as treatment of ADHD remain inconclusive whether supplementation can be used as a treatment for ADHD for the same reason (Belanger *et al*., 2009; Gustafsson *et al*., 2010; Hirayama *et al*., 2004; Johnson *et al*., 2009; Milte *et al*., 2012; Perera *et al*., 2012; Raz *et al*., 2009; Sorgi *et al*., 2007; Voigt *et al*., 2001). Gustafsson *et al*. show some improvement in clinical symptoms of ADHD patients, whereas Voigt *et al*. did not find effects on attention and impulsivity (Gustafsson *et al*., 2010; Voigt *et al*., 2001). Milte *et al*. found that the improvement in cognition was associated with n-3 PUFA levels in erythrocyte phospholipids and suggest that not only the dose of supplementation should be taken into account, but especially the erythrocyte n-3 PUFA status of the patient (Milte *et al*., 2012). They demonstrated that patients with an increased erythrocyte n-3 PUFA status were more likely to show improvements in cognitive function (Milte *et al*., 2012). The overall weak points in these studies seem to be the small sample size, short duration of supplementation, and short follow-up periods (Gillies *et al*., 2012; James *et al*., 2011).

All in all, the supplementation studies performed during neural development produce inconclusive findings. More research has to be done to elucidate the influence of LCPUFA in neural development in health and disease.

LCPUFA in normal aging

LCPUFA concentration in the brain decreases with age in both humans and rodents. In elderly subjects, LCPUFA have the potential to act as neuroprotective mediators and intervene in the mechanisms resulting in cognitive impairment or inflammation (Cole *et al*., 2009). DHA, for example, has the ability to act as a neurotrophic factor (Wu *et al*., 2008). It increases the level of brain-derived neurotrophic factor (BDNF), which is predominantly synthesized by hippocampal neurons. BDNF can act on tyrosine kinase B (Trk B) receptor signaling, resulting in activation of synaptic proteins such as synapsin-1 (Wu *et al*., 2008). This protein may contribute in enhancing synaptic plasticity and cognitive function. Synapsin-1 increases the synthesis of synaptic membranes leading to elevated levels of phosphatides and specific pre- and postsynaptic proteins (Wurtman, 2008). Via this pathway DHA increases the number of dendritic spines and possibly synapses on hippocampal neurons, particularly on excitatory glutamatergic synapses (Wurtman, 2008). These neurons are involved in learning and memory. Wu *et al*. have shown a synergistic effect of DHA and physical exercise on synaptic plasticity (Wu *et al*., 2008). Agrawal *et al*. have also demonstrated that an n-3 PUFA deficiency decreased synaptophysin and phosphorylation of synapsin-1 (Agrawal and Gomez-Pinilla, 2012). They showed that n-3 PUFA supplementation has the ability to normalize this effect, thereby restoring cognitive function (Agrawal and Gomez-Pinilla, 2012).

Normal aging is often accompanied by a decline in cognition, marked by decreased synaptic density, a decrease in neuronal survival, and loss of both gray and white matter volume

(Brown, 2009; Masliah *et al*., 2006; Masliah *et al*., 1993; Moretti *et al*., 2012; Raz *et al*., 1997; Salat *et al*., 1999; Tisserand *et al*., 2002). The number of aging individuals experiencing cognitive impairment is increasing rapidly worldwide. Age-related cognitive impairment is strongly correlated to the development of dementia (van de Rest *et al*., 2008). Factors contributing to this cognitive decline are obesity, cardio- and cerebrovascular disease, hypertension, and diabetes mellitus type 2 (DMII). All these factors are risk factors for vascular disorders and dementia as well and have in common that they can derive from the metabolic syndrome (MetS) that afflicts modern Western society (Singh *et al*., 2012). These risk factors can derive separately from MetS, though they can also act synergistically on each other exerting combined effects. A low n-3 PUFA intake and a high intake of n-6 PUFA, saturated fatty acids, or trans fatty acids are also risk factors in developing MetS (2003; Agrawal and Gomez-Pinilla, 2012; Singh *et al*., 2012). This leads to a high ratio between ARA and EPA/DHA that activates a pro-inflammatory state resulting in low-grade inflammation (Muskiet, 2010).

A sedentary lifestyle is another major risk factor for the development of MetS, besides a low n-3 PUFA intake, and is often accompanied by a high cholesterol intake (Singh *et al*., 2012). Cholesterol is absorbed from food or synthesized in the liver, intestine or brain. It is required for the formation of bile acids, steroid hormones and membrane synthesis, but excessive levels lead to hypercholesterolemia. Cholesterol circulating in the blood is not able to cross the blood brain barrier (BBB). Yet, the brain is the organ in the human body that contains the most cholesterol (Jenner *et al*., 2010). It is mainly synthesized by astrocytes and especially present in the myelin sheath and membranes of neurons and astrocytes (Donahue and Johanson, 2008). In general, increased serum levels of cholesterol (hypercholesterolemia) lead to increased blood levels of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) (Libby *et al*., 2013). LDL and VLDL invade the endothelium and start accumulating, initiating a state commonly known as atherosclerosis (Libby *et al*., 2013). The damage to the endothelium initiates an inflammatory response and monocytes and macrophages start accumulating, while platelets adhere to the affected area (Libby *et al*., 2013). Eventually, this will evolve into a chronic inflammatory state if the cholesterol intake is not changed. This situation increases hypoperfusion and ultimately the risk of cardiovascular disease.

Important causes of cardiovascular disease are hypertension and atherosclerosis. Atherosclerosis is induced by accumulation of cholesterol within the arterial walls, leading to plaques and consequently to narrowed arterial lumen and, occasionally, resulting in an acute myocardial infarction (AMI) or a cerebral stroke due to a stenosis, or rupture of a blood vessel in case of hemorrhages. Hypertension is thereby also a risk factor for stroke, ischemic white matter lesions, silent infarcts, general atherosclerosis, myocardial infarction, and often co-exists with other vascular risk factors, such as diabetes mellitus, obesity and hypercholesterolemia. Hypertension can predict both vascular dementia and Alzheimer's disease (AD) already 20 years before onset (Haan and Wallace, 2004; Launer and Hofman, 2000; Ruitenberg *et al*., 2001; Skoog and Gustafson, 2006). Atherosclerosis like hypertension, is a process that precedes dementia symptoms by many years. Both hypertension and atherosclerosis cause impaired blood flow and blood brain barrier function, hypoperfusion and blood vessel wall pathology which may initiate the underlying neurodegenerative processes leading to cognitive impairment and ultimately AD (de la Torre, 2012; Grammas *et al*., 2002; Kalaria *et al*., 2012; Muller *et al*., 2007; Skoog and Gustafson, 2006).

As mentioned before, consumption of the n-3 PUFA protects against cardiovascular disease (Hu *et al*., 2002; Kris-Etherton *et al*., 2003; Thies *et al*., 2003). These beneficial effects have been explained by the capacity to prevent arrhythmias, improving vasoreactivity, decreasing blood pressure and inflammation and decreasing atherosclerosis (Calder and Yaqoob, 2010; Geleijnse *et al*., 2002; Goodfellow *et al*., 2000; Harris *et al*., 1997; Leaf *et al*., 2003; Mozaffarian and Wu, 2012; von Schacky and Harris, 2007). This was already reported over 30 years ago in

1976 when studies on Greenland Inuit suggested that ingestion of n-3 PUFA protects against cardiovascular diseases (Bang *et al*., 1976). The Inuit consumed diets with large quantities of the long-chain polyunsaturated fatty acids EPA and DHA present in traditional Inuit food of seals, whales, and fish (Dyerberg and Bang, 1979). In other studies it has been found that eating fish once a week significantly decreased coronary heart disease mortality rates (He *et al*., 2004). Omega-3 fatty acids from fish oil might beneficially influence cardiovascular disease by decreasing blood pressure, and atherosclerosis formation (Connor, 2000; Geleijnse *et al*., 2002; Okuda *et al*., 2005; von Schacky and Harris, 2007). Growing evidence in literature points to the benefits of the Mediterranean diet on human health: it has been shown recently that extra-virgin olive oil containing diets rich in LCPUFA reduce the risk of not only cardiovascular disease, but also cancer, AD, and PD (Estruch *et al*., 2013; Sofi *et al*., 2013). All these findings suggest that LCPUFA containing diets resulted in a substantial reduction in the risk of major cardiovascular events among high-risk persons and support the benefits of the Mediterranean diet for the primary prevention of cardiovascular disease.

Clinical studies

Supplementation studies performed in healthy elderly subjects, and patients with either MCI or cerebrovascular disease are very scarce. Table 2 demonstrated that the studies of Yurko-Mauro *et al*., Witte *et al*., and Nilsson *et al*. are the only ones that show an effect of LCPUFA supplementation on cognition in healthy elderly (Nilsson *et al*., 2012; Witte *et al*., 2013; Yurko-Mauro *et al*., 2010). Yurko-Mauro *et al*. demonstrated that DHA supplementation during 24 weeks improved episodic memory in healthy elderly subjects (Yurko-Mauro *et al*., 2010). Witte *et al*. showed enhanced executive functions in healthy elderly after 26 weeks of supplementation, accompanied by improvements in white matter integrity, gray matter volume, and vascular parameters (Witte *et al*., 2013). Nilsson *et al*. found an improvement in working memory in a cross-over study after already 5 weeks of supplementation with n-3 PUFA (Nilsson *et al*., 2012). Stough *et al*. did not find effects on cognition after DHA supplementation during 90 days, but an improvement of visual acuity in elderly subjects with corrected vision was revealed (Stough *et al*., 2012). The studies performed by van de Rest *et al*. and Dangour *et al*. failed to show an effect of DHA+EPA on cognition in healthy elderly, both on a short (13 or 26 weeks) or long (24 months) supplementation period (Table 2) (Dangour *et al*., 2010; van de Rest *et al*., 2008). A possible explanation may lie in the relatively short duration of supplementation, and in addition starting supplementation earlier in life may have a stronger effect. Danthiir *et al*. describe the trial design and methodology of the ongoing study on the influence of n-3 PUFA supplementation in healthy elderly (Danthiir *et al*., 2011). They will supplement a large group of healthy elderly with 1720mg DHA and 600mg EPA daily during 18 months. Cognitive performance is assessed every 6 months. Other factors, such as dose of administration and mini-mental state examination (MMSE), differ between the studies, making it difficult to compare them to each other. In contrast to clinical studies on neural development, the clinical studies in normal aging only supplemented the subjects with n-3 PUFA. At adult age, ARA is no longer required for growth of the brain and high doses will only lead to the formation of pro-inflammatory eicosanoids.

Supplementation of LCPUFA in patients with MCI looks very promising, as (minor) improvements in cognition have been shown (Table 2) (Chiu *et al*., 2008; Lee *et al*., 2013a; Sinn *et al*., 2012). There are a few studies that have looked into the benefits of n-3 PUFA supplementation in patients with a history of cerebrovascular disease (Andreeva *et al*., 2011; Terano *et al*., 1999). While Terano *et al*. demonstrated a reduction of depressive symptoms in patients with thrombotic cerebrovascular disease, Andreeva *et al*. found no difference in cognition in patients with a history of cardio- and cerebrovascular disease (Andreeva *et al*., 2011; Terano *et al*., 1999).

Overall, duration of supplementation is again essential in setting a proper design for these types of studies. In general, the clinical studies performed in healthy elderly and MCI

Table 2: Overview of supplementation studies in normal aging, supplementation in healthy subject, MCI patients, and patients with a history of cerebrovascular disease.

Author	Year	N	Duration supplementation	Daily dose					Primary outcome
				DHA	EPA	ARA	ALA	LA	
Healthy subjects									
Witte *et al.*	2013	65	26 weeks	800mg	1320mg	-	-	-	Enhanced executive functions after 26 weeks; Improvement of white matter integrity, grey matter volume and vascular parameters
Nilsson *et al.*	2012	40	5 weeks	1050mg	1500mg	-	-	-	Improved working memory
Stough *et al.*	2012	74	90 days	252mg	-	-	-	-	No difference in cognitive function after 90 days; Improvement in visual acuity in participants with corrected vision
Danthiir *et al.*	2011	391	18 months	1720mg	600mg	-	-	-	Ongoing study
Dangour *et al.*	2010	867	24 months	500mg	200mg	-	-	-	No difference in cognitive function after 24 months
Yurko-Mauro *et al.*	2010	485	24 weeks	900mg	-	-	-	-	Improved episodic memory and learning after 24 weeks
Van de Rest *et al.*	2008	299	26 weeks	1) 847mg 2) 176mg	1) 1093mg 2) 226 mg	- -	- -	- -	No difference in cognitive function after 13 and 26 weeks
Mild cognitive impairment									
Sinn *et al.*	2012	40	6 months	1) 160mg 2) 1550mg 3) -	1) 1670mg 2) 400mg 3) -	- - -	- - -	1) - 2) - 3) 2200mg	Reduction of depressive symptoms; improvement of cognition
Lee *et al.*	2013	35	12 months	1300mg	450mg	-	-	-	Improvement of memory after 12 months
Chiu *et al.*	2008	29 (MCI + AD)		720mg	1080mg	-	-	-	Improvement in CIBIC-plus
Andreeva *et al.*	2011	1748	4 years	200mg	400mg	-	-	-	No difference in cognitive function in patients with a history of cardio- or cerebrovascular disease
Terano *et al.*	1999	20	12 months	720mg	-	-	-	-	Improvement of dementia scores in moderately severe dementia from thrombotic cerebrovascular disorder

patients indicate that supplementation should persist for at least 6 months. Furthermore, again it should be noted that differences in outcomes may occur to large variances in and between study populations.

LCPUFA in neurodegeneration

Life expectancy has increased enormously in the last century, from around 50 years to over 80 due to better medical care and better living conditions. However, increasing age is also the main risk factor for major life-threatening conditions, such as cardiovascular disease and neurodegenerative and age-related cognitive disorders. Both cerebrovascular and neurodegenerative diseases increase significantly after 60 years of age in almost all populations worldwide. Understanding exactly how ageing increases the risk of disease is necessary to fight this growing societal problem. The most frequent age related neurodegenerative diseases are AD and PD, characterized by the abnormal deposition of insoluble protein aggregates, and progressive death of neurons and loss of brain structures, associated with progressive, age-related decline in neuronal function.

AD is the most common age related neurodegenerative disorder and is widely recognized as the most important cause of dementia, while the second most common form of brain degenerative disorder leading to dementia, is caused by cerebrovascular disease. Vascular lesions are frequently found to co-exist with AD-type pathologies in older subjects and it is now evident that vascular and neurodegenerative lesions intensify each other, accelerating pathological mechanisms and increasing the risk that individuals with Alzheimer lesions will exhibit dementia (Kalaria, 2010). In agreement with these findings, major risk factors for AD are vascular related risk factors like hypertension and atherosclerosis (Wendell *et al*., 2012; Yarchoan *et al*., 2012). Future preventative interventions should take the proper time-window for intervention into account and the multifactorial nature of AD (Qiu, 2012).

A decreased level of plasma DHA is associated with cognitive impairment with aging (Conquer *et al*., 2000; Ikemoto *et al*., 2001; Kyle *et al*., 1999). Many animal, epidemiological and clinical studies have shown that high DHA consumption is associated with reduced AD risk (Bourre, 2007; Freemantle *et al*., 2006; Green *et al*., 2007a; Green *et al*., 2007b; Kiso, 2011; Luchtman and Song, 2013; Yurko-Mauro, 2010). In rat, a DHA containing diet enhanced the effects of exercise on cognition and BDNF-related synaptic plasticity (Fedorova and Salem, 2006; Wu *et al*., 2008). More recent studies showed that dietary DHA could be protective against β-amyloid (Aβ) production, deposition in plaques and cerebral amyloid angiopathy in an aged AD mouse model and increases cerebral blood volume (Hooijmans *et al*., 2007; Hooijmans *et al*., 2009). In other transgenic AD mouse models DHA also protects against dendritic pathology (Calon *et al*., 2004; Cole and Frautschy, 2006).

Both AD patient brain and the 3xTg-AD mouse exhibit reductions in DHA and the DHA derived NPD1 (Zhao *et al*., 2011). As mentioned, it has been shown that NPD1 has anti-inflammatory and neuroprotective, but also anti-amyloidogenic bioactivity (Bazan *et al*., 2011). Large multicentre randomized trials should still be executed, because studies performed up until now include various populations (for example Mediterranean, Australian, Dutch, and North American populations). These geographical differences cause a broad variation of dietary habits which may lead to large baseline differences. Many observational studies in elderly indicate that development of cognitive decline and dementia can be inhibited via healthy foods and dietary supplements (Calon, 2011; Cederholm and Palmblad, 2010; Cole *et al*., 2009; Jicha and Markesbery, 2010; Pauwels *et al*., 2009; Siegel and Ermilov, 2012). Furthermore, consumption of fish is related to lower risk of AD, maybe via inhibition of inflammation and enhancing vascular health and countering atherosclerosis (Frisardi *et al*., 2010; Gu *et al*., 2010; Scarmeas *et al*., 2007; Scarmeas *et al*., 2006).

PD is the second most common neurodegenerative disease after AD. It has been

estimated that 9 million individuals aged over 50 will have PD worldwide in 2030 (Dorsey *et al*., 2007). PD is a complex age related neurodegenerative disorder resulting in movement, balance, and fine motor control changes as a consequence of cell death of dopamine-containing neurons of the substantia nigra pars compacta (SNpc) (Warner and Schapira, 2003). The dopaminergic cell death is induced by oxygen reactive free radicals overproduction and mitochondrial dysfunction among other factors. This process can be mimicked by the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) model which affects mitochondria by inhibiting mitochondrial complex I or complex III, which induces specifically neuronal cell death in the SNpc (Warner and Schapira, 2003).

Currently, the main clinical treatment for PD is dopamine replacement therapy using l-dihydroxyphenylalanine (l-DOPA) and/or dopamine receptor agonists (Samii *et al*., 2004). In the early phase of the disease, treatment is generally highly effective, but medication becomes increasingly inadequate in controlling motor fluctuations and dyskinesia as the disease progresses. Moreover, pharmacotherapy cannot postpone the progression of the loss of dopaminergic neurons, and also cannot recover the lost dopaminergic neurons. Deep brain stimulation (DBS) is also seen as an alternative treatment suggested earlier in life and not just late in life as used nowadays (Desouza *et al*., 2013; Schupbach *et al*., 2007; Spieles-Engemann *et al*., 2010). However, still many surgical complications from the DBS procedure are reported such as infection, bleeding, stroke, neuropsychiatric adverse effects including depression, aggression, apathy, and anxiety (Desouza *et al*., 2013). Another possible beneficial clinical option for PD treatment could be cell transplantation therapy, but development of this therapy is still very premature (Nishimura and Takahashi, 2013).

It has been shown that n-3 PUFA have a modulatory effect on BDNF and glial cell-derived neurotrophic factor (GDNF) (Tanriover *et al*., 2010). In line with the positive effect of n-3 PUFA on BDNF regulation, is the finding that an ALA deficient diet decreased striatal BDNF content in mice (Miyazawa *et al*., 2010). Moreover, it has recently been shown that BDNF expression decreases in n-3 PUFA deficient rats and the upregulation of BDNF and its receptor has been recognized as a potential mechanism of action of n-3 PUFA as demonstrated in MPTP treated mice (Bousquet *et al*., 2009; Rao *et al*., 2007). Animals with MPTP-induced impaired balance and motor coordination showed diminished Parkinsonism symptoms and decreased dopaminergic neuronal death when fed a DHA diet (Hacioglu *et al*., 2012). Furthermore, it has been indicated that DHA supplementation may protect dopaminergic neurons in experimental PD models by targeting inflammatory signaling pathways and by enhancing the expression of GDNF and Neurturin (member of the same protein family as glial cell-derived neurotrophic factor). Both have been shown to benefit the dopaminergic neurons in the substantia nigra which is affected in PD (Bousquet *et al*., 2011; Cardoso *et al*., 2012; Hacioglu *et al*., 2012; Ji *et al*., 2012; Ozsoy *et al*., 2011; Tanriover *et al*., 2010). DHA supplementation in a nonhuman primate (MPTP) model reduces levodopa-induced dyskinesia, suggesting an innovative and safe approach to improve the quality of life of PD patients (Mahmoudi *et al*., 2009). Administration of n-3 PUFA could therefore be used as therapeutic strategy against PD via stimulation of cerebral BDNF production, which is supported by the observation of decreased post-mortem levels of BDNF in the brains of PD patients, and that neurotrophic factors are not able to cross the BBB (Mori *et al*., 1999; Parain *et al*., 1999).

Higher adherence to the Mediterranean diet consisting of whole grains, fish and olive oil and moderate consumption of alcohol is also associated with significant improvement in incidence of neurodegenerative disorders.

Table 3: Overview of supplementation studies in neurodegeneration, supplementation in AD and PD patients.

Author	Year	N	Duration supplementation	Daily dose					Primary outcome
				DHA	EPA	ARA	ALA	LA	
Alzheimer's disease									
Scheltens *et al.*	2012	258	24 weeks	1200mg	300mg	-	-	-	Improvement in memory performance in mild AD
Quinn *et al.*	2010	295	18 months	2000mg	-	-	-	-	No difference in ADAS-cog No difference in CDR
Chiu *et al.*	2008	29 (AD + MCI)	24 weeks	720mg	1080mg	-	-	-	No difference in ADAS-cog (AD) Improvement in CIBIC-plus (AD + MCI)
Freund-Levi *et al.*	2008	174	6 months rdbpc 6 months open label	1720mg	600mg	-	-	-	No difference in NPI
Freund-Levi *et al.*	2006	174	6 months rdbpc 6 months open label	1720mg	600mg	-	-	-	No difference in ADAS-cog No difference in MMSE
Kotani *et al.*	2006	8	90 days	240mg	-	240mg	-	-	No difference in RBANS
Boston *et al.*	2004	19	12 weeks rdbpc 12 weeks open label	-	1000mg ethyl-EPA	-	-	-	No difference in ADAS-cog
Yehuda *et al.*	1996	100	4 weeks rdbpc	-	-	-	LA/ALA: 4.5/1		Improvement of short term memory
Parkinson's disease									
Da Silva *et al.*	2008	29	3 months	480mg	720mg	-	-	-	Antidepressant effect in PD patients with depression

Clinical studies

Table 3 shows the LPCUFA supplementation studies that were performed in AD and PD patients. Strikingly, these studies show only slight improvement or no effect on cognition after supplementation (Boston *et al*., 2004; Chiu *et al*., 2008; da Silva *et al*., 2008; Freund-Levi *et al*., 2008; Freund-Levi *et al*., 2006; Kotani *et al*., 2006; Quinn *et al*., 2010; Scheltens *et al*., 2012; Yehuda *et al*., 1996). Chiu *et al*. were the only group to show a minor improvement in a clinician's interview-based impression of change scale which included caregiver-supplied information (CIBIC-plus). Though, no effect on the cognitive subscale of the Alzheimer's disease assessment scale (ADAS-cog) could be observed (Chiu *et al*., 2008). However, this study combined AD patients with patients with MCI and the improvement in CIBIC-plus was only found in the combined group. The AD patients did not show a difference in ADAS-cog after LCPUFA supplementation (Chiu *et al*., 2008). In a study performed by Freund-Levi *et al*., 174 AD patients (mean age of 74 years) were supplemented daily with 1.7 g DHA and 0.6 g EPA for 12 months, and tested on a number of standard cognitive assessments (Table 3) (Freund-Levi *et al*., 2006). In a subgroup with very mild cognitive dysfunction, a significant reduction in the cognitive decline rate was observed compared to placebo, suggesting that those with milder cognitive impairment may benefit from n-3 PUFA supplementation.

Da Silva *et al*. were the only group to study supplementation in PD patients and found that LCPUFA act as antidepressants in PD patients that were experiencing depression (Table 3) (da Silva *et al*., 2008). Overall, the clinical trials applied relatively short supplementation periods, with an exception of the trial performed by Quinn *et al*. (Quinn *et al*., 2010). They supplemented mild to moderate AD patients with DHA for 18 months, but still did not find an effect on ADAS-cog. Two other studies supplementing both DHA and EPA did find slight improvements in cognition after only 24 weeks of supplementation (Chiu *et al*., 2008; Scheltens *et al*., 2012).

Studies with transgenic mice and Aβ-infused rats did show improvements in cognition after LCPUFA supplementation (Arendash *et al*., 2007; Arsenault *et al*., 2011; Calon *et al*., 2005; Hashimoto *et al*., 2006; Hooijmans *et al*., 2009; Oksman *et al*., 2006). The duration of supplementation in these animal studies is relatively longer compared to the clinical studies. This may indicate that the supplementation should start earlier on in clinical studies to obtain LCPUFA effects on cognition in AD patients.

Conclusion

The wide range of studies available have shown that LCPUFA are able to influence the brain in many ways throughout life (Figure 3). In general, LCPUFA are essential in membrane fluidity and their function as inflammatory mediators. They are important at the start of life to support neural development and prevent neurodevelopmental disorders, and remain important throughout life for membrane fluidity, the prevention of inflammatory states, and cardiovascular health. Supplementation studies starting early in life show more potent results than those starting during aging or AD. This suggests that it is important to achieve a healthy LCPUFA status early on in life and maintain this status throughout life in order to have a beneficial effect.

The Mediterranean diet fits very well with a healthy LCPUFA status. Not only does it enclose a balanced n-6/n-3 ratio, due to the fact that it is rich in fish and lean meat, but it also contains other important nutrients, such as vitamins and antioxidants, originating from fruit, vegetables, and whole grains. This in contrast to Western diets that are rich in saturated fats, trans fats, sugar, and refined grains. Therefore, implementation of a Mediterranean diet contributes to a healthy lifestyle.

The overall weaknesses in clinical LCPUFA supplementation studies throughout life are the relatively short duration of supplementation, variance in populations, and the limitations of testing variables. In rodent studies, LCPUFA have shown potential to contribute to a healthy life, but in clinical studies they could not yet demonstrate their full potential due to these

shortcomings.

This review shows that LCPUFA have a beneficial effect on health, but the n-6/n-3 ratio is most important to establish a healthy and balanced diet. The key message in maintaining this healthy LCPUFA status is finding the proper n-6/n-3 balance, with a ratio of about 1/1 – 2/1.

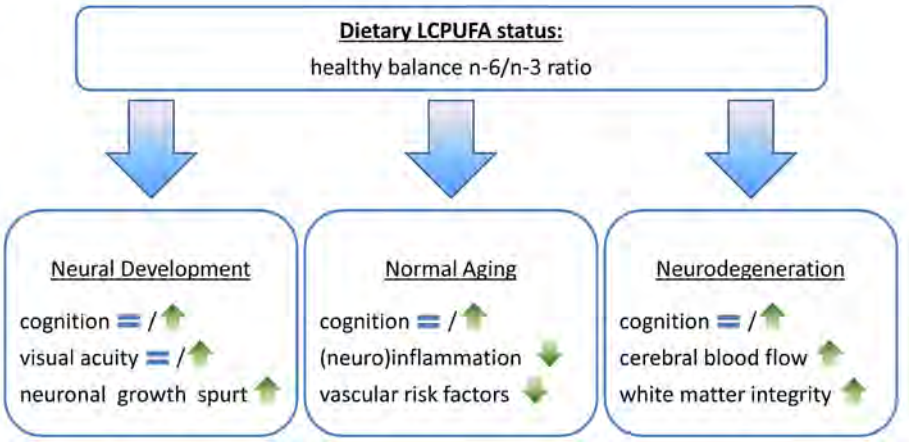


Figure 3: Concluding overview. Schematic overview of findings from clinical studies on LCPUFA supplementation in neural development, normal aging, and neurodegeneration. This review has shown that there are indications that LCPUFA (mainly a healthy balance n-6/n-3) support and improve brain structure and functioning. In neural development there is evidence that LCPUFA might improve cognition and visual acuity. Subsequently, LCPUFA are required during the perinatal neuronal (out) growth when the number of glial cells increase, outgrowth of axons and dendrites takes place, as well as the myelination of nerve fibers. During normal aging LCPUFA supplementation may improve cognition, decrease (neuro)inflammation, and reduce vascular risk factors. Furthermore, LCPUFA have also shown to improve white matter integrity and cerebral blood flow in neurodegeneration.

Chapter 3

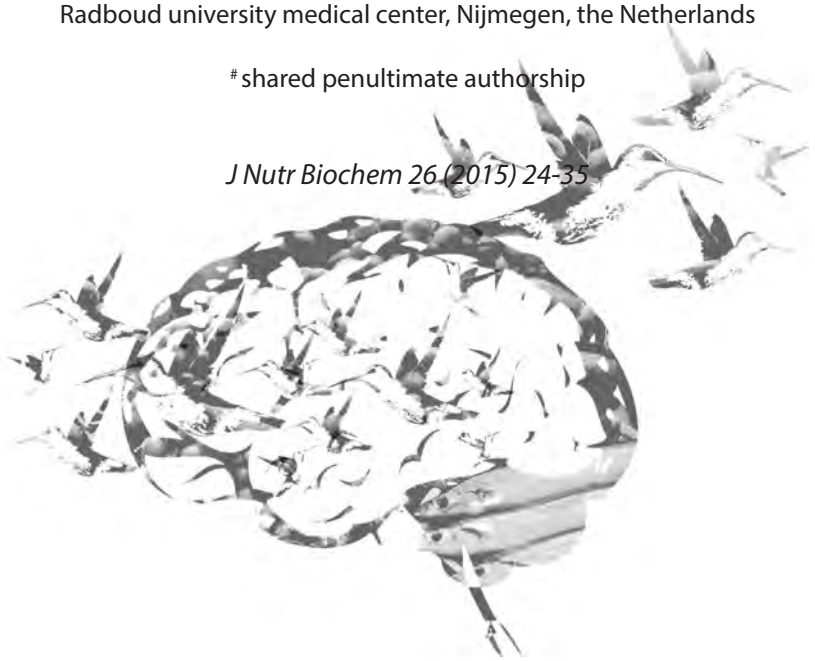
Impact of dietary n-3 polyunsaturated fatty acids on cognition, motor skills, and hippocampal neurogenesis in developing C57BL/6J mice

Carola IF Janssen[1], Valerio Zerbi[1], Martina PC Mutsaers[1], Bas SW de Jong[1], Maximilian Wiesmann[1,2], Ilse AC Arnoldussen[1], Bram Geenen[1], Arend Heerschap[3], Frits AJ Muskiet[4], Zeina E Jouni[5], Eric AF van Tol[5], Gabriele Gross[5], Judith R Homberg[6#], Brian M Berg[5#], Amanda J Kiliaan[1]

[1]Department of Anatomy, Donders Institute for Brain, Cognition, and Behaviour, Radboud university medical center, Nijmegen, the Netherlands
[2]Department of Geriatrics, Donders Institute for Brain, Cognition, and Behaviour, Radboud university medical center, Nijmegen, the Netherlands
[3]Department of Radiology, Donders Institute for Brain, Cognition, and Behaviour, Radboud university medical center, Nijmegen, the Netherlands
[4]Laboratory Medicine, University Medical Center Groningen, Groningen, the Netherlands
[5]Mead Johnson Pediatric Nutrition Institute, Nijmegen/Evansville, the Netherlands/IN, USA
[6] Department of Cognitive Neuroscience, Donders Institute for Brain, Cognition, and Behaviour, Radboud university medical center, Nijmegen, the Netherlands

[#] shared penultimate authorship

J Nutr Biochem 26 (2015) 24-35



Abstract

Maternal intake of omega-3 polyunsaturated fatty acids (n-3 PUFA) is critical during perinatal development of the brain. Docosahexaenoic acid (DHA) is the most abundant n-3 PUFA in the brain and influences neuronal membrane function and neuroprotection. The present study aims to assess the effect of dietary n-3 PUFA availability during the gestational and postnatal period on cognition, brain metabolism, and neurohistology in C57BL/6J mice. Female wild type C57BL/6J mice at day 0 of gestation were randomly assigned to either an n-3 PUFA deficient diet (0.05% of total fatty acids) or an n-3 PUFA adequate diet (3.83% of total fatty acids) containing preformed DHA and its precursor α-linolenic acid. Male offspring remained on diet and performed cognitive tests during puberty and adulthood. In adulthood, animals underwent 31P MR spectroscopy to assess brain energy metabolites. Thereafter, biochemical and immunohistochemical analyses were performed assessing inflammation, neurogenesis and synaptic plasticity. Compared to the n-3 PUFA deficient group, pubertal n-3 PUFA adequate fed mice demonstrated increased motor coordination. Adult n-3 PUFA adequate fed mice exhibited increased exploratory behavior, sensorimotor integration, and spatial memory, while neurogenesis in the hippocampus was decreased. Selected brain regions of n-3 PUFA adequate fed mice contained significantly lower levels of arachidonic acid and higher levels of DHA and dihomo-γ-linolenic acid. Our data suggest that dietary n-3 PUFA can modify neural maturation and enhance brain functioning in healthy C57BL/6J mice. This indicates that availability of n-3 PUFA in infant diet during early development may have a significant impact on brain development.

Introduction

Long-chain polyunsaturated fatty acids (LCPUFA) in the human diet are important for maintaining health. During evolution in some cultures, LCPUFA dietary intake has changed from an omega-3 polyunsaturated fatty acids (n-3 PUFA) rich diet to an n-3 PUFA deficient diet (Carlson *et al*., 2013; Muskiet, 2010; Simopoulos, 2011). Within the Western dietary pattern, n-6 PUFA, saturated fatty acid (SFA) and trans fatty acids levels have gradually increased, whereas n-3 PUFA levels have decreased (Muskiet, 2010). This shift in fatty acid composition is also reflected in human breast milk (Ailhaud *et al*., 2006; Garcia *et al*., 2011; Makrides *et al*., 1996; Storck Lindholm *et al*., 2013).

LCPUFA play an important role in brain development, especially during the growth spurt in the last trimester of pregnancy and the early postnatal period up to 2 years of age (Brenna, 2011; Gibson *et al*., 2011; Innis, 2009). Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are the most abundant LCPUFA in the brain (Innis, 2009). Placental fatty acids are dependent on the maternal supply. After birth, breast milk is the only source of essential fatty acids for breast-fed infants.

Deficiency studies in rodents have demonstrated the importance of n-3 PUFA such as DHA, eicosapentaenoic acid (EPA, 20:5n-3), and their precursor α-linolenic acid (ALA, 18:3n-3) for brain development and cognitive functioning (Fedorova *et al*., 2009a; Fedorova and Salem, 2006; McNamara *et al*., 2009; Moriguchi and Salem, 2003). Research has suggested that an n-3 PUFA deficiency may lead to a variety of neuronal and psychological abnormalities, such as attention-deficit hyperactivity disorder (ADHD), depression, schizophrenia, autism, and anxiety (Chalon, 2006; Fedorova *et al*., 2009a; Fedorova and Salem, 2006; Gustafsson *et al*., 2010; Lyall *et al*., 2013; Muskiet, 2010; Perera *et al*., 2012; Yui *et al*., 2012). N-3 PUFA deficiency in rodents can result in hyperactivity, a feature underlying these neuropsychiatric disorders (Levant *et al*., 2004; Moriguchi and Salem, 2003; Umezawa *et al*., 1995). It has been proposed that n-3 PUFA deficiency may affect neurotransmission, especially within the dopaminergic and serotonergic systems, as a consequence of altered membrane fluidity and related receptor functions (Chalon, 2006; Muskiet, 2010). Furthermore, n-3 PUFA are important for the regulation of synaptic plasticity, and learning and memory by their involvement in regulating gene expression and retinoid signaling pathways (Cao *et al*., 2009a; Dyall and Michael-Titus, 2008; Dyall *et al*., 2010; Kitajka *et al*., 2004; Sidhu *et al*., 2011).

A sufficient dietary intake of n-3 PUFA is necessary for maintaining a healthy LCPUFA status. This is due to the fact that both ALA and linoleic acid (LA, 18:2n-6; the precursor of n-6 PUFA) compete for the same conversion enzymes, namely delta-5-desaturase and delta-6-desaturase (Schmitz and Ecker, 2008; Simopoulos, 2008a). A high LA intake interferes with the desaturation and elongation of ALA and thereby with the conversion of ALA via EPA to DHA (Simopoulos, 2008a). This imbalance in the n-6/n-3 ratio also results in the production of the n-6 ARA, leading to the formation of more pro-inflammatory eicosanoids, while the n-3 EPA is the precursor of anti-inflammatory eicosanoids (Schmitz and Ecker, 2008; Simopoulos, 2011; Tassoni *et al*., 2008). DHA can also interfere with neuroinflammation, as it is the precursor for resolvins and neuroprotectins, such as neuroprotectin D1 (NPD1) (Tapiero *et al*., 2002; Tassoni *et al*., 2008). NPD1 induces signaling for homeostatic maintenance of cellular integrity and can inactivate pro-apoptotic and pro-inflammatory signaling (Schmitz and Ecker, 2008). Therefore, it has been proposed that a balanced n-6/n-3 ratio is critical for maintaining a healthy brain and immune status.

Clinical studies have shown that (perinatal) LCPUFA supplementation may be beneficial for healthy neural development in both preterm and full term infants (Birch *et al*., 2010; Dunstan *et al*., 2008; Fang *et al*., 2005; Fewtrell *et al*., 2004; Henriksen *et al*., 2008; Isaacs *et al*., 2011; Janssen and Kiliaan, 2014; Makrides *et al*., 2010; Smithers *et al*., 2011; van Goor *et al*., 2011b; Westerberg *et al*., 2011; Willatts *et al*., 2013). Studies in preterm infants underline the significance of the timing of supplementation, because these infants cannot fully benefit from the accumulation

of LCPUFA starting in the last trimester of gestation. Studies in full term infants show that both pre- and postnatal supplementation are able to improve cognition, corresponding with the findings in preterm infants that it is important to start supplementation in the last trimester of gestation (Birch *et al*., 2010; Birch *et al*., 2007; Birch *et al*., 2000; Dunstan *et al*., 2008; Helland *et al*., 2003a; Hoffman *et al*., 2003; Innis and Friesen, 2008; Jensen *et al*., 2005; Judge *et al*., 2007a, b; Willatts *et al*., 2013). However, in humans the outcome of infant n-3 PUFA supplementation on long-term brain development appears to be subtle (Bouwstra *et al*., 2006a; Dunstan *et al*., 2008; Fewtrell *et al*., 2002; Firmansyah *et al*., 2011; Helland *et al*., 2003a; Hoffman *et al*., 2003; Innis and Friesen, 2008; Jensen *et al*., 2005; Judge *et al*., 2007a, b; Lauritzen *et al*., 2005; Lucas *et al*., 1999; Malcolm *et al*., 2003; van Goor *et al*., 2011b; Wurtman, 2008), whereas rodent studies have shown more pronounced beneficial effects of n-3 PUFA supplementation (Cao *et al*., 2009a; Carrié *et al*., 2000b; Coluccia *et al*., 2009; Fedorova *et al*., 2009a; Maekawa *et al*., 2009; Niculescu *et al*., 2011; Wurtman, 2008). This may be explained by the fact that human supplementation studies are limited to non-invasive parameters and often encounter difficulties when studying a broad lifespan for long-term effects: these studies rarely exceed the infant age. Experiments with rodents offer the opportunity to overcome these limitations of human studies, and enable us to study the effect of n-3 PUFA availability starting during gestation on brain development in more detail throughout life.

Therefore, the aim of this study was to obtain detailed insights into the mechanisms underlying the long-term beneficial effects of n-3 PUFA availability during gestation and throughout life in mice. A broad combination of parameters was determined to assess effects on behavior, brain structure and function. With behavioral and cognitive tests, changes in cortical and hippocampal functionality were studied. Cerebral metabolite status was measured to assess energy metabolism and neuronal membrane turnover, immunohistochemistry evaluated neurogenesis and synaptic plasticity, and quantitative real-time polymerase chain reaction (qRT-PCR) was used to study gene expression of inflammatory markers.

Material and methods

Animals and diets

Mouse C57BL/6J dams (3-4 months old, Harlan Laboratories Inc., Horst, The Netherlands) were used for breeding and randomly assigned to either n-3 PUFA deficient (n-3 def; n=10) or n-3 PUFA adequate (n-3 adq; n=11) diets at the first day of gestation (GD 0). The isocaloric diets were based on AIN93m and only differed in n-3 PUFA composition (Table 1) (Fedorova *et al*., 2009b; Greiner *et al*., 2003; Reeves *et al*., 1993). As shown in table 1, the n-3 adq diet contained 2.55% ALA and 1.28% DHA of total fatty acids, while the n-3 def diet contained 0.05% ALA and 0.00% DHA (Research Diets Services, Wijk bij Duurstede, The Netherlands). Diets are comparable to those used in other studies so that the results can be easily compared (Fedorova *et al*., 2009b; Greiner *et al*., 2003; Moriguchi and Salem, 2003). It was demonstrated that n-3 PUFA supplementation contributed to increased spatial learning and memory, although the mechanisms involved were not elucidated (Fedorova *et al*., 2009b; Moriguchi and Salem, 2003). Offspring of the dams was maintained on the corresponding diet throughout the whole study. To normalize litter sizes, they were culled to 3 males and 3 females per dam. Six parallel groups of male offspring were used, which were either tested and sacrificed on postnatal day (PND) 30 (n-3 def = 13, n-3 adq = 12) or PND 60 (n-3 def = 11, n-3 adq = 12), including cognition and brain biochemistry parameters, or used for brain histology at PND 60 (n-3 def = 7, n-3 adq = 9). One male pup from each dam was represented in each parallel group for testing and histology (Figure 1).

Starting from GD 0, the dams were housed individually in Phenotypers (Noldus, Wageningen, The Netherlands) and remained there during birth and until weaning of the offspring. The male offspring was housed in groups of 3. All mice were housed in the central animal facility with temperature controlled at 21 °C, an artificial 12:12h light:dark cycle

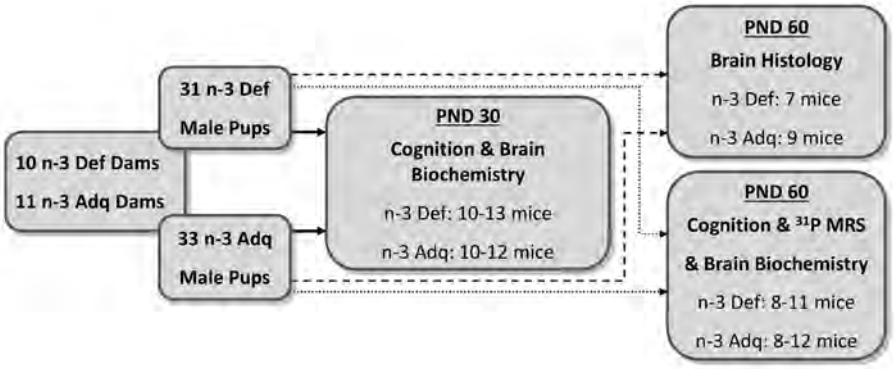


Figure 1: Schematic overview of the study design. C57BL/6J dams were randomly assigned to n-3 def or n-3 adq at GD 0. Offspring was maintained on the corresponding diet throughout the whole study. Male littermates were randomly assigned to one out of three parallel groups. One group was tested for behavioral and cognitive parameters on PND 30 and subsequently sacrificed for brain biochemistry. A parallel group was tested for behavioral and cognitive parameters on PND 60 and subsequently underwent 31P MRS and was also sacrificed for brain biochemistry. A third group was used for brain histology at PND 60. One male pup from each dam was represented in each parallel group for testing and histology. Due to some technical problems during the experiments, not all mice could be included for statistical analysis for each parameter.

(lights on at 7:00 am), continuous music playing in the background during the light period, and cage enrichment consisting of a plastic shelter and cotton nesting material. Food and water were available ad libitum. The experiments were performed according to Dutch federal regulations for animal protection and were ethically approved by the Veterinary Authority of the Radboud university medical center, Nijmegen, The Netherlands.
Due to some technical problems during the experiments, not all mice could be included for statistical analysis for each parameter. For example, some mice were excluded from further analysis of phosphorus MR spectroscopy, since the obtained spectra did not meet inclusion criteria.

Behavioral and cognitive tests
Dams and pups were monitored in Phenotyper cages for 24 hours per day during PND 0-21 (birth until weaning). Combined total activity was measured as well as administration on opening of the eyes. Pups were weighed on PND 14, 21, 30, and 60. Behavioral and cognitive tests on the male offspring were performed starting at PND 30 (pubertal age) and PND 60 (young adult age) (Figure 2). All tests were performed in the same order (open field, rotarod, prepulse inhibition) during the light cycle between 8:00 a.m. and 5:00 p.m. The Morris water maze (MWM) was only performed at young adult age, after animals had performed the behavioral tests.

Open field
The open field test was performed at PND 30 and PND 60 as a measure for locomotion and exploration. The mice were placed individually in a square open field (45 cm × 45 cm × 30 cm) with transparent Plexiglas walls. Their activity was recorded for 30 minutes. Locomotion was automatically registered with EthoVision XT8.5 (Noldus, Wageningen, The Netherlands), while exploration was manually scored. The duration (s) of walking, wall leaning, rearing, sitting, and grooming were scored and analyzed as previously described (Hooijmans *et al*., 2009; Streijger *et al*., 2005).

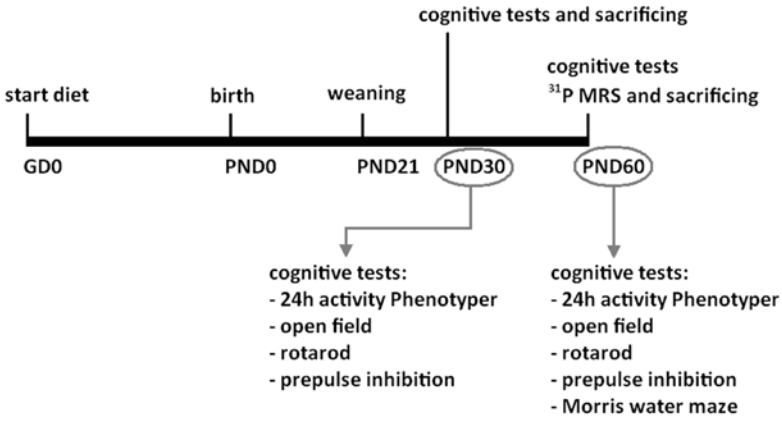


Figure 2: Time line of the experiment. On GD 0 dams were randomly assigned to n-3 PUFA adequate or n-3 PUFA deficient diet for the remainder of the experiment. Offspring are fed the diets throughout life. Behavioral and cognitive tests on the male offspring were performed starting at PND 30 (pubertal age) and PND 60 (young adult age) and they were sacrificed for brain biochemistry. Additionally, mice that performed behavior and cognition, also underwent 31P MRS before being sacrificed for brain biochemical purposes. In a parallel group, brain histology was performed at PND 60.

Rotarod

Motor coordination was studied using the rotarod at PND 30 and 60. The mice were placed on a rotating rod (3.18 cm in diameter, IITC Inc., Woodland Hills, CA, United States) and their ability to remain on the device was recorded as latency to fall (s). Trials were performed at both fixed and accelerated speed. All trials had a maximum duration of 300 s and the intertrial interval was 20 minutes. First, mice were accustomed to the task by placing them on a stationary drum for a minute followed by a test trial at 10 rpm. Next, the fixed speed trials were performed at 10, 15, and 20 rpm. Finally, two trials at accelerating speeds (4-40 rpm) followed.

Prepulse inhibition

The prepulse inhibition test was also performed at PND 30 and 60 to study sensorimotor integration as previously described by Streijger *et al*. (Streijger *et al*., 2005). Startle reactivity was measured in a startle response system, the SR-LAB (San Diego Instruments, San Diego, CA, United States). A non-restrictive Plexiglas restrainer (4 cm diameter) on a Plexiglas platform was placed inside a ventilated and sound attenuated startle chamber. A high-frequency speaker in this chamber produced the various acoustic stimuli and the background noise (set at 70 dB). The whole-body startle response of the mouse produced vibrations of the platform, which were detected and transduced by a piezoelectric accelerometer mounted underneath the platform, which was connected to an automated system.

The testing session started with a 5 minute habituation to the 70 dB background noise. Next, three blocks of startle pulses followed. The first block consisted of 5 pulses at 120 dB. Subsequently, the mice were presented with 5 blocks that all contained 2 startle pulse alone trials (120 dB), 4 prepulse trials, in which 72, 74, 78, or 86 dB startle stimuli are followed by a 120 dB pulse, and 1 no stimulus trial (70 dB background), in pseudo-randomized order. The last block consisted of 5 startle pulse trials at 120 dB again. The (pre)pulses had a duration of 20 ms, with a 100 ms interval between the onset of the prepulse and the onset of the pulse. The startle response was measured during 50 ms, starting at the onset of the pulse, with an amplitude read-out expressed in arbitrary units. The intertrial interval range was 10 – 20 s.

MWM

The effect of dietary n-3 PUFA availability on spatial learning and memory was tested using the MWM at PND 60. The young adult mice were placed at different starting positions in a circular pool (120 cm diameter) that was filled with water (21-22 °C, made opaque by adding milk

Table 1: Composition of experimental diets.

Ingredient	Amount (g/100g diet)	
	n-3 deficient	n-3 adequate
Protein: Alacid 710 (vit free casein)	20	20
Carbohydrate: cornstarch	15	15
Sucrose	10	10
Dextrose	19.9	19.9
Maltose-dextrin	15	15
Cellulose	5	5
Salt-mineral mix	3.5	3.5
Vitamin mix	1	1
L-cystine	0.3	0.3
Choline bitartrate	0.25	0.25
TBHQ	0.002	0.002
Fat: Hydrogenated coconut oil	8.1	7.45
Safflower oil	1.9	1.77
Flaxseed oil	-	0.48
DHASCO®	-	0.3
Fatty acid composition (%):		
Total saturated fatty acids	80.8	75.6
Monounsaturated fatty acids	4	4.8
18:2n-6 (LA)	15.1	15.7
18:3n-3 (ALA)	0.05	2.55
20:2n-6 (ARA)	0.05	0.06
22:6n-3 (DHA)	-	1.28
n-6/n-3	303	4.1
18:2n-6/18:3n-3	302	6.2

powder). The mice were trained to find a submerged platform (8 cm diameter) one centimeter below the water surface located in the north-east quadrant of the pool by using distant visual cues. The spatial cues were present on the four walls surrounding the pool at a distance of 0.5 m. During all trials, the observer was present in the room and always located at the same location (behind a curtain surrounding the set-up).

Acquisition phase of MWM

Mice performed 4 acquisition trials (maximal swimming time 120 s; 30 s on platform; intertrial interval 60 min) per day during 4 consecutive days. Starting positions were south, north, east, and west. All trials were recorded and the latency to find the platform (s) was used as measure for spatial learning.

Probe phase

All mice performed a single probe trial on day 5 (1 day after acquisition), in which the platform was removed from the pool. They were allowed to swim for 120 s and all trials were recorded and analyzed with EthoVision XT8.5 (Noldus, Wageningen, The Netherlands). Time spent searching in the target quadrant and the number of platform crossings were used as a measure for spatial memory.

^{31}P MRS

At the end of the behavioral testing, the young adult mice underwent phosphorus MR spectroscopy (^{31}P MRS) to quantify energy metabolites and phospholipid content in the brain. MR measurements were performed on a shielded 7T/300 mm horizontal-bore MR magnet

interfaced to a clinical console (ClinScan, Bruker Biospin, Ettlingen, Germany). A homemade probe was designed to fit the mouse head consisting of a ring shaped ^{31}P coil (121.7 MHz) for localized ^{31}P MRS and a surface ^{1}H coil (300.4 MHz) for MRI and localized field shimming. The coils were positioned with a perpendicular field orientation to avoid coupling. Mice were placed in a stereotactic holder to prevent unwanted movement during scanning. They were anaesthetized using 2% isoflurane (Abott, Cham, Switzerland) in a 2:1 O2:N2O mixture and body temperature was maintained at about 37 °C with a heated airflow and monitored with a rectal optical temperature probe. Respiration of the animal was monitored using a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc, NY, USA). MR images in the coronal, transversal, and longitudinal orientation were acquired to visualize the anatomy and morphology of the mouse brain.

Thereafter, first and second order shims were automatically adjusted with FASTMAP from a voxel placed over the entire mouse brain (24 x 24 x 24 mm3) (Gruetter, 1993). The ^{31}P spectra were acquired using a 3D MRSI sequence with the following parameters: field of view = 24 x 24 x 24 mm; matrix size= 8 x 8 x 8; voxel size = 3 x 3 x 3 mm3; repetition time (TR) = 1500 ms; BIR-4 45 degree excitation pulse; acquisition of 2048 data points over spectral width 10000 Hz; averages = 192, resulting in a total acquisition time of 1 hour and 52 minutes. The signals of interest were: phosphocreatine (PCr), γ- and α- adenosine triphosphate (ATP); inorganic phosphate (Pi); phosphomonoesters (PME), and phosphodiesters (PDE).

All spectra were fitted with software package jMRUI 5.0 (http://sermn02.uab.es/mrui/) and the metabolites were quantified as ratio compared to the metabolite PCr. For each animal, 3 to 4 spectra corresponding to voxels inside the brain were selected and their results averaged.

Immunohistochemistry

Brains were collected at PND 60 from a parallel group of littermates for immunohistochemical analysis (see Figure 1). Mice were transcardially perfused with 0.1 M phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PF). Next, mice were decapitated and whole brains were collected. The brains were post-fixed overnight in 4% PF at 4 °C and thereafter stored in 0.1 M PBS with 0.01% sodiumazide at 4 °C. Coronal brain sections were cut using a sliding microtome (Microm HM 440, Walldorf, Germany) equipped with an object table for freeze sectioning at -60 °C gaining 8 series of 30 µm thick sections which were used for immunohistochemical purposes (240 µm distance between the sections). The tissue was stained for immature neurons (measure for neurogenesis) with antibodies against doublecortin (DCX), for synaptophysin immunoreactive presynaptic boutons (SIPBs; measure for synaptic plasticity) with antibodies against synaptophysin, and for postsynaptic density (measure for synaptic plasticity) with antibodies against postsynaptic density protein 95 (PSD95).

Immunohistochemistry was performed using standard free-floating labeling procedures, using the following protocol. The sections were incubated with the primary antibody overnight at room temperature on a shaker table. After incubation, the sections were rinsed three times with 0.1 M PBS and incubated with the secondary antibody. After 90 minutes, the sections were rinsed three times again and transferred to 0.1 M PBS containing Vector ABC-elite (1:800; Vector Laboratories Inc., Burlingame, CA, USA) for another 90 minutes. After rinsing the sections three times, visualization of the marker was achieved by incubation with diaminobenzidine-nickel (DAB-Ni) solution. Stained sections were mounted on gelatin-coated glass slides, dried overnight in a stove at 37 °C, dehydrated in alcohol series, cleared with xylol and mounted in Entellan.

For DCX, polyclonal goat anti-doublecortin (1:4000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used as a primary antibody to assess neurogenesis. The secondary antibody was donkey anti-goat biotin (1:1500; Jackson ImmunoResearch, West Grove, PA, USA). In the synaptophysin staining, we used monoclonal rabbit anti-synaptophysin clone EP1098Y (1:10,000; Abcam Inc., Cambridge, UK) as a primary antibody to visualize synaptophysin present

in presynaptic boutons. For PSD95, polyclonal rabbit anti-PSD95 (1:2000; Abcam, Cambridge, UK) was used as a primary antibody to visualize the postsynaptic density. In both the synaptophysin staining and PSD95, the secondary antibody used was donkey anti-rabbit biotin (1:1500; Jackson ImmunoResearch, West Grove, PA, USA).

Stained brain regions were analyzed double blind by 2 independent observers using a Zeiss Axioscop microscope equipped with hardware and software of Microbrightfield (Williston, VT, USA). Quantified hippocampus regions were based on the mouse brain atlas of Franklin & Paxinos (third edition, 2008).

Quantification doublecortin

DCX positive cells were quantified in three succeeding sections of the hippocampus (-1.70, -2.18 mm, and -2.46 mm posterior to bregma) for each mouse. Contours were drawn along the borders of the hippocampus at 2.5× magnification using the program Stereo Investigator (Microbrightfield, Williston, VT, USA). The DCX positive cells in the subgranular zone of the hippocampus were manually counted at 40× magnification and the values of the three succeeding sections were averaged to obtain a single value for each animal.

Quantification synaptophysin and PSD95

The relevant regions in the hippocampus were digitized using Stereo Investigator. Contours of the inner molecular layer (IML), outer molecular layer (OML), stratum radiatum (SR), and stratum lucidum (SL) of the hippocampus (-2.18 mm up to -2.46 mm posterior to bregma) were drawn at 2.5× magnification. Per region, 2 photographs were taken at 100× magnification. The quantification of the staining was performed using the program Image J (National Institute of Health, Bethesda, MD, USA). Images were converted to 8-bit gray scale, followed by conversion to 16-bit gray scale, next the contrast was enhanced. For synaptophysin, only the SIPBs between 0.1 µm2 and 4.5 µm2 were considered to be 'normal' sized SIPB and included in the analysis (Mulder *et al*., 2007; Rutten *et al*., 2005). For PSD95, we did not set limits for the particle size.

Table 2: qRT-PCR primers.

Primers (mouse)	Forward	Reverse
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'
IL-1β	5'-GCAACTGTTCCTGAACTCAACT-3'	5'-ATCTTTTGGGGTCCGTCAACT-3'
IL-6	5'-CAAGTCGGAGGCTTAATTACACATG-3'	5'-ATTGCCATTGCACAACTCTTTTCT-3'
TNF-α	5'-CAGACCCTCACACTCAGATCATCT-3'	5'-CCTCCACTTGGTCCTTTGCTA-3'
CD36	5'-ATGGGCTGTGATCGGAACTG-3'	5'-GTCTTCCCAATAAGCATGTCTCC-3'
MCP-1	5'-CCCAATGAGTAGGCTGGAGA-3'	5'-TCTGGACCCATTCCTTCTTG-3'
synaptophysin	5'-TCTTTGTCACCGTGGCTGTGTT-3'	5'-TCCCTCAGTTCCTTGCATGTGT-3'
DCX	5'-GATGTCAACCGGGAAAGCAC-3'	5'-GTGGAACCACAGCAACTTTTC-3'

Biochemistry

Fatty acid analysis

The mice that were tested on behavior on PND 30 and those that were tested on behavior and underwent 31P MRS on PND60 were transcardially perfused with 0.1 M PBS. Subsequently, mice were decapitated and whole brains were collected and snap frozen in liquid nitrogen. The brains were stored at -80 °C up until analysis. The left cerebral hemispheres and erythrocytes collected from blood were processed to analyze fatty acid composition at (early) adult age. Brain tissue was divided into 4 regions; brain stem, cortex, subcortical region, and cerebellum. Samples were kept at -80 C until analysis. Preparation and analysis of fatty acid methyl esters (FAME)

was performed with a one-step homogenation/methylation procedure as described previously (Zhou *et al*., 2008). Briefly, about 50 mg of tissue was treated simultaneously with an aqueous and an organic solution that extract, separate and methylate fatty acids. FAME were quantified with a 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto,CA) equipped with a BPX70 fused silica column (25 m x 0.22 mm i.d. × 0.25 mm film; SGE Inc, Austin,TX). FAME structures were positively identified by covalent-adduct chemical ionization tandem mass spectrometry on a Saturn 2000 ion trap mass spectrometer (Varian, Inc., Walnut Creek,CA). An equal weight FAME mixture was used daily to measure response factors that were applied to the day's analyses. The concentration of the fatty acids is expressed as µg fatty acid per mg wet tissue weight.

qRT-PCR

The cortex of the right hemispheres of the brains collected at PND 30 and 60 were analyzed for inflammatory markers and synaptic plasticity with qRT-PCR. The subcortical area of the right hemispheres collected at PND 30 and 60 was analyzed for neurogenesis with qRT-PCR. Brain tissue was analyzed for interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), cluster of differentiation 36 (CD36), and monocyte chemoattractant protein-1 (MCP-1), synaptophysin, and DCX.

Brain tissue was collected in 1 ml cold Trizol (Invitrogen, Paisley, UK) and homogenized using a dispersing machine (ultra-Turrax, IKA Werke GmbH & Co. KG, Staufen, Germany). After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in 25 µl RNase-free diethylpyrocarbonate (DEPC)-treated water. The RNA concentration of the tissue was measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, DE, USA). First strand cDNA synthesis was performed using 1 µg RNA dissolved in a 10 µl solution containing RNase-free DEPC-treated water, 2 µl 5× iScript reaction mix and 0.5 µl iScript reverse transcriptase (iScript cDNA synthesis kit, Bio-Rad Laboratories B.V., Hercules, CA, USA) at 25 °C for 5 minutes, at 42 °C for 30 minutes and at 85 °C for 5 minutes and cooled down to 4 °C. The cDNA samples were stored at -80 °C.

qRT-PCR was performed in a total volume of 10 µl buffer solution containing 2 µl of template cDNA (diluted 1:10), 5 µl 2× Power SYBR Green Master mix (Applied Biosystems, Foster City, CA, USA), 2.92 µl RNase-free DEPC-treated water and 0.04 µl of each primer (100 µM). Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), IL-1β, IL-6, TNF-α, CD36, MCP-1, synaptophysin, and DCX were designed using Vector Primer Express software (Applied Biosystems) (Table 2). The optimal temperature cycling protocol was determined to be 95 °C for 10 minutes followed by 40 reaction cycles at 90 °C for 15 seconds and at 60 °C for 1 minute, using a StepOnePlus real time PCR system (Applied Biosystems, Foster City, CA, USA). Subsequently, the melt curve temperature protocol was determined to be 95 °C for 15 seconds, 60 °C for 20 seconds, and 90 °C for 15 seconds with a slope of 0.6 °C. Absolute quantities were determined using standard curves, and the validity of the results was confirmed by including appropriate negative controls. The quantity of cDNA was calculated for each sample with StepOne Software version 2.2.2. To evaluate differences, relative gene expression ratios were calculated according to the comparative CT method (also referred to as the 2-ΔCT method) (Schmittgen and

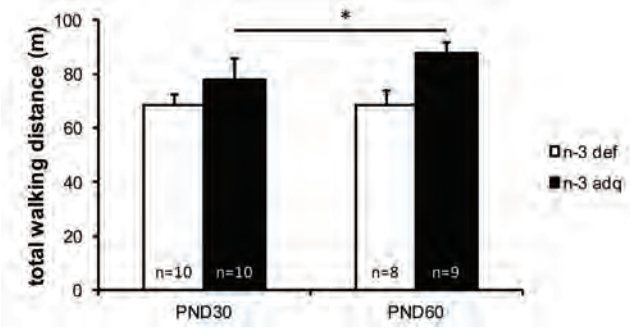


Figure 3: Locomotion in the open field. Activity (total walking distance) was measured over a 30 minute period. *p≤0.05.

Livak, 2008). Relative CT values were calculated by subtracting the CT value of the housekeeping gene GAPDH from the CT values of IL-1β, IL-6, CD36, MCP-1, TNF-α, synaptophysin, and DCX. For each primer, two independent qRT-PCR runs were performed, and the means of their relative values were used for statistical analysis.

Statistical analyses

Data are expressed as mean ± SEM and were analyzed with SPSS for Windows 20.0 software (SPSS Inc., Chicago, IL, USA). The repeated measures ANOVA was used for the open field parameters (with the repeated measure: 10 minute intervals), the first and last block of pulse-alone trials in the prepulse inhibition (with the repeated measure: time), and the acquisition phase of the MWM (with the repeated measure: acquisition days). A Student's t-test was performed for the parameters of the open field (total time), rotarod, prepulse inhibition, MWM parameters in the probe phase, energy metabolites measured with ^{31}P MRS, immunohistochemistry, and biochemistry. For readability reasons, F-values and degrees of freedom are not displayed in the text. Statistical significance was set at *p≤0.05 and #0.05<p<0.075.

Results

Cognitive tests

There were no differences between the two dietary groups in general developmental parameters such as opening of the eyes or activity of the litter in the Phenotypers (data not shown). Activity of the dam with litter increased over time for both diet groups (data not shown). Dietary intervention had no effects on body weight of the two groups throughout the study (data not shown).

Open field

The open field was used to assess locomotion (total walking distance) and exploration (walking, wall leaning, rearing, sitting) and

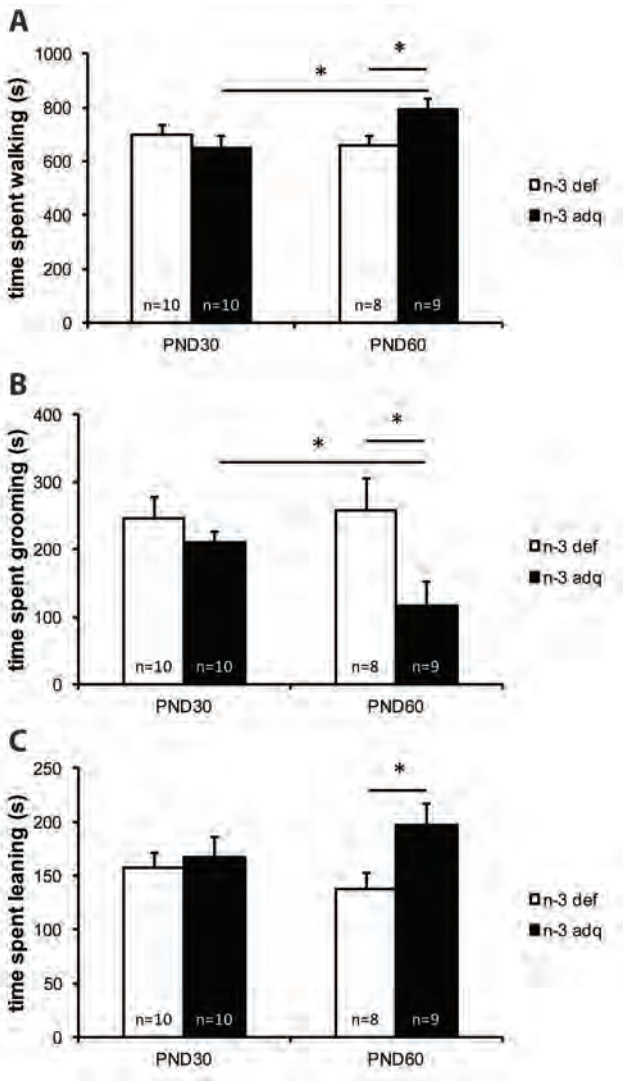


Figure 4: Exploration in the open field. Different open field parameters were measured in a 30 minute period. **A** n-3 adq diet increased time spent walking on PND 60 compared to PND 30. Furthermore, n-3 adq increased time spent walking compared to n-3 def on PND 60. **B** n-3 adq diet decreased time spent grooming on PND 60 compared to PND 30. Additionally, n-3 adq decreased time spent grooming compared to n-3 def on PND 60. **C** n-3 adq diet increased time spent leaning on PND 60 compared to PND 30. *p≤0.05.

grooming for 30 minutes.
In the open field no effects of diets could be detected on locomotion at both PND30 and PND60. However, the young adult mice fed n-3 adq diet displayed a higher total walking distance (p=0.016) than pubertal mice (Figure 3). This effect was not found in the n-3 def diet.

Furthermore, we observed no dietary effects in exploratory behavior in pubertal mice in the total 30 minutes. However, young adult mice on the n-3 adq diet spent more time walking (p=0.031) and less time grooming (p=0.027) than pubertal mice fed the n-3 adq diet (Figure 4A and B). Moreover, they showed more time wall leaning (p=0.033) and walking (p=0.024), but less time grooming (p=0.027) than their n-3 def fed littermates in across the 30 minutes of the test (Figure 4A-C). Subsequently, we observed that the n-3 adq fed young adult mice spent more time walking than their n-3 def fed littermates (p=0.024; Figure 4A). Lastly, the n-3 adq fed mice demonstrated less time grooming (p=0.027) than their n-3 def littermates over 30 minutes (Figure 4B).

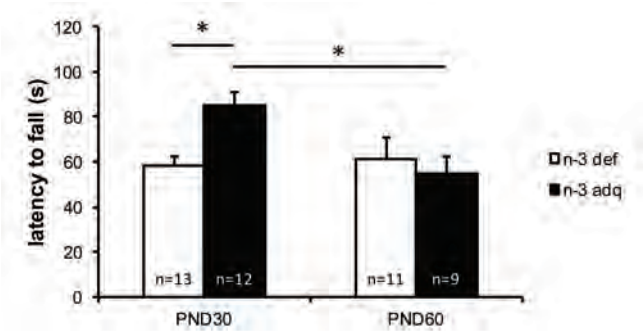


Figure 5: Latency to fall in the rotarod. On PND30, n-3 adq diet increased latency to fall at acceleration compared to n-3 def. On PND 60, n-3 adq diet displayed a decreased latency to fall compared to PND 30. *p≤0.05.

Rotarod

The rotarod is designed to assess motor coordination. Mice performed a motor task on a rotating rod at fixed speeds or acceleration and the latency to fall was recorded.

No dietary effect was observed at fixed speeds (10, 15, 20 rpm) in both pubertal and young adult mice (data not shown). However, n-3 adq fed pubertal mice demonstrated a higher latency to fall (p=0.001) compared to n-3 def fed pubertal mice at accelerated speed (4-40 rpm) (Figure 5). Young adult mice showed no diet effect at accelerated speed (data not shown). Yet, the n-3 adq fed mice on PND 60 display a lower latency to fall than n-3 adq fed mice on PND 30 (p=0.011, Figure 5). Comparing performances in all groups, the n-3 adq fed mice at pubertal age performed best among all groups.

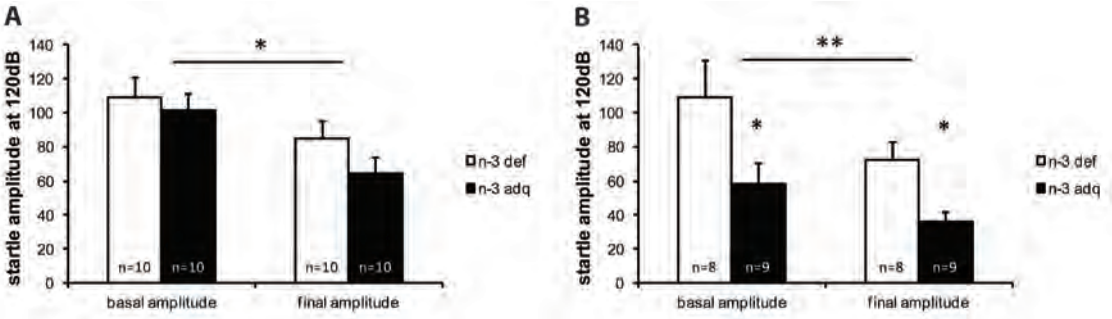


Figure 6: Startle response in the prepulse inhibition test. Mice were exposed to acoustic pulses, and the startle amplitude at 120 dB of the mice was measured at the start and at the end of the experiment. **A** The mice displayed an overall habituation effect on PND 30. **B** On PND 60, there was also a habituation, and additionally a diet effect. *p≤0.05, **p≤0.01.

Prepulse inhibition

The prepulse inhibition test studies sensorimotor integration by measuring the startle response in mice that are administered acoustic pulses with or without a preceding softer prepulse.

At both ages, no dietary effects could be detected (data not shown). However, an age effect was found in the n-3 def diet; mice fed the n-3 def diet at PND 60 demonstrated a stronger prepulse inhibition than at PND 30, which was not the case in n-3 adq fed mice. A habituation effect to the stimuli was observed in pubertal mice ($p<0.001$, Figure 6A) and in the young adult mice ($p=0.004$, Figure 6B). However, at young adult age, this habituation effect was stronger in the n-3 adq fed mice than in the n-3 def fed mice. Both diet groups showed a stronger habituation at PND 60 compared to PND 30 ($p=0.009$).

MWM

In the MWM task, spatial learning is tested in the acquisition phase where the mice have to find a hidden platform. Spatial memory is assessed in a subsequent trial where the platform is removed from the pool.

The MWM was only performed at PND 60. In the acquisition phase both diet groups showed a learning effect ($p=0.002$), but there was no significant difference between the two diet groups ($p>0.05$, Figure 7A). In the probe phase we focused on the first 30 seconds of the trial. The n-3 adq fed mice spent more time in the target quadrant than the n-3 def fed mice ($p=0.007$, Figure 7B) and also showed more platform crossings ($p=0.029$, Figure

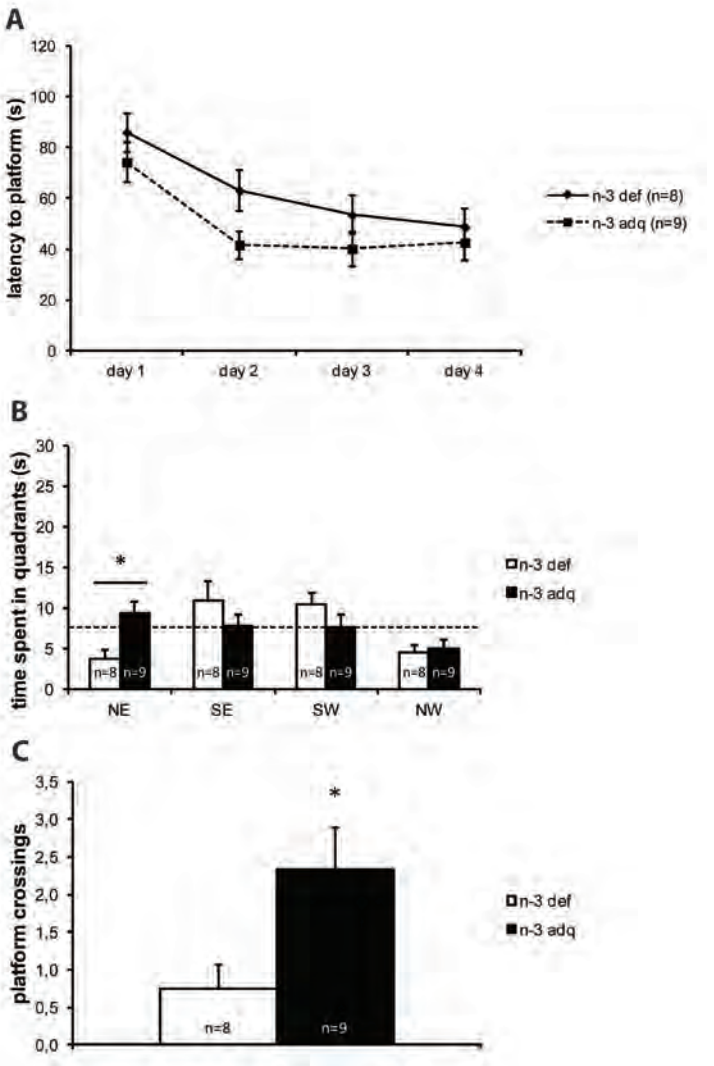


Figure 7: Morris water maze learning and memory in young adult mice (PND 60). Spatial learning was measured in a 4-day acquisition phase, by determining the latency to find a hidden platform in the NE target quadrant. Spatial memory was tested in a probe phase in which the percentage of time spent in the target quadrant and the total number of platform crossings (of the previous location of the platform) was measured. **A** MWM acquisition: both diets show a learning curve. **B** MWM probe target quadrant: n-3 adq diet increased time spent in the NE quadrant. The dotted line represents the 25% chance level of finding the target quadrant. The n-3 def mice performed below 25% chance level while n-3 adq performed at 25% chance level. **C** MWM probe platform crossings: n-3 adq increased platform crossings. *$p \leq 0.05$.

7C) over the area where the platform used to be located. The n-3 def fed mice performed below 25% chance level (p=0.004) while the n-3 adq fed mice performed at 25% chance level.

^{31}P MRS

After performing the MWM, the mice underwent ^{31}P MRS to measure the content of the main phosphorylated metabolites in the brain at PND 60. The metabolite levels (PCr, γ- and α-ATP; Pi; PME, and PDE) showed no differences between the diets for all metabolites in the mice (data not shown).

Immunohistochemistry

Immature neurons were visualized in young adult mice with a polyclonal antibody against DCX. DCX positive cells were counted in the subgranular zone of the hippocampus as a measure for neurogenesis (Figure 8A and B). Calculation of the DCX-positive cells in the hippocampus demonstrated significantly less DCX- positive cells in the subgranular zone of n-3 adq fed young adult mice compared to n-3 def fed mice (p=0.048, Figure 8C).

The amount of presynaptic boutons (size ranges from 0.1 μm2 to 4.5 μm2) was visualized with a monoclonal antibody against synaptophysin. Whereas, postsynaptic density was visualized with a polyclonal antibody against PSD95 in the hippocampus of young adult mice to reflect synaptic function. No significant diet effect in presynaptic (SIPB) and postsynaptic densities in the IML, OML, SL, and SR of the hippocampus were observed (data not shown).

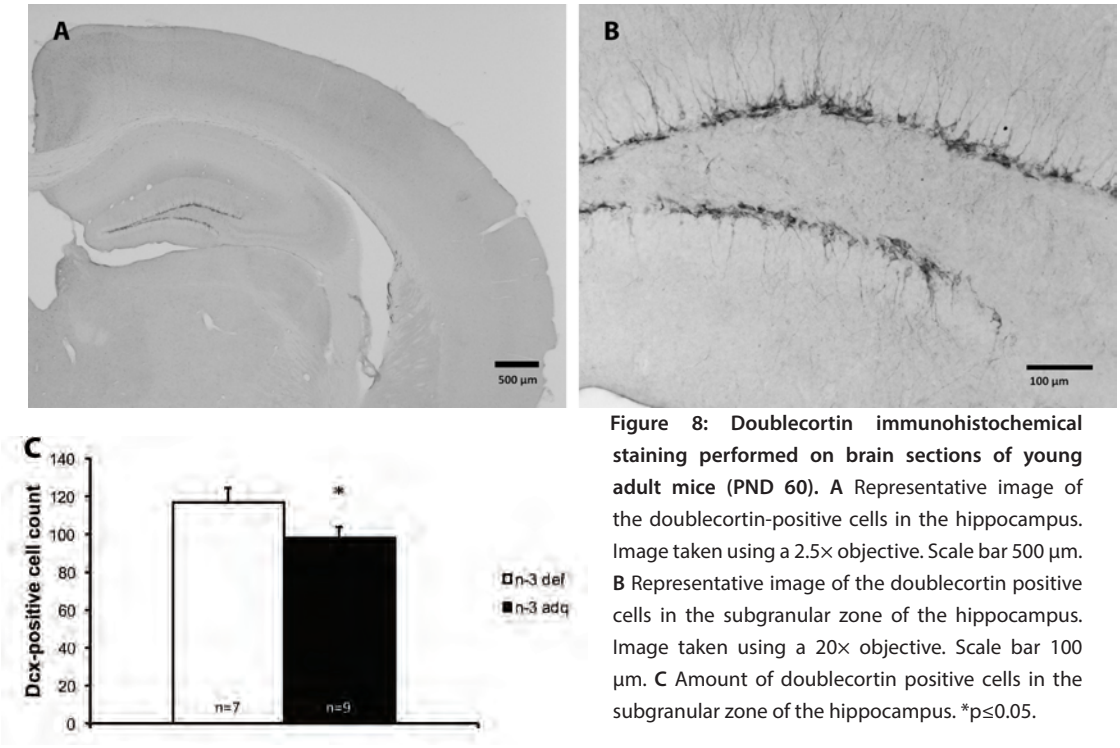


Figure 8: Doublecortin immunohistochemical staining performed on brain sections of young adult mice (PND 60). A Representative image of the doublecortin-positive cells in the hippocampus. Image taken using a 2.5× objective. Scale bar 500 μm. **B** Representative image of the doublecortin positive cells in the subgranular zone of the hippocampus. Image taken using a 20× objective. Scale bar 100 μm. **C** Amount of doublecortin positive cells in the subgranular zone of the hippocampus. *p≤0.05.

Biochemistry

Fatty acid analysis

Fatty acid analysis of erythrocytes confirmed increased levels of DHA in n-3 adq fed animals at young adult age as compared to n-3 def fed animals (p<0.01, Table 3). Analysis of all four brain

regions demonstrated lower levels of ARA ($p<0.01$) and higher levels of dihomo-γ-linolenic acid (DGLA) ($p<0.01$) and DHA ($p<0.01$) in n-3 adq fed mice as compared to n-3 def fed mice (Table 3).

Table 3: Fatty acid analysis of erythrocytes and brain tissue at (young) adult age.

	n-3 deficient %	n-3 adequate %	*p*-value
Eryhtrocytes	*n=10*	*n=9*	
ARA	12.41±1.31	9.13±0.94	NS
DGLA	1.24±0.14	1.35±0.07	NS
DHA	<0.001±0.00	6.80±0.97	≤0.01
DPA	1.55±0.28	0.13±0.03	≤0.01
Brain stem	*n=9*	*n=7*	
ARA	7.07±0.17	5.22±0.40	≤0.01
DGLA	0.42±0.03	0.83±0.09	≤0.01
DHA	4.86±0.31	8.84±1.10	≤0.01
DPA	4.33±0.31	0.07±0.01	≤0.01
Cortex	*n=10*	*n=9*	
ARA	10.03±0.31	7.97±0.45	≤0.01
DGLA	0.38±0.04	0.64±0.04	≤0.01
DHA	6.09±0.59	15.88±1.03	≤0.01
DPA	8.43±0.29	0.39±0.11	≤0.01
Subcortical region	*n=10*	*n=9*	
ARA	10.25±0.12	8.36±0.12	≤0.01
DGLA	0.42±0.02	0.72±0.02	≤0.01
DHA	6.77±0.27	14.48±0.26	≤0.01
DPA	7.02±0.46	0.31±0.03	≤0.01
Cerebellum	*n=10*	*n=9*	
ARA	6.94±0.15	5.19±0.18	≤0.01
DGLA	0.41±0.01	0.62±0.04	≤0.01
DHA	5.43±0.28	9.90±0.61	≤0.01
DPA	4.22±0.47	0.17±0.08	≤0.01

qRT-PCR

Analysis of gene expression of inflammatory markers (IL-1β, IL-6, TNF-α, CD36, MCP-1), and synaptophysin in the cortical region of the brains of both pubertal and young adult mice was performed with qRT-PCR. Analysis of doublecortin expression as a measure of neurogenesis was quantified in the subcortical region of the brains of pubertal and young adult mice. No significant differences were detected between n-3 adq and n-3 def fed animals at pubertal or young adult age for the expression of inflammatory markers (data not shown). The n-3 adq fed animals demonstrated a trend for increased synaptophysin mRNA expression (synaptic plasticity) in the cortex at pubertal age ($p=0.054$), but no longer in adulthood (Figure 9). Furthermore, the n-3 adq fed mice displayed a decrease in synaptophysin mRNA expression on PND 60 compared to PND 30 ($p=0.001$). No diet effects were detected in DCX mRNA expression of the subcortical region at either PND 30 or 60 (data not shown).

Discussion

The present study aimed to investigate the influence of dietary n-3 PUFA availability during the development of C57BL/6J mice on behavior, cognition, cerebral metabolism, and brain plasticity using behavioral and cognitive tests, ^{31}P MRS, immunohistochemistry, and biochemical

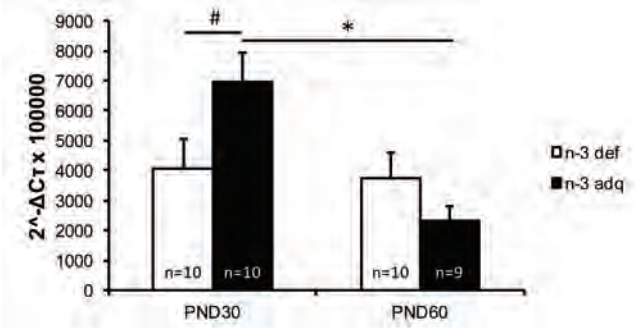


Figure 9: Synaptophysin mRNA expression measured with qRT-PCR in the cortical region of the brain in pubertal (PND 30) and young adult mice (PND 60). On PND30, n-3 adq diet increased expression of synaptophysin compared to n-3 def. On PND 60, n-3 adq decreased synaptophysin expression compared to PND30. *p≤0.05, #0.05<p<0.075.

approaches. An overview of the main results is shown in Table 4. Overall, significant effects on motor coordination, exploratory behavior, sensorimotor integration, spatial memory, and neurogenesis were observed. These findings indicate that dietary intake of both ALA and preformed DHA during early development has a significant impact on neural and brain development as well as on cognition and behavior.

Regarding behavioral and cognitive outcomes, n-3 PUFA availability had no effects on locomotion, although total walking distance and activity of the pups increased as they reached weaning age (PND 21). This observation is in agreement with other studies (Carrié *et al*., 2000a; Coluccia *et al*., 2009; Enslen *et al*., 1991; Nakashima *et al*., 1993). Significantly more exploratory behavior, reflected by more time spent walking and wall leaning, was found in the open field in the n-3 adequate fed mice at adult age, but not at pubertal age. Carrié *et al*. demonstrated increased locomotion and an increase in the exploratory parameter rearing in n-3 PUFA supplemented adult female mice compared to control mice (Carrié *et al*., 2000b). However, Coluccia *et al*. observed no difference in rearing between n-3 PUFA supplemented and control rats at both pubertal and adult age (Coluccia *et al*., 2009). In yet another study, rearing and horizontal movement were decreased in n-3 PUFA deprived rats (Enslen *et al*., 1991). These studies define exploratory parameters purely as rearing or sometimes vertical movement when drawing their conclusions on exploration. In the current study no dietary effect in the time spent rearing could be observed, but indeed an increase in time spent on wall leaning in animals with an adequate n-3 PUFA intake was demonstrated, which is also a vertical movement. Additionally, n-3 PUFA adequate fed young adult mice also displayed more walking, which is considered as an exploratory horizontal movement (Enslen *et al*., 1991). Furthermore, grooming has been suggested as a measure for anxiety, because mice are known to display grooming under stressful conditions (O'Leary *et al*., 2013). We observed less grooming in the adequate n-3 PUFA group at adult age, but not in puberty, while the n-3 PUFA deficient mice did not display age differences. This indicates that n-3 PUFA supplementation may decrease anxiety-related behavior, however, no specific tests of anxiety were included in the current study.

In the prepulse inhibition test of the acoustic startle reflex, a habituation effect to the 120 dB pulses was found in pubertal and young adult mice. At young adult age, this habituation effect was significantly stronger in animals with adequate n-3 PUFA intake. This reflects improved habituation to startle stimuli and therefore our data point to an improvement of cortical development by adequate n-3 PUFA intake (Geyer and Dulawa, 2001). Fedorova *et al*. also measured prepulse inhibition in 8 week old mice on n-3 PUFA deficient and n-3 PUFA supplemented diets (Fedorova *et al*., 2009a). In that study, the n-3 PUFA supplemented mice showed a stronger inhibition to the acoustic pulses than mice on the deficient diet. Additionally, a habituation process was observed in supplemented, but not in deficient animals, which is in line with the results of this present study. In this experiment, we observed a stronger habituation process in our n-3 PUFA adequate mice. In a schizophrenia rat model, LCPUFA have been shown to be able to ameliorate the impaired prepulse inhibition (Maekawa *et al*., 2009). Schizophrenia is

Table 4: Summary of the main results found in the behavioral and cognitive tests in pubertal and young adult mice that on an n-3 PUFA adequate diet throughout life.

Effect of n-3 PUFA adequate diet		PND 30	PND 60
Open field	Behavior and activity	NS	Increased exploration
			Decreased anxiety
Rotarod	Motor coordination	Increased motor coordination	NS
Prepulse inhibition	Sensorimotor integration	NS	NS
	Habituation	NS	Increased habituation
Morris water maze	Learning and memory	n.a.	Increased memory
31P MRS	Cerebral metabolites	n.a.	NS
DCX expression	Subcortical region	NS	NS
Immature neurons	Hippocampus	n.a.	Fewer immature neurons
Synaptophysin expression	Cortex	Increased synaptic plasticity	NS
Presynaptic boutons	Hippocampus	n.a.	NS
Postsynaptic density	Hippocampus	n.a.	NS
Inflammatory markers	Cortex	NS	NS
Fatty acid analysis	Brain	n.a.	Increased DGLA
			Increased DHA
	Erythrocytes	n.a.	Decreased ARA
			Increased DHA

a condition that mostly develops during early adulthood and has been associated with LCPUFA deficiency (Levant *et al*., 2004; Moriguchi and Salem, 2003; Umezawa *et al*., 1995). These data suggest that LCPUFA supplementation supports dopaminergic neurotransmission processes, by improving membrane fluidity. More testing at time points later in life could provide more information on the full potency of LCPUFA. It has been reported that high levels of n-3 PUFA were associated with a negative effect on neural development by prolonging auditory brain stem conduction times and a delay in auditory startle reflex in rodents (Haubner *et al*., 2007; Saste *et al*., 1998). These studies show the importance of dosage, but indirectly also stress the relevance of the n-6/n-3 ratio, which deserves further attention.

In the current study, n-3 PUFA adequate mice displayed a higher latency to fall in the accelerating rotarod test at pubertal age, but not at young adult age. A higher latency reflects improved performance, since the animals were able to stay longer on the rotarod. The n-3 PUFA deficient mice did not demonstrate differences between puberty and adulthood. The increased rotarod latency in n-3 PUFA adq mice suggests that the n-3 PUFA adequate diet was associated with an accelerated development of motor coordination. In contrast to our results, Collucia *et al*. showed that n-3 PUFA supplementation in both juvenile and adult rats improved performance in the rotarod at fixed but not accelerating speeds (Coluccia *et al*., 2009). During development, Purkinje cells are innervated by multiple climbing fibers. Around PND 21, elimination of synapses occurs, leading to mono-innervation of Purkinje cells (Bearzatto *et al*., 2005). Bearzatto *et al*. compared three common wild type mouse strains and suggested a delay in the elimination of multiple climbing fibers innervating the Purkinje cells in adult C57BL6/J mice (Bearzatto *et al*., 2005). Our findings have led us to postulate that the possible delay in elimination of multiple climbing fibers in C57BL6/J mice as described by Bearzatto *et al*. might be shortened by adequate dietary n-3 PUFA availability. As a result, the plateau in the development of motor coordination may be reached already before adulthood.

The results of the MWM test indicate that spatial memory at PND 60 was improved in n-3

PUFA adq animals, but spatial learning remained unaffected. Several studies have stated the probable relation of learning and memory functions with brain fatty acid status (Fedorova and Salem, 2006; Moriguchi and Salem, 2003). Moriguchi *et al*. showed that n-3 PUFA deficiency in second and third generation deprived adult rats caused an impairment in performing the MWM task (Moriguchi *et al*., 2000). The second generation n-3 adequate fed rats showed improved spatial learning and memory as compared to the deficient rats, and the effect was even stronger in the third generation. Yet, Carrié *et al*. found no effects on spatial learning or memory in adult female mice demonstrated in both an n-3 PUFA deficiency model as well as in an n-3 PUFA supplementation study (Carrié *et al*., 2000a; Carrié *et al*., 2000b). A study performed by Kavraal *et al*. underlined the importance of n-3 PUFA availability starting early on at gestation in rats (Kavraal *et al*., 2012). In that study, both dams and offspring were supplemented with n-3 PUFA, and offspring was also supplemented with n-3 PUFA from gestation and throughout life. However, in contrast to our results, an increase in spatial learning was demonstrated, whereas there was no significant effect on spatial memory. The limitation of that study was that the supplemented diet was compared to a standard rat chow, which contained 1.9% ALA, preventing complete n-3 PUFA depletion (Kavraal *et al*., 2012). These studies show the importance of choosing the right starting point for supplementation and composition of diets.

In the present study, ^{31}P MRS demonstrated no effects of n-3 PUFA availability on phosphorylated energy and lipid metabolites. As neurogenesis was decreased in n-3 PUFA adequate animals, a change in the PME levels reflecting membrane formation was expected. A clinical ^{31}P MRS study in epilepsy patients showed that n-3 PUFA supplementation reduced membrane phospholipid breakdown, whereas it improved brain energy metabolism (Puri *et al*., 2007). However, a post mortem observational study of schizophrenia patients revealed no changes in phospholipid metabolism measured by ^{31}P MRS (Pearce *et al*., 2009). Studies using ^{1}H MRS have further elucidated the effect of n-3 PUFA supplementation on cerebral metabolite status (McNamara *et al*., 2009; McNamara *et al*., 2013b). McNamara *et al*. demonstrated a decrease of myo-inositol in the prefrontal cortex of n-3 PUFA deficient adult rats (McNamara *et al*., 2009). It is hypothesized that this reduction may be caused by impaired astrocyte maturation and deficits in osmotic regulation. Another study using ^{1}H MRS revealed that ethyl-EPA has the ability to protect the human brain from early psychosis by increasing glutathione levels in the temporal lobes and increasing glutamate/glutamine levels in the left hippocampus (Berger *et al*., 2008). These studies indicate that ^{1}H MRS may provide in a more sensitive method to detect effects on cerebral metabolite status that would help to understand the changes in behavioral and cognitive tests in the present experimental setup, such as improvement of spatial memory in the MWM and neuronal functionality as reflected by decreased neurogenesis. Furthermore, we were only able to measure whole brain metabolite status with our ^{31}P MRS method. Possibly, measurements distinguishing between different brain regions such as hippocampus and cortex would be more sensitive to detect metabolite changes.

The qRT-PCR data on neurogenesis revealed no diet effect on DCX mRNA expression in the subcortical area (containing hippocampus and subcortical structures. This may have led to a weakening of possible effects in DCX mRNA expression). Another explanation may be that n-3 PUFA already exert their effect before puberty. Strikingly, immunohistochemical staining for doublecortin demonstrated decreased hippocampal neurogenesis in n-3 PUFA adequate fed young adult mice. To our knowledge, this is the first study to show diminished neurogenesis in young adult mice due to altered n-3 PUFA availability during development. Contrarily, several studies have illustrated an increase of neurogenesis after LCPUFA supplementation (Crupi *et al*., 2012; Maekawa *et al*., 2009; Niculescu *et al*., 2011). Niculescu *et al*. assessed hippocampal neurogenesis of n-3 PUFA supplemented mice at the end of the lactation period (PND 19) and detected an increase in cell proliferation (Niculescu *et al*., 2011). In another study, dietary LCPUFA supplementation led to increased neurogenesis in juvenile rats (Maekawa *et al*., 2009). Dietary

DHA has been suggested to promote neurogenesis and synaptogenesis during embryonic development (Cao *et al*., 2009a). It should be noted that these studies were performed at an earlier age than the immunohistochemical stainings in our experiment. Additionally, n-3 PUFA supplementation counteracted the reduction of hippocampal neurogenesis in an immune deprived mouse model (Crupi *et al*., 2012). Combined, these data suggest that n-3 PUFA availability may promote earlier neurogenesis, possibly already before pubertal age.

In the current study, immunohistochemical stainings for synaptophysin and PSD95 showed no effect on pre- and postsynaptic density, respectively, indicating that synaptogenesis remained unaffected in adulthood. However, our results obtained by qRT-PCR indicate that synaptic plasticity may be increased at pubertal age in the cortex, while it remains unaffected at adult age. Several studies demonstrated an increased expression of pre- and postsynaptic proteins after n-3 PUFA supplementation, hinting at increased synaptogenesis either early on in life or during ageing (Cao *et al*., 2009a; Crupi *et al*., 2012; Wurtman, 2008). Our data on synaptophysin expression led us to hypothesize that synaptogenesis is stimulated at adolescent age on an n-3 PUFA adequate diet, and reaches a plateau before adult age. We postulate that neurogenesis may already be accelerated before puberty, as our data show unaffected DCX mRNA expression during puberty and adulthood. Our immunohistochemistry data on hippocampal neurogenesis strengthen the idea that n-3 PUFA induce a plateau in neurogenesis before adulthood. In line with these results, the n-3 PUFA adequate animals showed enhanced performance on the rotarod in puberty, but not in adulthood. At young adult age, n-3 PUFA adequate fed mice still demonstrated improved spatial memory in the MWM. Additional gene expression analysis (for example cAMP response element binding protein: involved in long-term potentiation, or peroxisome proliferator-activated receptor and retinoid X receptor: involved in regulating synaptic plasticity, and learning and memory) may further support our findings.

In the present study, only DHA levels in erythrocyte membrane were affected by the dietary intervention, while all brain regions tested displayed decreased levels of ARA and increased levels of DHA and DGLA as well. This decrease in ARA might be explained by the competition of n-3 and n-6 PUFA for the conversion enzymes delta-5 and delta-6 desaturase, which favor the conversion of n-3 PUFA (Schmitz and Ecker, 2008; Simopoulos, 2008a). Another explanation could be a competition in tissue uptake, due to the administration of preformed DHA (Brenna *et al*., 2009). These changes in n-3 and n-6 PUFA are in the same range as observed in previous studies in rats (Moriguchi *et al*., 2000; Moriguchi and Salem, 2003). The results acquired in the current study underline the importance of brain fatty acid status during development. Besides, the expression of inflammatory markers remained unaffected by n-3 PUFA availability during development. N-3 PUFA supplementation has been shown to decrease neuroinflammation in immune deprived and aged mice (Crupi *et al*., 2012; Labrousse *et al*., 2012). It is not likely that the young, healthy C57BL/6J mice used in our experiments are prone to inflammation at this stage in life, which is supported by the fact that n-3 PUFA availability did not affect inflammatory markers.

In summary, the current study indicates that adequate dietary n-3 PUFA levels early in life improve neural development in the cerebellar cortex during puberty, as reflected by improved motor coordination. Additionally at adult age, we observed cortical and hippocampal changes with increased habituation in the prepulse inhibition, increased exploratory behavior and decreased anxiety in the open field, increased spatial memory in the MWM, a decrease in neurogenesis and unaltered synaptogenesis in immunohistochemistry. qRT-PCR revealed that n-3 PUFA increased synaptogenesis in the cortex at pubertal age, but did not change neurogenesis in the subcortical area. The overall level of cerebral phosphorylated metabolites as measured by *in vivo* ^{31}P MRS was not altered by n-3 PUFA. Expression of inflammatory markers also remained unaffected in both puberty and young adulthood. Finally, we demonstrated that dietary modifications led to increased n-3 PUFA and decreased n-6 PUFA in cerebral fatty acid status, and increased DHA in erythrocyte membranes.

We postulate that perinatally administered dietary n-3 PUFA have the potential to improve cortical and hippocampal development and enhance cognitive functioning. While the present study focused on dietary n-3 PUFA availability in a healthy mouse model, it already yields a range of beneficial effects in brain functioning. Overall, our findings indicate that ensuring adequate dietary n-3 PUFA levels starting early in life may support optimal neural development in healthy full term infants. Furthermore, our results implicate that it would be interesting to study the effect of a sufficient n-3 PUFA intake in disorders caused by an n-3 PUFA deficiency more extensively.

Acknowledgements
The authors would like to thank Professor Thomas Brenna for the fatty acid analysis of brain and erythrocytes. We would like to acknowledge Janneke Mulders and the Central Animal Laboratory of the Radboud university medical center for taking excellent care of our mice. Furthermore, we would like to thank Jos Dederen, Inge Smeets, Annelies van Nuland, Maarten van Dijk, Sarita Dam, and Elina Samani for their laboratory work. This work was sponsored by Mead Johnson Pediatric Nutrition Institute.

Chapter 4

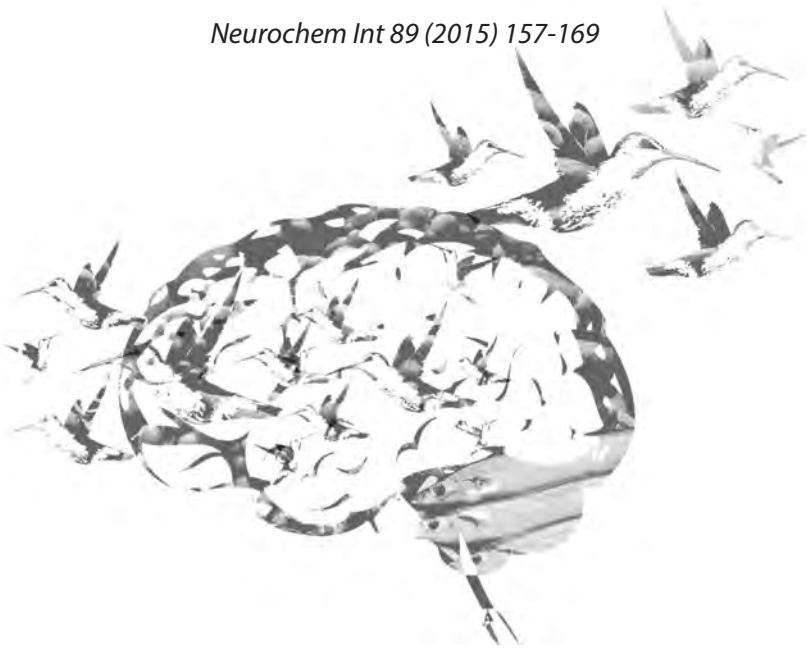
Effect of Perinatally Supplemented Flavonoids on Brain Structure, Circulation, Cognition, and Metabolism in C57BL/6J Mice

Carola IF Janssen[1], Valerio Zerbi[1], Martina PC Mutsaers[1], Mieke Jochems[1], Claudia A Vos[1], Julle O Vos[1], Brian M Berg[2], Eric AF van Tol[2], Gabriele Gross[2], Zeina E Jouni[2#], Arend Heerschap[3#], Amanda J Kiliaan[1]

[1] Department of Anatomy, Donders Institute for Brain, Cognition, and Behaviour, Radboud university medical center, Nijmegen, The Netherlands
[2] Mead Johnson Pediatric Nutrition Institute, Nijmegen/Evansville, the Netherlands/USA
[3] Department of Radiology, Donders Institute for Brain, Cognition, and Behaviour, Radboud university medical center, Nijmegen, The Netherlands

[#] shared penultimate authorship

Neurochem Int 89 (2015) 157-169



Abstract

Evidence suggests that flavanol consumption can beneficially affect cognition in adults, but little is known about the effect of flavanol intake early in life. The present study aims to assess the effect of dietary flavanol intake during the gestational and postnatal period on brain structure, cerebral blood flow (CBF), cognition, and brain metabolism in C57BL/6J mice. Female wild-type C57BL/6J mice were randomly assigned to either a flavanol supplemented diet or a control diet at gestational day 0. Male offspring remained on the corresponding diets throughout life and performed cognitive and behavioral tests during puberty and adulthood assessing locomotion and exploration (Phenotyper and open field), sensorimotor integration (Rotarod and prepulse inhibition), and spatial learning and memory (Morris water maze, MWM). Magnetic resonance spectroscopy and imaging at 11.7T measured brain metabolism, CBF, and white and grey matter integrity in adult mice. Biochemical and immunohistochemical analyses evaluated inflammation, synaptic plasticity, neurogenesis, and vascular density. Cognitive and behavioral tests demonstrated increased locomotion in Phenotypers during puberty after flavanol supplementation ($p=0.041$) but not in adulthood. Rotarod and prepulse inhibition demonstrated no differences in sensorimotor integration. Flavanols altered spatial learning in the MWM in adulthood ($p=0.039$), while spatial memory remained unaffected. Additionally, flavanols increased diffusion coherence in the visual cortex ($p=0.014$) and possibly the corpus callosum ($p=0.066$) in adulthood. Mean diffusion remained unaffected, a finding that corresponds with our immunohistochemical data showing no effect on neurogenesis, synaptic plasticity, and vascular density. However, flavanols decreased CBF in the cortex ($p=0.001$) and thalamus ($p=0.009$) in adulthood. Brain metabolite levels and neuroinflammation remained unaffected by flavanols. These data suggest that dietary flavanols results in subtle alterations in brain structure, locomotor activity and spatial learning. Comparison of these data to published findings in aging or neurodegeneration suggests that benefits of dietary flavanols may increase with advancing age and in disease.

Introduction

Nutrition plays an important role in developing and maintaining a healthy brain. For example, there is increasing evidence that dietary polyphenols can support brain health. Polyphenols are fruit and plant constituents that are well-known for their antioxidant properties. A class of polyphenols that has been associated with improved cognition are flavonoids (Corcoran *et al*.; Ghosh and Scheepens, 2009; Hollenberg *et al*., 2009; Rendeiro *et al*., 2009; Spencer, 2009). Studies indicate that flavonoid consumption can beneficially affect normal cognitive function and cerebral blood flow in adults (Francis *et al*., 2006; Sorond *et al*., 2008; Vauzour, 2012).

In our current study, we focus on flavanols, a group of flavonoids that are present in apple and grape seed extract. Flavanols are the most abundant flavonoids in the human diet and are mostly provided by fruits (such as apple and grapes), cocoa, tea, wine, nuts, and beans (Arola-Arnal *et al*., 2013). The daily intake of flavanols such as catechins and proanthocyanidins is estimated at 18-50 mg/day in humans, while intake of the flavonol quercetin has been estimated to be 20-35 mg/day (Manach *et al*., 2005). These substances have the ability to improve health by exerting cardioprotective, anti-inflammatory, and antioxidant effects (Arola-Arnal *et al*., 2013; Vauzour, 2012). The most well-known biological actions of flavanols are their antioxidant effects as shown in *in vitro* conditions. Due to donation of a hydrogen atom from hydroxyl groups to reactive oxygen species (ROS), phenoxyl radicals arise which can be stabilized by reactions with other radicals and stabilized oxygen species (Gomez-Pinilla and Nguyen, 2012). After ingestion, the largest portion of dietary flavonoids are metabolized in the small and large intestine, in the liver, and in cells (Rendeiro *et al*., 2012). They form substrates for phase I and II enzymes and are de-glucosylated and further metabolized into glucuronides, sulphates, and O-methylated derivatives (Rendeiro *et al*., 2012). In order to be able to influence brain health directly, flavonoids or their metabolites must pass the blood-brain barrier (BBB). The ability of structures to cross the BBB depends on their systemic availability and lipophilicity (Faria *et al*., 2014; Rendeiro *et al*., 2012). As a result, theoretically the O-methylated metabolites would be more potent to pass the BBB than sulphates and glucuronides, which has been shown in *in vitro* studies (Rendeiro *et al*., 2012; Vauzour, 2012; Youdim *et al*., 2003).

While the effects of flavonoid supplementation have been studied in normal aging and neurodegenerative models, little is known about their influence during development. In neurodegeneration, flavonoids have shown to exert neuroprotective properties such as the modulation of neuroinflammation and improvement of cognition (Vauzour, 2012). It has been shown that flavonoids modulate neuronal signaling pathways involved in synaptic plasticity and neurogenesis (Spencer, 2010; Vauzour, 2012). *In vivo*, flavonoids are able to affect these pathways by activation of cyclicAMP-response element binding (CREB) receptors and brain derived neurotrophic factor (BDNF), mechanisms that are involved in memory acquisition and consolidation (Spencer, 2010). Potential effects in neural development may be expected, but this has not been studied yet.

In the current study, the impact of flavanol availability during gestation and the postnatal period was assessed in mice to understand the mechanisms underlying potential long-term beneficial effects throughout life. A broad spectrum of parameters was investigated in order to evaluate effects on brain structure and function. Changes in cortical and hippocampal functionality were studied with behavioral and cognitive tests. Proton magnetic resonance spectroscopy (^{1}H MRS) was used to assess brain metabolism, while cerebral blood flow (CBF) was measured with arterial spin labeling (ASL), and diffusion tensor imaging (DTI) was performed to study brain integrity. Neurogenesis, synaptic plasticity and capillary density were evaluated immunohistochemically, and quantitative real-time polymerase chain reaction (qRT-PCR) was used to study gene expression of inflammatory markers, synaptic plasticity and angiogenesis.

Material and methods

Animals and diets

C57BL/6J mouse dams (3-4 months old, Harlan Laboratories Inc., Horst, the Netherlands) were used for breeding. The dams obtained the control diet upon arrival in the Central Animal Facility (Radboud university medical center, Nijmegen, the Netherlands) and were randomly assigned to the control diet (n=14), or a flavanol supplemented diet (apple + grape seed extract; apple+GSE), n=11) at the first day of gestation (GD 0). The diets were isocaloric and based on AIN93m; they only differed in flavonoid concentrations (Table 1A and B; Research Diets Services, Wijk bij Duurstede, the Netherlands) (Reeves *et al*., 1993). Offspring of all dams were maintained on the corresponding diets throughout the whole experiment. Litter sizes were normalized by culling the litters to 3 males and 3 females per dam. Nine parallel groups of male offspring were tested, the groups were either tested and sacrificed on postnatal day (PND) 30 (control n=11, apple+GSE, n=13) or PND 60 (control n=9, apple+GSE, n=10), including behavioral testing and brain biochemistry parameters, or used for brain histology at PND 60 (control n=11, apple+GSE, n=11) (Figure 1).

The dams were housed individually in Phenotypers (Noldus, Wageningen, the Netherlands) from GD 0 onwards and remained there during delivery of the pups until weaning of the offspring. After weaning, male offspring were housed in groups of 3 in individually ventilated cages. Only male offspring were used in these experiments to avoid influence of hormonal fluctuations in the female offspring. All mice were housed in the Preclinical Imaging Centre (PRIME) in the central animal facility with temperature controlled at 21 °C, an artificial 12:12h light:dark cycle (lights on at 7:00 a.m.), continuous music playing in the background during the light period, and cage enrichment consisting of a plastic shelter and cotton nesting material. Food and water were available ad libitum. The experiments were performed according to Dutch federal regulations for animal protection and were ethically approved by the Veterinary Authority of the Radboud university medical center. Data of some mice were excluded from further analysis of magnetic resonance (MR) spectroscopy and imaging due to movement errors (i.e. obtained spectra did not meet inclusion criteria).

Behavioral and cognitive tests

Dams and pups were monitored in Phenotyper cages (EthoVision XT 8.5, Noldus, Wageningen, the Netherlands) for 24 hours a day during PND 0 – 21 (from birth until weaning). Locomotor activity of the litters was monitored as well as opening of the eyes. The offspring were weighed on PND 14, 21, 30, and 60. Male offspring performed behavioral and cognitive tests starting on PND 30 (puberty) and PND 60 (young adulthood) (Figure 1). Tests were performed in the same order (Phenotypers open field, rotarod, prepulse inhibition) during the light period between 8:00 a.m. and 5:00 p.m. The Morris water maze (MWM) was only performed in young adulthood, after the animals had exerted the other behavioral tests.

Phenotypers

Locomotor activity was measured in Phenotypers at PND 30 and PND 60. Mice were individually monitored in Phenotyper cages during 24 hours.

Open field

Locomotion and explorative behavior was evaluated in the open field at PND 30 and PND 60 as previously described (Jansen *et al*., 2013a; Jansen *et al*., 2014; Janssen *et al*., 2015). Locomotion was automatically recorded with EthoVision XT8.5 (Noldus, Wageningen, The Netherlands), while exploration was manually scored and analyzed as described previously (Hooijmans *et al*., 2009; Streijger *et al*., 2005).

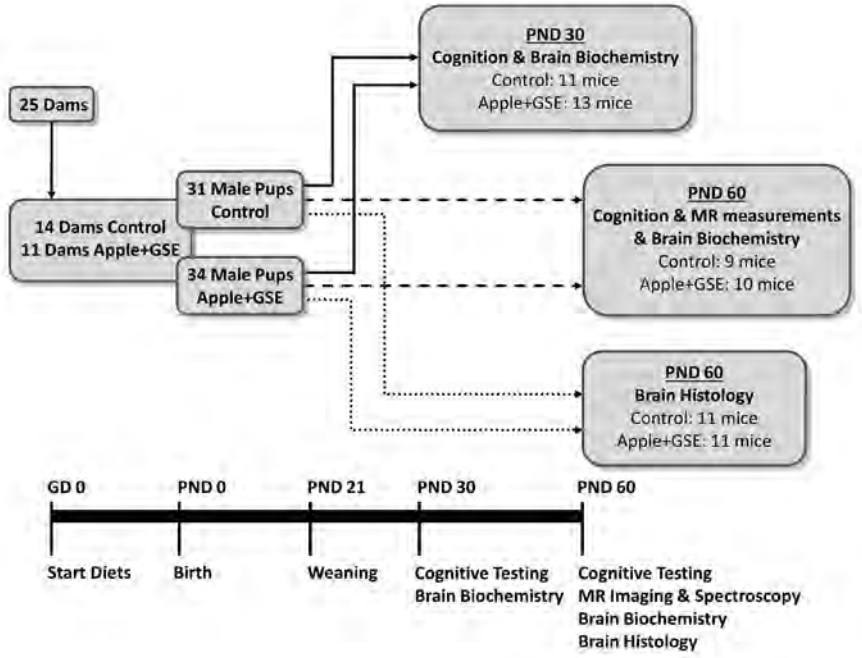


Figure 1: Schematic overview of the study design and timeline of the experiment. C57BL/6J dams were randomly assigned to remain on a control diet or switch to an apple+GSE supplemented diet on GD 0. Offspring were maintained on the corresponding diets throughout the whole study. Male littermates were randomly assigned to one of three parallel groups. One group was tested for behavioral and cognitive parameters (24h activity, open field, Rotarod, and prepulse inhibition) on PND 30 and subsequently sacrificed for brain biochemistry. A parallel group was tested for behavioral and cognitive parameters (24h activity, open field, Rotarod, prepulse inhibition, and MWM) on PND 60 and subsequently underwent MR imaging and spectroscopy (ASL, 1H MRS, and DTI) and was sacrificed afterwards for brain biochemistry. A third group was sacrificed at PND 60 for brain histology. One male pup from each dam was represented in each parallel group for testing.

Rotarod

The Rotarod was performed to study motor coordination at PND 30 and 60 (for more detailed information see (Janssen *et al*., 2015)). Mice were placed on a rotating drum and their ability to remain on the rotating drum was recorded as latency to fall (s).

Prepulse inhibition

The prepulse inhibition test was performed at PND 30 and 60 to evaluate sensorimotor integration as previously described (Janssen *et al*., 2015; Streijger *et al*., 2005). The whole-body startle response of the mouse was measured during three blocks of startle pulses.

Morris water maze

Spatial learning and memory was tested in the MWM at PND 60 (for more detailed information see (Jansen *et al*., 2013a; Jansen *et al*., 2014; Janssen *et al*., 2015)). The young adult mice were trained to find a submerged platform located in the north-east quadrant of the pool by using distant visual cues. The acquisition phase was used as a measure for spatial learning, while the probe phase evaluated spatial memory.

Table 1A: Composition of diets.
Both diets were isocaloric and based on AIN93M (Reeves *et al*., 1993).

Ingredient	Amount (g/kg diet)	
	Control	Apple+GSE
Cornstarch	397.486	388.186
Casein (alcohol extracted)	200	200
Maltose	132	132
Sucrose	100	100
Soybean oils (no additives)	28	28
Hydrogenated coconut oil (no additives)	42	42
Cellulose BW 200	50	50
Mineral mix (AIN-93-mix)	35	35
Vitamin-mix (AIN-93-mix)	10	10
L-cystine	3	3
Choline bitartrate (4.1% choline)	2.5	2.5
TBHQ	0.014	0.014
Flavanols	ND	9.3
Flavonoid composition (μg/g)**:**		
Catechin	ND	243.61±7.13
Epicatechin	ND	478.26±5.84
Epigallocatechin	ND	2.24±0.13
Epicatechin gallate	ND	18.87±0.14
Procyanidin B1	ND	146.16±4.63
Procyanidin B2	ND	144.53±0.29
Quercetin	ND	13.58±0.16
Quercetin 3 glucoside	ND	68.48±0.30

Table 1B: Flavonoid composition of extract.
We used a 2:1 ratio of apple: grape seed extract. The apple extract used was Diana 9080 (Diana Naturals Inc, Rennes, France) and the grape seed extract was Ajinomoto 20R (Ajinomoto Omnichem, Louvain-la-Neuve, Belgium). The combined extract was incorporated into the diets at 9.3g/kg of the total diet (as referenced in Table 1A).

Compound	2:1 Apple:Grape Seed mix Compound (mg) / Extract (g)
Catechin	21.06
Epicatechin	40.24
Epigallocatechin	0.19
Epicatechin gallate	3.44
Quercetin	0.93
Quercetin 3 glucoside	16.14
Procyanidin B1	17.65
Procyanidin B2	32.27
Other Procyanidins	9.74
Other Procyanidins	1.64

MR measurements

After behavioral and cognitive testing on PND 60, mice were measured on an 11.7T BioSpec Avance III small animal MR system (Bruker BioSpin, Ettlingen, Germany) equipped with an actively shielded gradient set of 600 mT/m and operated by Paravision 5.1 software. A circular polarized volume resonator was used for signal transmission and an actively decoupled mouse brain quadrature surface coil with integrated combiner and preamplifier was used for signal reception (Bruker BioSpin). Mice were placed in a stereotactic holder to immobilize the head and prevent unwanted movement during scanning. During the MR measurements, mice were anaesthetized with isoflurane (Nicholas Primal (I) Limited, London, United Kingdom; 3.5% for induction and 1.8% for maintenance) in a 2:1 oxygen and N2O mixture through a nose cone. Body temperature was measured using a rectal optimal temperature probe and maintained at 37 °C with heated airflow. Respiration of the mouse was monitored using a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc., NY, United Stated). Gradient echo (GE) images in the axial, sagittal, and coronal orientation were acquired to visualize the anatomy and morphology of the mouse brain (see Table 2A for details).

Table 2A: Imaging parameters ASL, DTI.

	Anatomical scans	ASL	DTI
Method	Gradient echo	FAIR-ASL	SE-EPI
Echo time (TE)	5 ms	11.8 ms	21.4 ms
Repetition time (TR)	630 ms	13.75 s	7.75 s
Image matrix	512 x 512	128 x 128	128 x 128
Field of view (FOV)	40 x 40 mm	30 x 30 mm	20 x 20 mm
Spatial resolution	0.078 x 0.078 x 0.034 mm/pixel	0.234 x 0.234 x 1 mm/pixel	0.156 x 0.156 x 0.5 mm/pixel
Acquisition time	~8 min	~12 min	~18 min

ASL

Cerebral blood flow was measured under resting conditions using an ASL method with flow-sensitive alternating inversion recovery (FAIR) (Kim, 1995; Kwong *et al*., 1991). A spin-echo planar imaging sequence preceded by a 180° hyperbolic secant (sech80) RF inversion pulse was used. The inversion slab thickness was 6 mm and the slice margin was 1.5 mm. Twenty-five images with increasing inversion times (40 ms – 3000 ms) were obtained for the T1 calculations. Inversion recovery data from the imaging slice were acquired after selective inversion interleaved with non-selective inversion (see Table 2A for details). For each mouse, FAIR images were processed and analyzed as described previously (Zerbi *et al*., 2014a). Regional perfusion was evaluated in the cerebral cortex, hippocampus, and thalamus according to the mouse brain atlas of Franklin & Paxinos (third edition, 2008).

Table 2B: imaging parameters ^{1}H MRS.

	Anatomical scans
Method	Single voxel
Echo time (TE)	10.905 ms
Repetition time (TR)	2500 ms
T1	6.31 ms
T2	4.59 ms
Signal averages	800
Acquisition time	~27 min

^{1}H MRS

Metabolite concentrations in the hippocampus were determined by ^{1}H MRS (Jansen *et al*., 2013a) in a single voxel of 1 x 1 x 2 mm, positioned unilaterally in the right hippocampus guided by anatomical images. Water-suppressed ^{1}H MRS spectra were acquired with a point-resolved spectroscopy sequence (PRESS) with a short echo time (TE) (see Table 2B for details). Quantification of metabolite concentrations was performed with LCModel™ software (Linear Combination Model, S. Provencher, Oakville, Canada) as described previously (Jansen *et al*., 2013a). As a criterium for a reliable metabolite signal fit a Cramér-Rao lower bound (CRLB) ≤ 20% was used (Cavassila *et al*., 2001). Ten metabolites fulfilled this criterium: N-acetylaspartate (NAA), glycerophosphocholine (GPC), *myo*-Inositol (*m*I), taurine (Tau), glutamate (Glu), glutamine (Gln), choline + glycerophosphocholine + phosphocholine (tCho), N-acetylaspartate + N-acetylaspartateglutamate (NAA+NAAG), *myo*-Inositol + glycine (*m*I+Gly), glutamate + glutamine (Glu+Gln).

DTI

Diffusion of water was measured by the protocol originating from Harsan *et al*. and modified by Zerbi *et al*. (Harsan *et al*., 2010; Zerbi *et al*., 2014a; Zerbi *et al*., 2013). In short, 31 axial slices covering the whole brain were acquired with by spin-echo planar imaging (SE-EPI). B0 shift compensation, navigator echoes and an automatic correction algorithm to limit the occurrence of ghosts and artefacts were implemented. Encoding b-factors of 0 s/mm2 (b0 images; 5x) and 1000 s/mm2 were used and diffusion-sensitizing gradients were applied along 30 non-collinear directions in three-dimensional space (see Table 2A for details). Pre-processing was performed as previously described (Zerbi *et al*., 2013). Maps of mean diffusivity (MD) and fractional anisotropy (FA) were evaluated for diet differences with a region of interest (ROI) approach. Eight white and grey matter tracts were selected bilaterally across the brain according to the mouse brain atlas of Franklin & Paxinos (third edition, 2008). These regions include the corpus callosum (CC), fornix (F), optic tract (OT), hippocampus (HC), motor cortex (MC), somatosensory cortex (SSC), auditory cortex (AUC), and visual cortex (VC).

Biochemistry

qRT-PCR

Mice that were tested on behavior on PND 30 and those that were tested on behavior and underwent MR imaging and spectroscopy on PND60 were transcardially perfused with 0.1 M phosphate buffered saline (PBS). Subsequently, mice were decapitated and whole brains were collected and snap frozen in liquid nitrogen. The brains were stored at -80 °C until further analysis. The right hemisphere was divided into 4 regions; brain stem, cortex, subcortical region, and cerebellum. Samples were kept at -80 C until analysis.

The subcortical areas (containing hippocampus) of the right hemispheres of the brains collected at PND 30 and 60 were analyzed for inflammatory markers, synaptic plasticity, and angiogenesis with qRT-PCR. Inflammatory markers included interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), cluster of differentiation 36 (CD36), and monocyte chemoattractant protein-1 (MCP-1) (Table 3). Synaptophysin was used as a measure for synaptic plasticity and vascular endothelial growth factor (VEGF) was used to assess angiogenesis (Table 3).

qRT-PCR was performed as previously described by Janssen *et al*. (Janssen *et al*., 2015). To evaluate differences, relative gene expression ratios were calculated according to the comparative CT method (also referred to as the 2-ΔCT method) (Schmittgen and Livak, 2008). Relative CT values were calculated by subtracting the CT value of the housekeeping gene GAPDH from the CT values of IL-1β, IL-6, CD36, MCP-1, and TNF-α. For each primer, two independent qRT-PCR runs were performed, and the means of their relative values were used for statistical analysis.

Table 3: qRT-PCR primers (Janssen et al., 2015).

Primers (mouse)	Forward	Reverse
GAPDH	5′-AGGTCGGTGTGAACGGATTTG-3′	5′-TGTAGACCATGTAGTTGAGGTCA-3′
CD36	5′- ATGGGCTGTGATCGGAACTG-3′	5′-GTCTTCCCAATAAGCATGTCTCC-3′
IL-1β	5′-GCAACTGTTCCTGAACTCAACT-3′	5′-ATCTTTTGGGGTCCGTCAACT-3′
IL-6	5′-CAAGTCGGAGGCTTAATTACACATG-3′	5′-ATTGCCATTGCACAACTCTTTTCT-3′
MCP-1	5′-CCCAATGAGTAGGCTGGAGA-3′	5′-TCTGGACCCATTCCTTCTTG-3′
TNF-α	5′-CAGACCCTCACACTCAGATCATCT-3′	5′-CCTCCACTTGGTCCTTTGCTA-3′
synaptophysin	5'- TCTTTGTCACCGTGGCTGTGTT-3'	5'-TCCCTCAGTTCCTTGCATGTGT-3'
VEGF	5′-GGAGATCCTTCGAGGAGCACTT-3′	5′-GGCGATTTAGCAGCAGATATAAGAA-3′

Enzyme-linked immunosorbent assay (ELISA)

Mature BDNF concentration in the left hemispheres of the brains collected at PND 30 and 60 was measured with a commercially available high-sensitivity enzyme-linked immunosorbent assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instruction.

Immunohistochemistry

Brains were collected at PND 60 from the parallel group of littermates for immunohistochemical analysis (Figure 1). Mice were transcardially perfused with 0.1M phosphate buffered saline (PBS; room temperature), followed by 4% paraformaldehyde (PF; 4°C). Subsequently, mice were decapitated and whole brains were collected. The brains were post-fixated overnight in 4% PF at 4 °C and thereafter stored in 0.1M PBS with sodiumazide at 4 °C. Coronal brain sections were cut using a sliding microtome (Microm HM 440, Walldorf, Germany) equipped with an object table for freeze sectioning at -60 °C gaining 8 parallel series of 30 µm thick sections. These sections were used for immunohistochemical purposes (240 µm distance between the sections). The tissue was stained for immature neurons (measure for neurogenesis) with antibodies against doublecortin (DCX), for postsynaptic density (measure for synaptic plasticity) with antibodies against postsynaptic density protein 95 (PSD95), for glucose transporters (measure for capillary density) with antibodies against glucose transporter 1 (GLUT-1), and for activated microglia (measure for neuroinflammation) with antibodies against ionized calcium-binding adapter molecule 1 (Iba1) (Figure 2).

Immunohistochemistry was performed using standard free-floating labeling procedures, using the protocol described by (Jansen *et al*., 2012; Jansen *et al*., 2014; Janssen *et al*., 2015). For DCX, polyclonal goat anti-doublecortin (1:4000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used as a primary antibody to assess neurogenesis. In the Iba1 staining, polyclonal goat anti-Iba1 (1:1500, Abcam, Cambridge, United Kingdom) was used as a primary antibody to assess neuroinflammation. The secondary antibody for both DCX and Iba1 was donkey anti-goat biotin (1:1500; Jackson ImmunoResearch, West Grove, PA, USA). For PSD95, polyclonal rabbit anti-PSD95 (1:2000; Abcam, Cambridge, UK) was used as a primary antibody to visualize the postsynaptic density. In the GLUT-1 staining, polyclonal rabbit anti-GLUT-1 (1:10,000; Chemicon International Inc., Temecula, CA, United States) was used as a primary antibody to assess capillary density. The secondary antibody used for both PSD95 and GLUT-1 was donkey anti-rabbit biotin (1:1500; Jackson ImmunoResearch, West Grove, PA, USA).

Stained brain regions were analyzed double blind by 2 independent observers using a Zeiss Axioscop microscope equipped with hardware and software of Microbrightfield (Williston, VT, USA). Quantified hippocampus regions were based on the mouse brain atlas of Franklin & Paxinos (third edition, 2008).

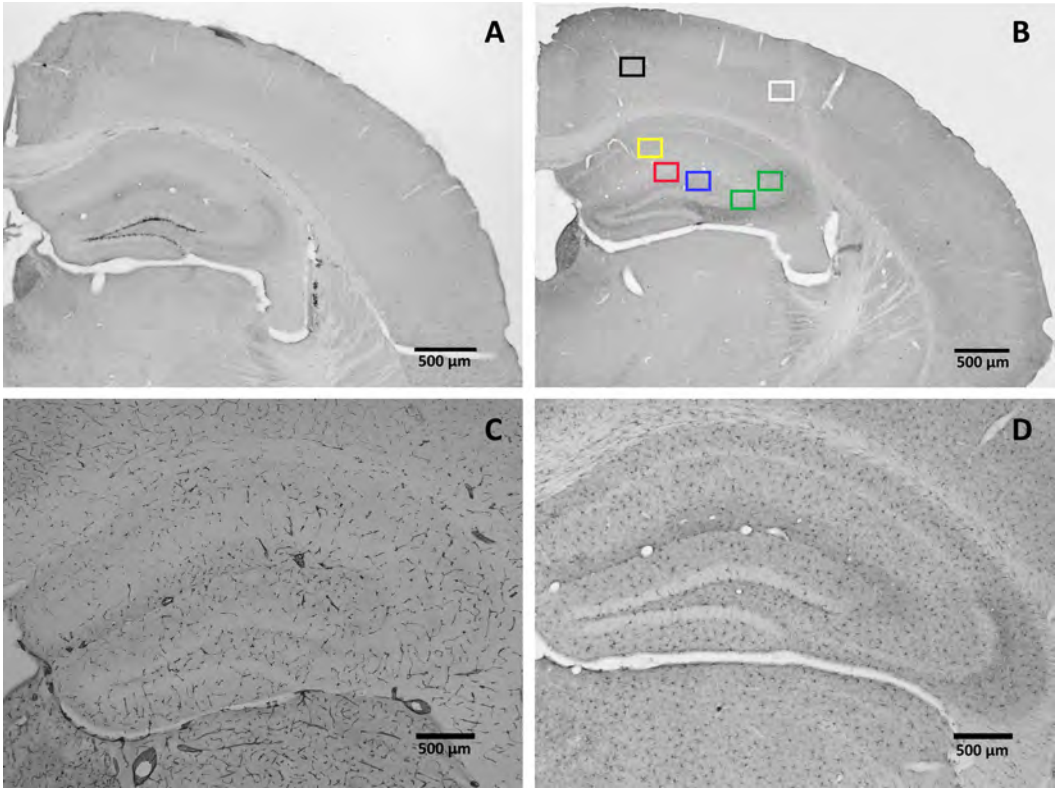


Figure 2: Immunohistochemistry in young adult mice. Representative images of (**A**) DCX staining (2.5× objective), (**B**) PSD95 staining (2.5× objective). Black = visual cortex, white = sensory cortex, yellow = stratum radiatum, blue = inner molecular layer, red = outer molecular layer, green = stratum lucidum, (**C**) Iba1 staining (5× objective), and (**D**) GLUT-1 staining (5× objective). Scale bars represent 500 µm.

Quantification doublecortin

DCX positive cells were quantified in three succeeding sections of the hippocampus (-1.70, -2.18 mm, and -2.46 mm posterior to bregma) for each mouse. Contours were drawn along the borders of the hippocampus at 2.5× magnification using the program Stereo Investigator (Microbrightfield, Williston, VT, USA). The DCX positive cells in the subgranular zone of the hippocampus were manually counted at 40× magnification and the values of the three succeeding sections were averaged to obtain a single value for each animal.

Quantification PSD95

The relevant regions in the hippocampus were digitized using Stereo Investigator. Contours of the inner molecular layer (IML), outer molecular layer (OML), stratum radiatum (SR), and stratum lucidum (SL) of the hippocampus, and the sensory and visual cortex (-2.18 mm up to -2.46 mm posterior to bregma) were drawn at 2.5× magnification (Figure 3B). Photographs were taken at 100× magnification. The quantification of the staining was performed using the program Image J (National Institute of Health, Bethesda, MD, USA). Images were converted to 8-bit grey scale, followed by conversion to 16-bit grey scale, next the contrast was enhanced and the amount of tissue stained was measured with a threshold-based approach. We did not set limits for the particle size.

Quantification Iba1 and GLUT-1

Relevant regions in the hippocampus were digitized using Stereo Investigator. Photographs of the whole hippocampus (-2.18 mm up to -2.46 mm posterior to bregma) were taken at 5x magnification. The quantification of the staining was performed using the program Image J. Images were converted to 8-bit grey scale, contours of the CA1, CA3, and DG were drawn, and the amount of tissue stained was measured with a threshold-based approach. Measurement of Iba1 and GLUT-1 density was defined as the area covered by either Iba1 or GLUT-1 immunoreactivity per mm2 of the total area of the region measured.

Statistics

Data are expressed as mean ± SEM and were analyzed with SPSS for Windows 20.0 software (SPSS Inc., Chicago, IL, USA). The repeated measures ANOVA was used for the first and last block of pulse-alone trials in the prepulse inhibition (with the repeated measure: time), and the acquisition phase of the MWM (with the repeated measure: acquisition days). A Student's t-test was performed for the parameters of the open field (total time), rotarod, prepulse inhibition, MWM parameters in the probe phase, parameters of MR imaging, immunohistochemistry, and biochemistry. For readability reasons, F-values and degrees of freedom are not displayed in the text. Statistical outliers were removed from the dataset for further analysis and statistical significance was set at $p \leq 0.05$ and a trend at $0.05 < p \leq 0.07$.

Results

There were no differences between the two dietary groups in general developmental parameters such as opening of the eyes or litter activity in the Phenotypers (data not shown). Activity of the dam with litter increased over time for both dietary groups (data not shown). Dietary intervention had no effect on body weight in the two groups on PND 30 (control 16.03±0.86; apple+GSE 15.06±1.09), PND 60 (control 23.07±0.58; apple+GSE 22.88±0.74) or at PND 60 in the parallel group for immunohistochemistry (control 24.52±0.84; apple+GSE 23.45±0.55).

Behavioral and cognitive tests

Phenotypers

At pubertal age (PND 30), the apple+GSE fed mice displayed increased mean walking velocity during 24 hours in the Phenotypers ($p=0.041$; Figure 3A). The total walking distance only showed a tendency for increased walking in apple+GSE fed mice ($p=0.060$; Figure 3B). In young adulthood (PND 60), the mice did not demonstrate differences in 24h activity in the Phenotypers. We also

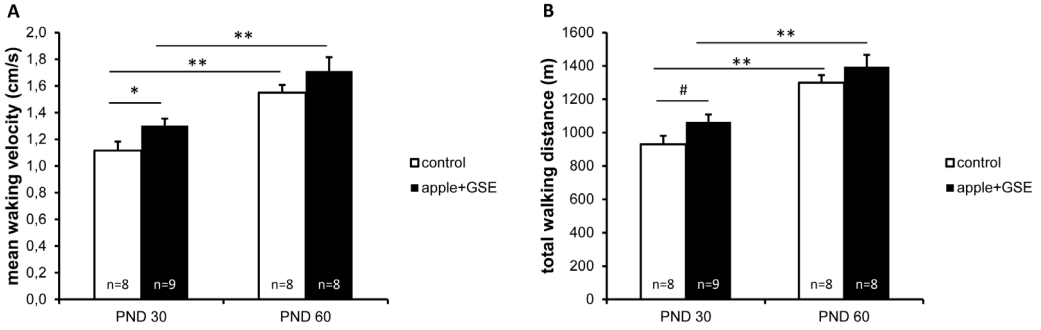


Figure 3: 24h activity in Phenotypers. Mice were placed in Phenotypers for 24 hours to measure locomotor activity. **A** Apple+GSE fed pubertal mice demonstrated increased mean walking velocity. Both diets showed increased mean walking velocity when comparing adulthood to puberty. **B** Apple+GSE fed pubertal mice showed a tendency ($p=0.060$) for increased total walking distance. Both diets showed increased total walking distance when comparing adulthood to puberty. *$p \leq 0.05$, **$p \leq 0.01$, and #$p \leq 0.07$.

observed increased activity over time between PND 30 and 60 in both control (total walking distance p=0.000, mean walking velocity p=0.000) and apple+GSE (total walking distance p=0.001, mean walking velocity p=0.006) fed mice (Figure 3A and B).

Open field

At pubertal age, there were no differences observed between the diets on any of the parameters (locomotion, behavior, and regional preferences). However, the young adult mice on apple+GSE diet showed increased grooming (p=0.033), but no differences in any other parameter (Figure 4A).When comparing pubertal and young adult mice, we found that young adult control fed mice demonstrated increased time spent walking compared to pubertal mice (p=0.000), this effect was not significant in apple+GSE fed mice (p=0.061; Figure 4B). Age comparisons also showed that young adult control fed mice spent decreased time grooming (p=0.002), while young adult apple+GSE fed mice did not (Figure 4A). The control fed mice displayed increased locomotion in adulthood compared to puberty (total walking distance p=0.004, mean walking velocity p=0.004; Figure 5A and B). However, this aging effect was not observed in the apple+GSE fed mice.

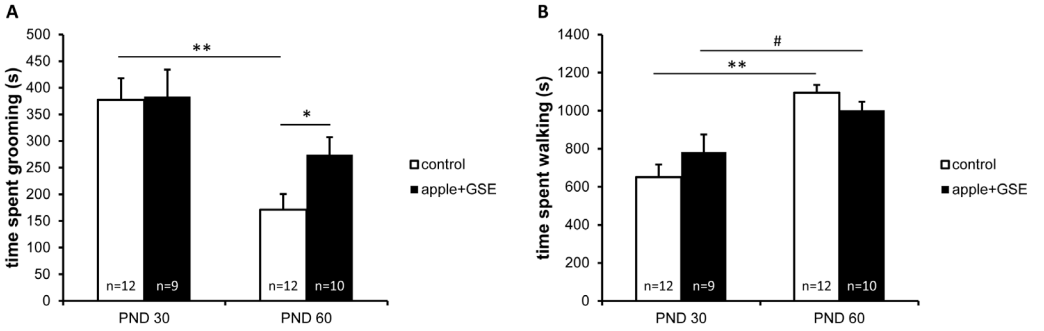


Figure 4: Behavior in the open field. Locomotion, behavior, and regional preferences were measured in the open field. **A** Apple+GSE fed young adult mice displayed increased time spent grooming in the open field. Furthermore, young adult control fed mice demonstrated decreased time spent grooming compared to their pubertal littermates. **B** Young adult control fed mice demonstrated increased time spent walking compared to their pubertal littermates. A tendency (p=0.061) for the same aging effect was shown in apple+GSE fed mice. *p≤0.05, **p≤0.01, and #p≤0.07.

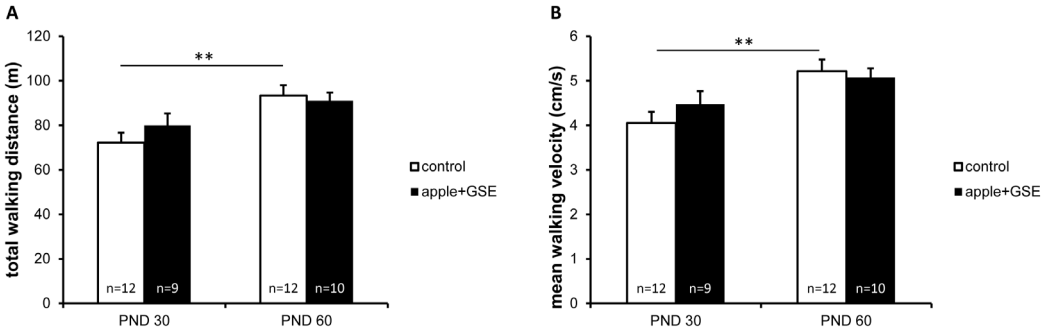


Figure 5: Locomotion in the open field. Locomotion was measured in the open field. **A** Young adult control fed mice showed increased total walking distance in the open field, this effect was not demonstrated in the apple+GSE fed mice. **B** Young adult control fed mice displayed increased mean walking velocity in the open field, this effect was not shown in the apple+GSE fed mice. **p≤0.01.

Rotarod

Motor coordination as measured with the Rotarod did not differ between the diet groups neither during puberty nor in young adulthood. However, young adult control fed mice demonstrated a decreased latency to fall compared to pubertal mice (p=0.016; Figure 6). This effect was not observed in apple+GSE fed mice.

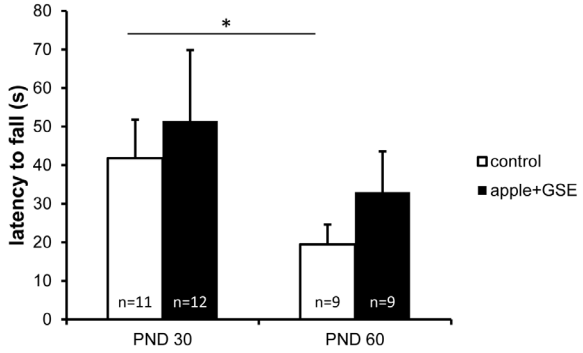


Figure 6: Motor function in the Rotarod. Young adult control fed mice demonstrated a decreased latency to fall compared to their pubertal control fed littermates. No differences were observed in the apple+GSE fed mice. *p≤0.05.

Prepulse inhibition

Sensorimotor integration as measured in the prepulse inhibition test remained unaffected by apple+GSE diet in pubertal mice. Furthermore, a habituation effect to the 120dB pulses was determined (p=0.000), but no diet effect could be observed (Figure 7). In young adulthood, the mice did not display differences in prepulse inhibition. Surprisingly, the mice also did not demonstrate a habituation to the 120dB pulses. Both young adult control fed mice and apple+GSE fed mice showed a decreased habituation effect compared to their pubertal littermates (control p=0.002, apple+GSE p=0.002; Figure 7).

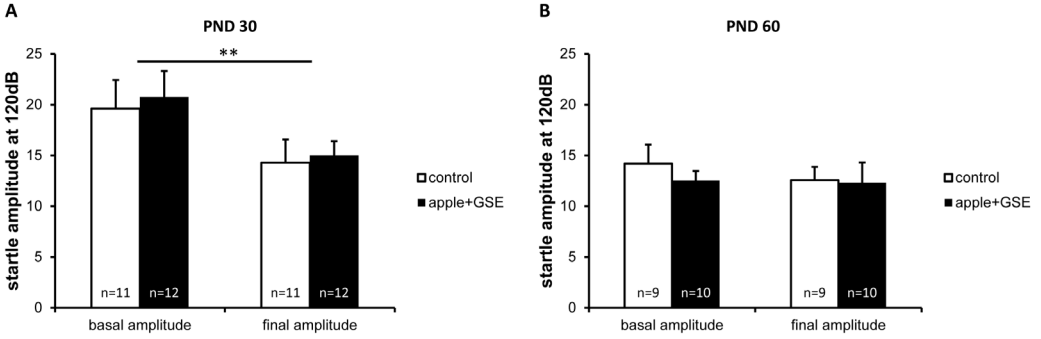


Figure 7: Startle response in the prepulse inhibition test. Mice were exposed to acoustic pulses, and the startle amplitude at 120 dB of the mice was measured at the start and the end of the experiment. **A** The mice displayed an overall habituation effect to the acoustic pulses on PND 30. **B** No differences were observed on PND 60, but both diets showed a decreased habituation effect in adulthood compared to puberty. **p≤0.01.

MWM

The young adult mice were also tested in the Morris water maze. All groups showed a learning curve during the acquisition phase (p=0.000; Figure 8). However, the learning curve in the apple+GSE fed mice deviated from control (p=0.039) fed mice. The apple+GSE group started with a higher escape latency on day 1. But while control fed mice only showed a significant learning effect from day 1 to day 2 (p=0.040), the apple+GSE fed mice also showed a learning effect from day 2 to day 3 (day 1–2: p=0.004; day 2–3: p=0.023). Spatial memory was not affected by apple+GSE diet (data not shown).

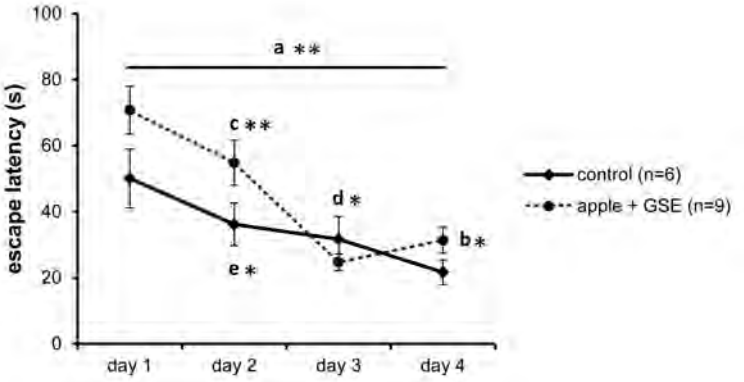


Figure 8: Morris water maze learning in young adult mice (PND 60). Spatial learning was measured in a 4-day acquisition phase, by determining the latency to find a hidden platform in the NE target quadrant. All groups showed a learning curve during the acquisition phase (a**). However, the learning curve in the apple+GSE fed mice deviated from control fed mice (b*). The apple+GSE group started with a higher escape latency on day 1. Apple+GSE fed mice also showed a learning effect from day 1 to 2 (c**) and day 2 to day 3 (d*), while control fed mice only showed a significant learning effect from day 1 to day 2 (e*). *p≤0.05, **p≤0.01.

MR measurements

ASL

After the behavioral tests on PND 60, the cerebral blood flow was measured with ASL (Figure 9A and B). In the apple+GSE fed mice, the CBF was decreased in the cortex (p=0.001) and the thalamus (p=0.009; Figure 9C). No effect on CBF was found in the hippocampus.

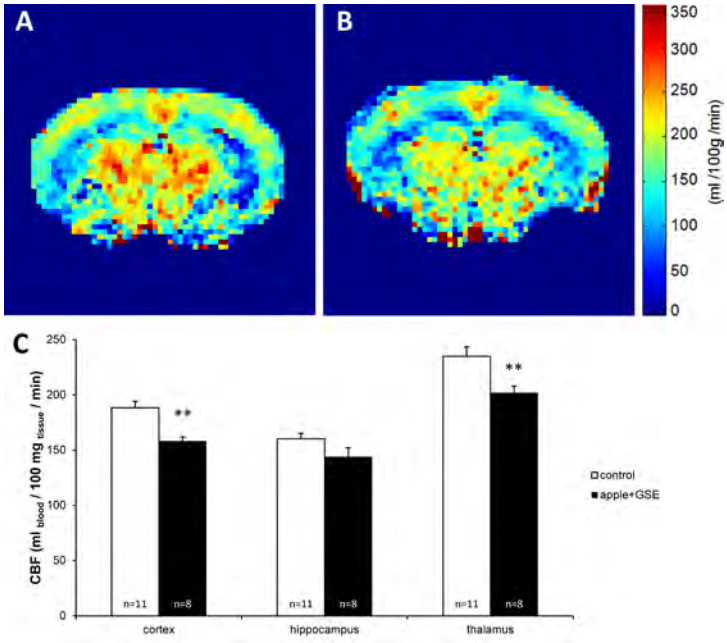


Figure 9: Cerebral blood flow in young adult mice. CBF was measured in the cortex, hippocampus, and the thalamus using ASL in control **(A)** and flavanol **(B)** fed young adult mice. **C** Apple+GSE fed young adult mice demonstrated decreased CBF in the cortex and the thalamus. **p≤0.01.

^{1}H MRS

^{1}H MRS was used to measure tissue levels of cerebral metabolites that represent neuronal integrity, occurrence of proceses associated with inflammation, membrane turnover, energy metabolism, osmoregulation, and neurotransmitters. No differences between the diet groups were observed in cerebral metabolites (data not shown).

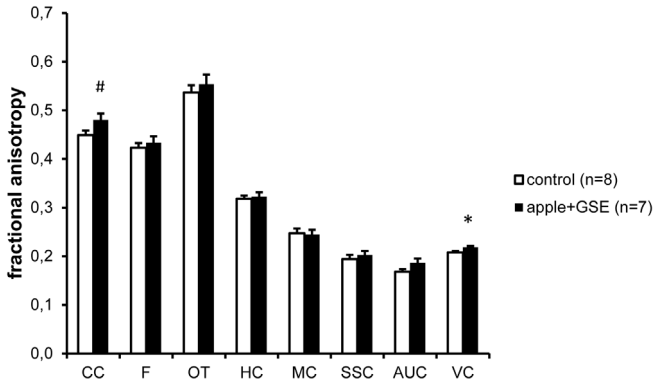


Figure 10: Diffusion coherence in young adult mice. Apple+GSE fed young adult mice displayed increased diffusion anisotropy in the visual cortex and a tendency to increased activity in the corpus callosum. *p≤0.05 and #p≤0.07.

DTI

Grey and white matter diffusion coherence was measured with DTI. The apple+GSE groups displayed a significant increased diffusion anisotropy in the visual cortex (p=0.014) and a trend in the corpus callosum (p=0.066; Figure 10). No differences between the diet groups were observed in mean diffusivity in all selected ROIs.

Biochemistry

qRT-PCR

After finalizing cognitive and motor function tests and imaging, brains of the mice were collected for biochemical analysis after perfusion with 0.1M PBS. We measured inflammatory markers, synaptic plasticity, and angiogenesis in the subcortical region (includes hippocampus) of the right hemispheres with qRT-PCR in pubertal and young adult mouse brains.

Synaptic plasticity did not change after apple+GSE supplementation, nor did angiogenesis. IL-1β expression showed a tendency for a decrease in the apple+GSE fed mice at both pubertal (p=0.067) and young adult age (p=0.065; Figure 11. Expression of the other inflammatory markers (CD36, IL-6, MCP-1, and TNF-α) did not show any differences.

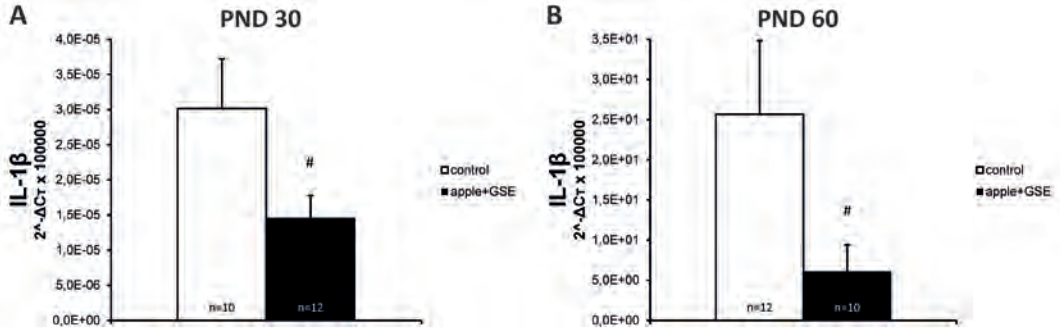


Figure 11: Neuroinflammation. Both pubertal **(A)** and young adult **(B)** apple+GSE fed mice showed a tendency for decreased IL-1β gene expression. #p≤0.07.

ELISA

BDNF concentrations were measured with ELISA in the left hemispheres of the pubertal and young adult mouse brains. BDNF concentration was not affected by apple+GSE supplementation in neither pubertal nor young adult mice (Figure 12). However, BDNF levels increased in adulthood compared to puberty in both control fed mice (p=0.000) and apple+GSE fed mice (p=0.002).

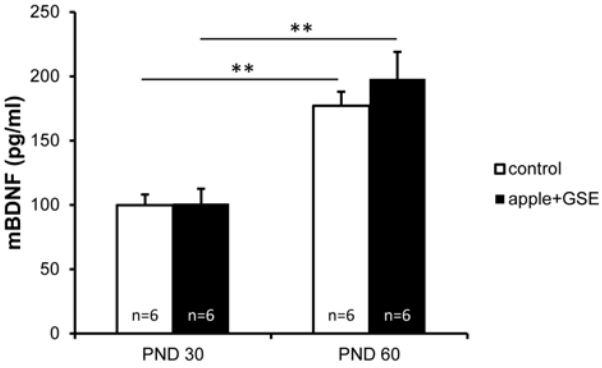


Figure 12: BDNF concentration. Mature BDNF levels were measured and demonstrated no diet effect of apple+GSE supplementation. Measurement do show an aging effect with significantly increased mBDNF concentration when comparing young adult mice to their pubertal littermates in both diets. **p≤0.01.

Immunohistochemistry

Brain tissue of young adult mice was stained for immature neurons (measure for neurogenesis) with doublecortin, postsynaptic density (measure for synaptic plasticity) with PSD95, and glucose transporter 1 (measure for capillary density) with GLUT-1. No differences in any of the parameters were found (data not shown).

Discussion

Overall, dietary flavanol supplementation in early life led to increased locomotor activity and a tendency for decreased neuroinflammation during puberty. In adulthood, we demonstrated increased grooming behavior, altered spatial learning and increased white matter integrity. Furthermore, CBF was decreased and we found a tendency for decreased neuroinflammation during adulthood. See Table 4 for an overview of the main results of the present study.

Both pubertal and young adult apple+GSE fed mice displayed increased locomotor activity in the Phenotypers. Barros *et al*. have shown that anthocyanins, a different class of flavonoids, also have the ability to increase locomotion (Barros *et al*., 2006). It has been suggested that locomotion was increased due to improved motor function (Barros *et al*., 2006; Joseph *et al*., 1999; Joseph *et al*., 1998). Our Rotarod data do not support this theory though, as we did not find an effect of flavanol supplementation on motor function.

Our PPI results, both in adult control and apple+GSE fed mice, were comparable to other studies from literature (Fedorova *et al*., 2009a; Foldi *et al*., 2011; Matsuo *et al*., 2010). While the pubertal mice showed a habituation response to the 120 dB pulses, the young adult mice failed to do so. This is in contrast to other studies showing a habituation response in adulthood (Fedorova *et al*., 2009a; Janssen *et al*., 2015). An explanation might be that the habituation response is decreased due to hearing loss. C57BL/6J mice are prone to early onset hearing loss, but not starting at such an early age (Johnson *et al*., 1997; Yu *et al*., 2011). This makes it unlikely that hearing loss caused the absence of the habituation effect. Similarly, impairment of prepulse inhibition has been observed in schizophrenia models (Dashti *et al*., 2013; Singer *et al*., 2013). However, the C57BL/6J mouse model is not known to be prone for schizophrenia. It has been reported that spatial memory in schizophrenia models was also impaired, which is also not the case in the present study (Singer *et al*., 2013). Therefore, our finding may need further research.

Spatial learning and memory may be affected by flavanol supplementation. Other studies on models for ageing have demonstrated that flavanols can improve both spatial learning and memory (Rendeiro *et al*., 2009; Rendeiro *et al*., 2013; Rodrigues *et al*., 2013; Sasaki *et al*., 2013; Zeng *et al*., 2012). In our study, all young adult mice showed a learning effect in the MWM, but the learning curve of the apple+GSE fed animals differed from the control diet. The apple+GSE fed mice showed a higher escape latency on day 1, and a learning effect from day 1 to day 2 (as in control), and also from day 2 to day 3 finishing with the same escape latency on day 4 as the control group. Therefore, the apple+GSE fed animal showed a different learning curve

Table 4: Summary of main results.

Effect of Apple+GSE diet		PND 30	PND 60
Phenotyper	Locomotor activity	Increased 24h activity (p=0.041)	NS
Open field	Behavior and activity	NS	Increased grooming (p=0.033)
Rotarod	Motor coordination	NS	NS
Prepulse inhibition	Sensorimotor integration	NS	NS
	Habituation	NS	NS
Morris water maze	Learning and memory	n.a.	Altered spatial learning (p=0.039)
ASL	Cerebral blood flow	n.a.	Decreased CBF: cortex (p=0.001) and thalamus (p=0.009)
1H MRS	Cerebral metabolites	n.a.	NS
DTI	Grey and white matter integrity	n.a.	Increased diffusion anisotropy visual cortex (p=0.014); tendency increased diffusion anisotropy corpus callosum (p=0.066)
Immature neurons	Hippocampus	n.a.	NS
Synaptic plasticity	Hipppcampus	n.a.	NS
	Subcortical region	NS	NS
Inflammatory markers	Hippocampus	n.a.	NS
	Subcortical region	Tendency increased IL-1β (p=0.067)	Tendency decreased IL-1β (p=0.065)
Vascular density	Hippocampus	n.a.	NS
	Subcortical region	NS	NS
BDNF	Subcortical region	NS	NS

than controls but both groups reached optimal performance by day 4. In the probe phase of the MWM, we did not find differences between the groups. Thus, while flavanols might contribute to spatial learning, they do not influence spatial memory.

The young adult mice did not display differences in cerebral metabolism, as measured with ^{1}H MRS in the hippocampus. This indicates that neuronal function and inflammation are not affected by flavanol supplementation in this young healthy mouse model. NAA, for example, functions as a neuronal marker as it is produced by healthy neurons (Govindaraju *et al*., 2000). We did not find changes in NAA concentration indicating that neural function was not affected by flavanol supplementation. Doublecortin (a marker for neurogenesis) and BDNF (a neurotrophin) concentrations also showed no effect of flavanol supplementation. MI acts as a glial marker and can be used as a marker for neuroinflammation (Govindaraju *et al*., 2000). We did not find changes in mI concentration with ^{1}H MRS, suggesting that neuroinflammation remained unaffected after flavanol supplementation. This is supported by our data collected with qRT-PCR for expression of inflammatory markers and IHC staining for Iba1 which both did not show significant effects on neuroinflammation.

Surprisingly, apple+GSE fed animals demonstrated decreased CBF in the cortex and thalamus, but not the hippocampus. There is little supporting literature for this effect, since most studies describe increased CBF after flavanol supplementation (Francis *et al*., 2006; Nehlig, 2013; Shah *et al*., 2010; Sorond *et al*., 2008). Quercetin, for example, has shown to not only protect

against microvascular damage, but also against white matter damage in a hypoperfusion rat model (Takizawa *et al*., 2003). While most studies use a specific flavanol component, we used a mixture of flavanols which may a reason for the discrepancy in results. Possibly, there is an interaction between components that is responsible for our findings. Furthermore, there are studies indicating that flavanols may inhibit angiogenesis (Tian *et al*., 2014; Zhao *et al*., 2013). We hypothesized that decreased CBF in an optimal (young healthy mice) state would be accompanied by an increase in GLUT-1 expression to assure a sufficient glucose transport across the blood-brain barrier. Furthermore, VEGF expression would be decreased due to reduced angiogenesis. However, we did not find an effect of flavanol supplementation on VEGF expression, indicating that angiogenesis remained unaffected in adulthood. Additionally, the GLUT-1 staining was used as a measure for capillary density, but also did not reveal differences in GLUT-1 expression, suggesting that capillary density also remained unaffected. It has also been suggested that CBF is regulated by glutamate (Attwell *et al*., 2010). 1H MRS measurements did not display changes in glutamate concentration and therefore do also not explain the decrease found in CBF. Therefore, an explanation to our results may be that alterations in blood pressure occur, ultimately leading to a decrease in CBF. Flavanols are mainly known to have blood pressure lowering properties, but epigallocatechine gallate (EGCG) for example demonstrated both vasorelaxant (Alvarez *et al*., 2006; Chen *et al*., 2000; Lorenz *et al*., 2009) and vasoconstrictive properties (Sanae *et al*., 2002; Shen *et al*., 2003) in *ex vivo* studies. This effect appears to be dose-dependent, as the vasoconstrictive properties were only apparent at low doses (Shen *et al*., 2003). Wightman *et al*. also found a reduced CBF in healthy volunteers after a single dose of 135 mg EGCG (Wightman *et al*., 2012). This low dose of EGCG resulted in decreased CBF in the frontal cortex during cognitive task performance (Wightman *et al*., 2012). Further research is necessary to explore these dose-dependent effects and underlying mechanisms.

In the apple+GSE fed animals we demonstrated a trend for increased diffusion anisotropy in the corpus callosum as measured with DTI. Increased white matter fiber coherence could lead to improved cognition and motor function, but currently we only observed mild changes in spatial learning in the MWM of the apple+GSE fed mice. Furthermore, we found increased FA in the visual cortex. However, we did not find any changes in synaptic plasticity in the visual cortex as measured in the PSD95 staining. Mean diffusivity, as a measure for grey matter integrity, remained unaffected by flavanol supplementation, a finding that corresponds to our immunohistochemical data that show no effect on neurogenesis, synaptic plasticity, and capillary density. We would expect that the changes found in spatial learning, would be reflected by structural changes in the hippocampus.

The brains of young adult mice were stained immunohistochemically for neurogenesis, synaptic plasticity, and capillary density. There were no differences observed in neurogenesis and capillary density after flavanol supplementation. We neither found differences in synaptic plasticity in the other regions of the dentate gyrus, nor in the visual and sensory cortex. Flavanols have been shown to support synaptic plasticity during ageing, which leads us to hypothesize that while flavanols do not have an impact during development, they show minor indications of a protective effect during ageing (Gomez-Pinilla and Nguyen, 2012; Hu *et al*., 2008; Rendeiro *et al*., 2009; Zeng *et al*., 2012). These results also support the findings in the behavioral tests, that flavanols do not have a major effect on cognition or motor function.

Last, we performed qRT-PCR to measure inflammatory markers and synaptic plasticity in the brains of young adult mice. We found a trend for decreased IL-1β gene expression in apple+GSE. All other inflammatory markers (CD36, IL-6, MCP-1, and TNF-α) did not show differences between the diet groups. It should be noted that IL-6, TNF-α, and IL-1β were barely detectable in all dietary groups, indicating that young C57BL/6J mice do not experience inflammation. Taken together, these data suggest that flavanols only have a minor influence on inflammatory processes in a healthy young mouse model. Studies on ageing (AD) and flavanols have shown anti-inflammatory

effects of flavanols (Esposito *et al*., 2014; Lee *et al*., 2013b; Nishizawa *et al*., 2011; Vauzour, 2013). The qRT-PCR for synaptophysin expression did not demonstrate changes in synaptic plasticity, supporting our findings in the PSD95 staining that flavanols do not affect synaptic plasticity during neurodevelopment.

Several studies have shown that flavanols are metabolized by the body after administration (de Boer *et al*., 2005; Faria *et al*., 2014). For this reason, it is important to evaluate the biodistribution of flavanols and its metabolites in both dams and offspring. In the current study, we are not able to distinguish if any of the found effects can be accounted to flavanols or to their metabolites. However, we have shown that both methylated and non-methylated metabolites of catechin and epicatechin were detectable at postnatal day 14, in brains and plasma of both dams and pups fed the same dietary supplements (unpublished results). These data suggest that flavonoids undergo biodegradation and that their metabolites may be absorbed in brain and plasma. The formation of these metabolites will most likely depend on mouse microbiota composition which may still had been in development in early life, but that has not been determined in the current study. It is known from studies in humans that microbiota composition has a high inter-individual heterogeneity, thus microbiota-mediated polyphenol conversion may differ among individuals and related health effects (Bolca *et al*., 2013; van Duynhoven *et al*., 2011).

Furthermore, the dose of flavanols also appears to be very important. As mentioned before, low doses may exert opposite effects compared to high doses of flavanols (Lorenz *et al*., 2009; Shen *et al*., 2003). We should also keep in mind that supplementation of a mixture of flavanols as used in this study may act differently than a single component.

All in all, the present study demonstrates that supplementation with dietary flavanols early in life may exert mild effects on activity, spatial learning, cerebral blood flow, and brain structural integrity during neural development. The changes observed appear to be very subtle in the healthy mouse model used. Comparison of these data to published findings in aged or neurodegenerating animals suggests that benefits of dietary flavanols may increase with advancing age and might be more pronounced in a challenged animal model.

Acknowledgements

The authors would like to thank Professor Mario Ferruzzi for the analysis of dietary flavanols. We would like to acknowledge Karin de Haas-Cremers, Henk Arnts, Bianca Lemmers-van de Weem, Kitty Lemmens-Hermans, Iris Lamers and the Central Animal Laboratory of the Radboud university medical center for taking excellent care of our mice. Furthermore, we would like to thank Jos Dederen and Bram Geenen for their laboratory work, dr. Judith Homberg for the use of her behavioral equipment, and Andor Veltien for help with the MR equipment (supported by NOW, VISTA project). This work was sponsored by Mead Johnson Pediatric Nutrition Institute.

Chapter 5

The Effect of a High-Fat Diet on Brain Plasticity, Inflammation and Cognition in ApoE4-knockin and ApoE-knockout Mice

Carola IF Janssen[1], Diane Jansen[1], Martina PC Mutsaers[1], Pieter JWC Dederen[1], Bram Geenen[1], Monique T Mulder[2], Amanda J Kiliaan[1]

[1] Department of Anatomy, Donders Institute for Brain, Cognition, and Behaviour, Radboud university medical center, Nijmegen, The Netherlands
[2] Department of Internal Medicine, Laboratory of Vascular Medicine, Erasmus University Medical Center, Rotterdam, The Netherlands

Submitted

Abstract

Apolipoprotein E4 (ApoE), one of three common isoforms of ApoE, is a major risk factor for late-ons*et al*zheimer disease (AD). ApoE-deficient mice, as well as mice expressing human ApoE4, display impaired learning and memory functions and signs of neurodegeneration. Moreover, ApoE protects against high-fat (HF) diet induced neurodegeneration by its role in the maintenance of the integrity of the blood-brain barrier. The influence of a HF diet on the progression of AD-like cognitive and neuropathological changes was assessed in wild-type (WT), human ApoE4 and ApoE-knockout ($ApoE^{-/-}$) mice to evaluate the modulatory role of ApoE in this process. From 12 months of age, female WT, ApoE4, and $ApoE^{-/-}$ mice were fed either a standard or a HF diet (19% butter, 0.5% cholate, 1.25% cholesterol) throughout life. At 15 months of age mice performed the novel object recognition task and the Morris water maze, evaluating short-term memory, and spatial learning and memory respectively. No significant differences were found in short term memory. $ApoE^{-/-}$ showed increased spatial learning compared to WT mice ($p=0.009$). HF diet improved spatial learning in WT mice ($p=0.045$), but did not affect ApoE4 and $ApoE^{-/-}$ mice. Immunohistochemical analyses of the hippocampus demonstrated increased neuroinflammation (CD68) in the cornu ammonis 1 (CA1) region in ApoE4 ($p=0.001$) and in $ApoE^{-/-}$ ($p=0.032$) mice on standard diet. HF diet tended to increase CD68 in the CA1 in WT mice ($p=0.052$), while it decreased in ApoE4 ($p=0.009$), but ApoE-/- remained unaffected. A trend towards increased neurogenesis (DCX) was found in both ApoE4 ($p=0.052$) and $ApoE^{-/-}$ mice ($p=0.068$). In conclusion, these data suggest that HF intake induces different effects in WT mice compared to ApoE4 and $ApoE^{-/-}$ with respect to markers for cognition and neurodegeneration. We propose that HF intake inhibits the compensatory mechanisms of neuroinflammation and neurogenesis in aged female ApoE4 and $ApoE^{-/-}$ mice.

Introduction

Apolipoprotein E4 (ApoE4) is a major genetic risk factor for Alzheimer disease (AD). Carriers of ApoE4 have a much higher prevalence and earlier age of onset of Alzheimer disease (AD) than non-carriers, There are 3 common APOE isoforms: ApoE2, ApoE4 and ApoE3 which is the most prevalent (Knouff *et al*., 1999; Stojakovic *et al*., 2004). While ApoE4 seems to stimulate AD pathogenesis, ApoeE2 appears to protect against AD pathology (Kim *et al*., 2009). Besides being a risk factor for AD, ApoE4 is also associated with an increased risk of cardiovascular disease. Because of its relatively high affinity for very low density lipoproteins, it leads to a more pro-atherogenic lipoprotein profile compared to ApoE3 (Mahley *et al*., 2006; Spinney, 2014; Tanzi and Bertram, 2001). Cardiovascular disease, type 2 diabetes mellitus, hypertension and a high fat intake at middle age all have been identified as risk factors for cerebrovascular disease including AD. All of these factors can be aggravated by a sedentary lifestyle and a high-fat intake (de la Torre, 2004; Grammas *et al*., 2002; Kalaria *et al*., 2012; Skoog and Gustafson, 2006). Atherosclerosis similar to hypertension, is a process that precedes dementia symptoms by many years. Both hypertension and atherosclerosis cause impairments in blood flow and in blood brain barrier function, as do hypoperfusion and blood vessel wall pathology which may initiate the underlying neurodegenerative processes leading to cognitive impairment and ultimately AD (de la Torre, 2012; Grammas *et al*., 2002; Kalaria *et al*., 2012; Muller *et al*., 2007; Skoog and Gustafson, 2006). A decline in regional cerebral blood flow (rCBF) over time in human nondemented ApoE4 carriers compared to noncarriers was shown (Thambisetty *et al*.). Another study showed that decreased CBF in patients with metabolic syndrome, a collection of cardiovascular risk factors including high triglyceride and low HDL cholesterol levels is associated with impaired cognition (Birdsill *et al*., 2013). Additionally, Zerbi *et al*. have shown that ApoE4 and ApoE knockout mouse models display reduced CBF (Zerbi *et al*., 2014b).

ApoE is expressed in most tissues in the body, with the brain producing the second highest amount after the liver, predominantly in astrocytes [8]. As in the rest of the body, in the brain ApoE plays a critical role in cholesterol transport, during development, synaptic remodelling, regeneration after injury and inflammatory responses. *In vitro* studies have also demonstrated that ApoE4 leads to a reduced cholesterol synthesis by astrocytes and neurons in comparison with ApoE3 (Jansen *et al*., 2009; Michikawa *et al*., 2000; Rapp *et al*., 2006). ApoE4 may therefore contribute to the development of AD in various manners, including modulation of cerebral lipid homeostasis, vascular function and cerebral blood flow. However, it was indicated that sex differences occur in neurodegeneration and cardiovascular disease (Levi and Michaelson, 2007; Rijpma *et al*., 2013). Therefore, the focus of the current study will be on the effect of a high-fat diet in female mice.

ApoE-deficient and human ApoE4-knockin mice have shown cognitive impairment and vascular changes (Knouff *et al*., 1999). ApoE-deficient mice and also, to a lesser extent, human ApoE4 knock-in mice display a pro-atherogenic lipoprotein profile leading to atherosclerosis. When fed a high fat diet, ApoE-deficient mice display signs of a dysfunctional blood-brain barrier and neurodegeneration. (Jansen *et al*., 2013b; Libby *et al*., 2013; Piedrahita *et al*., 1992). In the present study, we tested the hypothesis that female ApoE4 and ApoE-deficient mice are vulnerable for high-fat diet induced neurodegeneration and cognitive impairment. Clarity in underlying mechanisms may support the development of a dietary approach in the prevention of AD, in particularcarriers of ApoE4.

Material and methods

Animals and diets

Female ApoE4-knockin mice were obtained from Taconic Transgenic Models (Hudson, NY, USA). These mice were created by targeting the murine APOE gene for replacement with the human APOE4 alleles (4/4) in 129P2/OlaHsd-derived E14TG2a ES cells and injecting the targeted cells

into blastocysts. Resultant chimeras were backcrossed to C57BL/6J for 8 generations. The line was derived by embryo transfer and is maintained by incrossing homozygous mice.
ApoE deficient female mice (B6,129P2-Apoetm1Unc/J) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The mice were created by targeting the APOE gene in 129P2/OlaHsd-derived E14TG2a ES cells and injecting the targeted cells into blastocysts. Resultant chimeras were backcrossed to C57BL/6J for 11 generations. This line was derived by embryo transfer and is maintained by incrossing homozygous mice (Piedrahita *et al*., 1992).

The C57BL/6J wild-type female mice, used as control mice in the present study, were the non-transgenic wild-type littermates of a colony of AβPPswe-PS1dE9 mice originally obtained from Johns Hopkins University (Bar Harbor, ME, USA) and subsequently established in our lab at Radboud university medical center, Nijmegen, the Netherlands (Jankowsky *et al*., 2004; Jankowsky *et al*., 2001). At 12 months of age, the female mice were randomly assigned to either a standard rodent chow diet (3.3% fat, ssniff Spezialdiäten GmbH, Soest, Germany: CTRL), or a high fat cholesterol enhanced diet (19% butter, 0.5% cholate, 1.25% cholesterol: HF) (Mulder *et al*., 2001) and fed for the remainder of the experiments. Starting at 15 months of age, the mice underwent cognitive tasks. Afterwards, they were sacrificed by transcardial perfusion with 0.1M phosphate buffered saline (PBS) and brains were collected for immunohistochemical purposes. Mice were weighed at the start of the cognitive task and on the day that they were sacrificed.

Throughout the experiments, animals were housed in groups of 6-8 per cage in a controlled environment at the central animal facility of the Radboud university medical center. Room temperature was controlled at 21 °C, with an artificial 12:12h light:dark cycle (lights on at 7:00 a.m.), continuous music was playing in the background during the light period, and cage enrichment consisted of a plastic shelter and cotton nesting material. Food and water were available ad libitum. The experiments were performed according to Dutch federal regulations for animal protection and were ethically approved by the Veterinary Authority of the Radboud university medical center. In total, 5 ApoE$^{-/-}$ (3 on CTRL and 2 on HF diet) died during the experiment from undisclosed causes. The experiments were performed according to Dutch federal regulations for animal protection and were ethically approved by the Veterinary Authority of the Radboud university medical center (Permit Number: RU-DEC 2009-182). All efforts were made to minimize suffering of the animals.

Cognitive tasks

Novel object recognition task (NORT)

The NORT was performed to assess short term memory. The apparatus consisted of a square arena (45 cm × 45 cm × 30 cm) with transparent Plexiglas walls. The different objects were (1) a black aluminium cylinder with a roughened surface (diameter 7 cm, height 10 cm), (2) a dark transparent glass cube (5 cm × 5 cm × 5.5 cm), (3) a ceramic egg (diameter 4 cm, height 6 cm), and (4) a set-up of 3 Duplo™ blocks stacked as a pyramid (6.32 cm × 6.32 cm × 4.52 cm). The mice were not able to move any of the objects. The order of objects used per subject per session was determined using a randomization scheme.

In the habituation phase, the mouse was allowed to explore the empty arena in 4 min trials, twice a day, for two days. On the third day, the NORT started. The mouse was presented two similar objects in the first trial (T1) and was allowed to explore for 4 min. Subsequently, the mouse was returned to the home cage until the second trial. After a predetermined delay interval, the mouse was returned to the arena for a second 4 min trial (T2). Now the arena contained two dissimilar objects: the familiar object and a new object. The experimental phase was performed on 4 consecutive days, each day with a different predetermined delay interval of 0.5h, 1h, 2h, and 6h. The time spent exploring the objects was recorded manually. Sitting on an object was not considered as exploratory behavior. The discrimination index (d2) in T2 was calculated as a measure for object memory:

$$d2 = \frac{(\text{exploration time of novel object in T2}) - (\text{exploration time of familiar object in T2})}{T2}$$

Morris water maze (MWM)

The MWM was used to assess spatial learning and memory. The mouse was placed at different starting positions in a circular pool (diameter 104 cm) that was filled with water (21-22 °C, made opaque by adding milk powder). The mouse was trained to find the platform (diameter 8 cm) which was submerged 1 cm below the water surface and located in the north-east quadrant of the pool by using distant visual cues. The visual cues were present on the four walls surrounding the pool at a distance of 0.5 m. During all trials, the observer was present in the room and always located at the same position (behind a curtain surrounding the set-up).

Acquisition phase

The mouse performed 4 acquisition trials (maximal swimming time 120 s; 30 s on platform; inter-trial interval 60 min) per day during 4 consecutive days. Starting positions were south, north, east, and west. All trials were recorded and latency to find the platform (s) was used as a measure for spatial learning.

Probe phase

The mouse performed a single probe trial at the end of day 4 of acquisition, in which the platform was removed from the pool. The mouse was allowed to swim freely for 120 s and trials were recorded and analyzed at 30 and 120 s with EthoVision XT 7.0 (Noldus, Wageningen, the Netherlands). The time spent searching in the target quadrant (north-east) and the number of platform crossings were used as a measure for spatial memory.

Immunohistochemistry

Brains were collected for immunohistochemical analysis. Mice were transcardially perfused with 0.1M PBS and subsequently, brains were collected and cut midsagitally. The left hemisphere was used for immunohistochemistry and the right hemisphere for biochemistry. The left hemisphere was post-fixed overnight in 4% paraformaldehyde at 4 °C and thereafter stored in 0.1M PBS with 0.01% sodiumazide at 4 °C. After cryoprotection with 30% sucrose, the hemisphere was cut in coronal sections with a sliding microtome (Microm HM 440, Walldorf, Germany) equipped with an object table for freeze sectioning at -60 °C gaining 6 parallel series of 40 µm thick sections (240 µm distance between the sections). The sections were used to visualize and quantify immature neurons (measure for neurogenesis) with antibodies against doublecortin (DCX), to determine postsynaptic density (measure for synaptic plasticity) with antibodies against postsynaptic density protein 95 (PSD95), glucose transporters (measure for vascular density) with antibodies against glucose transporter 1 (GLUT-1), and microglia (measure for inflammation) with antibodies against cluster of differentiation 68 (CD68).

Immunohistochemistry was performed using standard free-floating labeling procedures, using the following protocol. Sections were incubated overnight with the primary antibody on a shaker table at room temperature (RT). After this incubation, sections were rinsed three times with 0.1M PBS and afterwards incubated with the secondary antibody. After 90 minutes, sections were rinsed three times again and transferred to 0.1M PBS containing Vector ABC-elite (1:800; Vector Laboratories Inc., Burlingame, CA, USA) for another 90 minutes. After rinsing three times, the sections were incubated with diaminobenzidine-nickel (DAB-Ni) solution to visualize the marker. Last, stained sections were mounted on gelatin-coated glass slides, dried overnight in a stove at 37 °C, dehydrated in alcohol series, cleared with xylol and mounted in Entellan.

For DCX, polyclonal goat anti-doublecortin (1:3,000; sc-8066; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used as a primary antibody to assess neurogenesis. The secondary antibody was donkey anti-goat biotin (1:1,500; Jackson ImmunoResearch, West Grove, PA, USA). In the PSD95, polyclonal rabbit anti-PSD95 (1:2,000; ab18258; Abcam Inc., Cambridge, UK) as a

primary antibody to visualize the postsynaptic density. The secondary used was donkey anti-rabbit biotin (1:1,500; Jackson ImmunoResearch, West Grove, PA, USA). For GLUT-1, polyclonal rabbit anti-GLUT-1 (1:20,000; AB1340; Chemicon International Inc., Temecula, CA, USA) was used as a primary antibody and donkey anti-rabbit biotin (1:1,500; Jackson ImmunoResearch, West Grove, PA, USA) was used as a secondary antibody. In the CD68, polyclonal rat anti-CD68 (1:15,000; ab53444; Abcam Inc., Cambridge, UK) as a primary antibody to visualize the postsynaptic density. The secondary used was donkey anti-rat biotin (1:1,500; Jackson ImmunoResearch, West Grove, PA, USA).

Quantification doublecortin

DCX positive cells were quantified in three succeeding sections of the hippocampus (-1.70, -2.18 mm, and -2.46 mm posterior to bregma) for each mouse (Franklin and Paxinos, 2008). Contours were drawn along the borders of the hippocampus at 2.5× magnification using the program Stereo Investigator (Microbrightfield, Williston, VT, USA). The DCX positive cells in the subgranular zone of the hippocampus were manually counted at 40× magnification and the values of the three succeeding sections were averaged to obtain a single value for each animal.

Quantification PSD95

The relevant regions in the hippocampus were digitized using Stereo Investigator. Contours of the visual and sensory cortex and the inner molecular layer (IML), outer molecular layer (OML), stratum radiatum (SR), and stratum lucidum (SL) of the hippocampus (-2.18 mm up to -2.46 mm posterior to bregma) were drawn at 2.5× magnification (Franklin and Paxinos, 2008). Per region, 2 photographs were taken at 100× magnification. The quantification of the staining was performed using the program Image J (National Institute of Health, Bethesda, MD, USA). Images were converted to 8-bit gray scale, followed by conversion to 16-bit gray scale, next the contrast was enhanced and the amount of tissue stained was measured with a threshold-based approach. We did not set limits for the particle size.

Quantification GLUT-1 and CD68

Relevant regions in the hippocampus were digitized using Stereo Investigator. Photographs of the whole hippocampus (-2.18 mm up to -2.46 mm posterior to bregma) were taken and contours of the CA1, CA3, and DG were drawn at 5x magnification (Franklin and Paxinos, 2008). The quantification of the staining was performed using the program Image J. Images were converted to 8-bit gray scale and the amount of tissue stained was measured with a threshold-based approach. Measurement of GLUT-1 and CD68 density was defined as the area covered by either GLUT-1 or CD68 immunoreactivity per mm2 of the total area of the region measured.

Statistical analyses

Data are expresses as mean ± standard error of the mean with SPSS for Windows 20.0 software (SPSS Inc., Chicago, IL ,USA). The set-up of the present study was designed to determine the effect of diets and the extent to which ApoE4 and ApoE$^{-/-}$ mice develop cognitive deficits. Therefore, the statistical analyses was performed separately for ApoE4 and ApoE$^{-/-}$ mice (ApoE4 versus WT and ApoE$^{-/-}$ versus WT).

The repeated measures ANOVA was used for the acquisition phase of the MWM. A univariate ANOVA was used for analysis of the DCX staining. The probe phase of the MWM, the ORT, and stainings for PSD95, GLUT-1 and CD68 were analyzed with a multivariate ANOVA. If the Bonferroni post hoc test indicated a significant interaction between genotype and diet (and trial day in the MWM), the data were split for the concerning factor and analyzed again. For readability reasons, F-values and degrees of freedom are not displayed in the text. Statistical outliers were removed from the dataset. Statistical significance was set at * $p \leq 0.05$ and a trend at # $0.05 < p < 0.07$.

Results

The effect of the high fat intake on body weight was assessed prior to cognitive testing when the mice were 15 months old. A high fat diet led to an increased body weight in both the ApoE4 and WT mice (Figure 1; $p<0.001$), but not in the ApoE$^{-/-}$ mice. After the cognitive testing period, body weight of the WT and ApoE$^{-/-}$ mice both on standard and on HFD remained unchanged ($p>0.05$), while body weight of the HF fed ApoE4 mice had further increased ($p=0.002$). There were no differences in brain weight immediately after brain collection ($p>0.05$, Table 1).

Table 1: Brain weights for immunohistochemistry.

Values represent mean ± SEM

	WT	ApoE4	ApoE$^{-/-}$
CTRL diet	0.49 ± 0.01	0.43 ± 0.02	0.48 ± 0.02
HF diet	0.43 ± 0.02	0.49 ± 0.01	0.44 ± 0.02

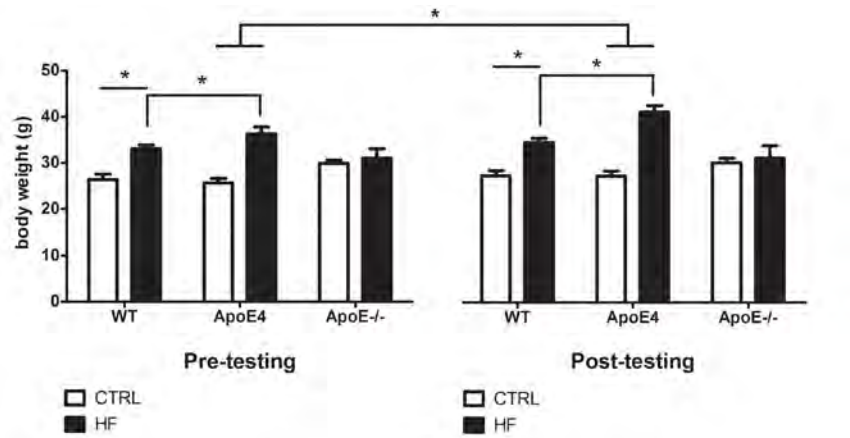


Figure 1: Body weight during the experimental phase. HF diet increased body weight pre- and post-testing in both ApoE4 and WT mice compared to the CTRL diet, but not in ApoE$^{-/-}$. Furthermore, the body weight increase due to HF diet was significantly stronger in ApoE4 compared to WT.

WT CTRL n=8, WT HF n=8, ApoE4 CTRL n=8, ApoE4 HF n=8, ApoE$^{-/-}$ CTRL n=5, ApoE$^{-/-}$ HF n=6.

*p≤0.05

Cognitive tasks

NORT

Short term memory was assessed in the NORT. No differences were observed between the 3 groups (ApoE4 compared to WT and ApoE$^{-/-}$ compared to WT) nor was there any effect of the HF diet ($p>0.05$; data not shown).

MWM

Mice were subjected to the MWM to examine spatial learning and spatial memory (Figure 2). The increased body weights in the wild-type and ApoE4 mice, caused by the HF diet, did not affect mean swimming velocity or total swimming distance ($p>0.05$). For this reason, we did not correct for body weight in the statistical analysis. In the acquisition phase, all groups displayed a learning effect (Figure 2A and B; $p<0.001$). Furthermore, HF fed WT mice displayed a decreased

escape latency compared to the animals on standard diet (p=0.045). ApoE$^{-/-}$ mice demonstrated a decreased escape latency compared to WT mice (p=0.009). We did not observe significant differences in the probe phase of the MWM at 30 and 120 s (p>0.05).

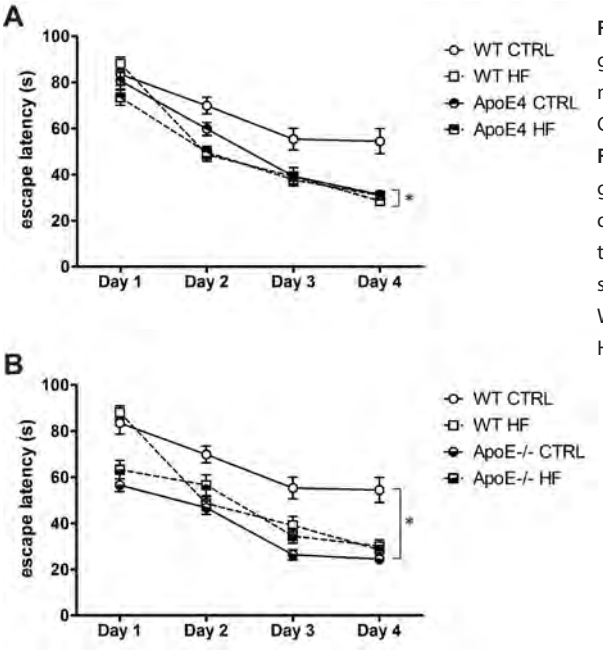


Figure 2A: MWM acquisition ApoE4 versus WT. Both groups displayed a learning effect (p=0.000). HF fed mice showed improved spatial learning compared to CTRL diet (p=0.045).

Figure 2B: MWM acquisition ApoE$^{-/-}$ versus WT. Both groups displayed a learning effect (p=0.000). ApoE$^{-/-}$ demonstrated improved spatial learning compared to WT mice (p=0.009). There was no difference in spatial learning between diets.

WT CTRL n=6, WT HF n=7, ApoE4 CTRL n=8, ApoE4 HF n=8, ApoE$^{-/-}$ CTRL n=6, ApoE$^{-/-}$ HF n=6. *p≤0.05

Immunohistochemistry

DCX

The DCX staining was used as an indicator of neurogenesis. The data suggest a genotype effect as we observed a trend for increased adult neurogenesis in ApoE4 and ApoE$^{-/-}$ mice (Figure 3; ApoE4 vs WT p=0.052, ApoE$^{-/-}$ vs WT p=0.068). No diet effects were detectable (p>0.05).

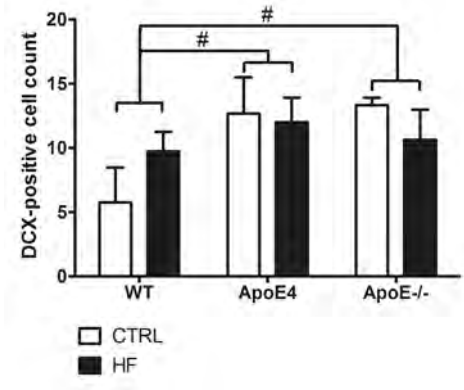


Figure 3: DCX-positive cells in the DG. ApoE4 and ApoE$^{-/-}$ mice display a trend for increased adult neurogenesis compared to WT mice (p=0.052 and p=0.068 respectively).

Values represent mean±SEM; WT CTRL n=4, WT HF n=6, ApoE4 CTRL n=4, ApoE4 HF n=7, ApoE$^{-/-}$ CTRL n=4, ApoE$^{-/-}$ HF n=7. #0.05<p<0.07

CD68

CD68 expression is used as a measure for neuroinflammation as, in the brain, it reflects the presence of microglia (Figure 5). We demonstrated a genotype × diet interaction in the CA1 (p=0.001), CA3 (p=0.024), and DG (p=0.047) when comparing ApoE4 to WT. When fed a standard diet, the ApoE4 mice showed significantly more inflammation in the CA1 than WT mice (p=0.001). We observed the same pattern in the CA3 and the DG although these results were not significant (p>0.05). A HF diet decreased CD68 immunoreactivity in the CA1 of ApoE4 mice (p=0.009). When comparing ApoE$^{-/-}$ to WT, we also demonstrated a genotype × diet interaction in the CA1 (p=0.010) and CA3 (p=0.023). ApoE$^{-/-}$ mice on standard diet showed significantly increased inflammation in the CA1 when compared to WT mice (p=0.032). The HF diet did not affect inflammation in ApoE$^{-/-}$ mice. Furthermore, significantly increased inflammation in the DG for ApoE$^{-/-}$ mice was observed (p=0.029). In contrast, HF fed WT mice displayed a trend towards an increase in inflammation in the CA1 (p=0.052).

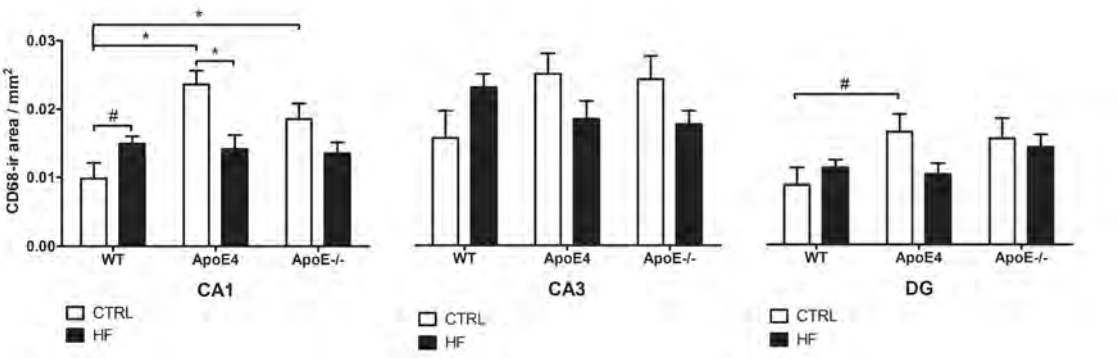


Figure 4: CD68 immunoreactivity. In the CA1, we observed a genotype × diet interaction (p=0.001), there was a trend for increased inflammation in WT after HF supplementation (p=0.052). Inflammation in ApoE4 mice significantly decreased after HF supplementation (p=0.009). ApoE4 mice on CTRL demonstrated increased inflammation compared to WT mice on CTRL (p=0.001). ApoE$^{-/-}$ compared to WT mice showed a genotype × diet interaction (p=0.010). ApoE$^{-/-}$ on CTRL demonstrated increased inflammation when compared to WT mice on CTRL (p=0.032).

In the CA3, a genotype × diet interaction was observed (p=0.024) but no significant effect on inflammation caused by genotype or diet when comparing ApoE4 to WT mice. Furthermore, ApoE$^{-/-}$ compared to WT mice showed a genotype × diet interaction (p=0.023). No significant effects on inflammation caused by genotype or diet were found though.

In the DG, we observed a genotype × diet interaction (p=0.047) when comparing ApoE4 to WT mice. There was a trend for increased inflammation in ApoE4 mice on CTRL diet compared to WT mice on CTRL (p=0.063). In addition, ApoE$^{-/-}$ displayed increased inflammation when compared to WT mice (p=0.029).

WT CTRL n=5, WT HF n=7, ApoE4 CTRL n=7, ApoE4 HF n=5, ApoE$^{-/-}$ CTRL n=4, ApoE$^{-/-}$ HF n=6. *p≤0.05, #0.05<p<0.07

PSD95

PSD95 staining was analyzed as an indicator for synaptic plasticity in the visual and sensory cortex, and the IML, OML, SR and SL of the hippocampus. No genotype or diet effects were found for synaptic plasticity (p>0.05, data not shown).

GLUT-1

GLUT-1 density is used as a measure for capillary density as it reflects the glucose transport across the BBB. We did not observe significant differences in capillary density, as determined by GLUT-1 staining, caused by either genotype or diet (p>0.05, data not shown).

Discussion

Overall, our data suggest increased signs of neurodegeneration in ApoE4 mice and in ApoE$^{-/-}$ mice in comparison with WT mice. A HF diet reduces signs of neurodegeneration in both ApoE4 mice and, to a lesser extend, in ApoE$^{-/-}$ mice, while it increases it in WT mice.

Our data show that the NORT does not reveal any differences in short term memory performance in 15 months old female ApoE4, ApoE$^{-/-}$ and WT mice. All groups displayed a spatial learning effect in the MWM task, with the ApoE$^{-/-}$ performing better than the WT mice. A beneficial effect of a HF diet on acquisition of the MWM task was observed in the WT mice, while no effect of HF diet was observed in ApoE4 and ApoE$^{-/-}$ mice.

In the current study, the ApoE$^{-/-}$ mice displayed better spatial learning in the MWM when compared to WT mice. Previously, male ApoE$^{-/-}$ have been shown to display impaired performance in the MWM in comparison with WT mice, but their performance improved under certain (stressful) conditions (Champagne *et al*., 2002; Grootendorst *et al*., 2001; Grootendorst *et al*., 2002; Oitzl *et al*., 1997). In contrast with our observations impaired performance in the MWM was observed in female ApoE-/- mice at advanced age (>14 months) (Grootendorst *et al*., 2004), but not in younger animals. Results from a study by Champagne *et al*. indicate that male ApoE$^{-/-}$ mice are not able to learn the procedural component of how to get to the platform as opposed to knowing where the platform is located (Champagne *et al*., 2002). This deficit was much larger in older (12 months old) than in younger (3 months old) ApoE$^{-/-}$ mice. These older male mice also displayed deficits in their ability of knowing where the platform is located. Yin *et al*. have shown similar age-related deficits for male ApoE4 mice (Yin *et al*., 2014). In the current study, it appears that female ApoE$^{-/-}$ are impaired in the procedural component of knowing where the platform is located. Other studies have shown spatial memory deficits in male ApoE4 mice and even more in ApoE4 female mice which have been attributed to estrogen (Bour *et al*., 2008; Raber *et al*., 1998). However, the females in our study have already reached (post-)menopausal stages where androgen levels are comparable to those of male mice (Rijpma *et al*., 2013; Suckow *et al*., 2001). In addition, Andrews-Zwilling *et al*. have shown that female ApoE4 mice display an age-dependent decrease in hilar GABAergic interneurons, correlating with the extent of ApoE4-induced learning and memory deficits in aged mice (Andrews-Zwilling *et al*., 2010). As stress was shown to affect performance in the MWM, the results may be complicated by the stress induced by the forced swimming. Additionally, differences in the temperature of the water may affect performance. Therefore, the Barnes maze may be a less stressful alternative test for ApoE$^{-/-}$ mice for assessment of spatial learning and memory.

ApoE is known for its anti-inflammatory effect. Addition of exogenous ApoE and its mimetics down-regulate activation of microglia and peripheral macrophages *in vitro* (Dorey *et al*., 2014). The absence of ApoE and also the ApoE4 isoform are therefore associated with secretion of inflammatory factors and increased neurinflammation (Dorey *et al*., 2014). ApoE4 enhances neuroinflammation either due to dysregulation of the nuclear factor-κB signaling cascade or due to an increase in cytokine levels (Dorey *et al*., 2014; Ophir *et al*., 2005). The HF diet increases the number of microglia in WT mice exclusively. Strikingly, the ApoE$^{-/-}$ animals showed increased neuroinflammation when fed standard rodent chow, but a decrease when fed a HF diet. The same was found in ApoE4 mice.

We hypothesized that the HF diet would decrease neurogenesis and synaptic plasticity in ApoE4 and ApoE$^{-/-}$ animals due to decreased levels of brain-derived neurotrophic factor (Dhungana *et al*., 2013; Molteni *et al*., 2002). However, on a control diet the ApoE$^{-/-}$ mice and also to a lesser extend in the ApoE4 mice already displayed a tendency for increased DCX expression. Increased DCX levels leads to more neurons, however we do not know if these neurons are actually surviving and/or functional. This may indicate that a repair mechanism for neurogenesis and synaptic plasticity is active in the control diet, but that this is no longer effective on HF diet. Our data are in line with results from Jansen *et al*. who have previously shown increased adult

neurogenesis in aged male ApoE$^{-/-}$ mice (Jansen *et al*., 2013b).

ApoE-containing lipoprotein particles secreted by astrocytes are supportive of synaptogenesis and maintenance of synaptic connections (Levi *et al*., 2003). Therefore, synaptic plasticity might be decreased in mice deficient for ApoE. Though, we did not find alterations in synaptic plasticity, as indicated by PSD95 staining, in both ApoE4 and ApoE$^{-/-}$ mice as compared to WT mice. This finding suggests that the proposed compensation mechanism for neurodegeneration appears to be sufficient at this age. A HF diet did not detectably affect synaptic plasticity in any of the groups. It has previously been shown that ApoE4 expression leads to reduced cerebral vascularization that is accompanied by thinner vessel walls and decreased glucose uptake (Alata *et al*., 2015). Under suboptimal conditions the number of GLUT-1 transporters may increase to ensure sufficient glucose transport. Strikingly, we found no effects in the number of GLUT-1 transporters in the hippocampus of both ApoE4 and WT mice after HF supplementation. In addition, ApoE$^{-/-}$ also did not show any differences in the number of GLUT-1 transporters compared to WT mice. In line with these findings, Alata *et al*. were also not able to show a difference in GLUT-1 expression in ApoE4 compared to WT mice (Alata *et al*., 2015).

Overall, our data suggest that HF intake induces a different effect in WT versus ApoE4 and ApoE$^{-/-}$ mice with respect to markers for neurodegeneration and cognition. We propose that HF intake inhibits the compensatory mechanisms of neuroinflammation and neurogenesis in aged female ApoE4 and ApoE$^{-/-}$ mice. Underlying mechanisms for neurogenesis, synaptic plasticity and neuroinflammation need to be elucidated in order to develop the dietary management for APOE4-carriers.

Acknowledgements

The authors would like to thank Henk Arnts and Janneke Mulders of the Central Animal Laboratory of the Radboud university medical center for taking excellent care of our mice. Furthermore, we would like to thank Liën Coolen for her laboratory support.

Chapter 6

General discussion and concluding remarks

A wide range of studies have shown that nutrients are able to affect the brain in many ways throughout life. Omega-3 polyunsaturated fatty acids (n-3 PUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been shown to have beneficial effects on cardiovascular health and brain function (Bang *et al*., 1971; Bang *et al*., 1980; Estruch *et al*., 2013; Hoevenaar-Blom *et al*., 2012; Hooijmans *et al*., 2009; Jansen *et al*., 2014). In recent years, it has become clear that the Mediterranean diet, which contains high levels of long-chain polyunsaturated fatty acids (LCPUFA) and low levels of saturated and trans-fatty acids, contributes in maintaining a healthy brain (Estruch *et al*., 2013; Feart *et al*., 2009; Gardener *et al*., 2011; Hooijmans *et al*., 2009; Jansen *et al*., 2014). This diet includes a balanced n-6/n-3 ratio, due to the fact that it is rich in fish, lean meat, and olive oils, but it also contains other important nutrients, such as vitamins, antioxidants and polyphenols, originating from fruits, vegetables, and whole grains (Feart *et al*., 2009; Gardener *et al*., 2011; Hoevenaar-Blom *et al*., 2012). This in contrast to Western diets that are rich in saturated fats, trans fats, sugar, and refined grains (Hooijmans *et al*., 2009; Jansen *et al*., 2012; Kanoski and Davidson, 2011).

In this thesis, we aimed to determine the influence of several nutrients on neural development and neurodegeneration. We studied the effect of perinatal availability of LCPUFA (chapter 3) and flavanols from fruits (chapter 4) on neural development in C57Bl/6J mice. Furthermore, we studied the effect of a cholesterol enhanced diet, mimicking a bad Western diet, on neurodegeneration in mouse models at risk for atherosclerosis and Alzheimer's disease (AD) (chapter 5).

LCPUFA

LCPUFA have been shown to act beneficial in obtaining and maintaining a healthy brain. They are important already at the start of life supporting neural development, and preventing neurodevelopmental disorders. Throughout life LCPUFA remain important for membrane fluidity, synaptic plasticity, neurogenesis, as anti-inflammatory mediators, and for cardiovascular health (Hooijmans *et al*., 2009; Jansen *et al*., 2014; O'Connor *et al*., 2001; van Goor *et al*., 2011b; Westerberg *et al*., 2011; Wurtman, 2008; Zerbi *et al*., 2014a). Supplementation studies in animals, starting early in life, show more potent influence on these mechanisms than those starting during aging or in AD mouse models (Carrié *et al*., 2000b; Fedorova and Salem, 2006; Hooijmans *et al*., 2009; Jansen *et al*., 2014; Maekawa *et al*., 2009; Niculescu *et al*., 2011). This suggests that it is important to achieve an optimal LCPUFA status early on in life and maintain this status throughout life to attain beneficial effects. In our review (**chapter 2**) we showed that LCPUFA have beneficial effects on brain health, such as increased neurogenesis and decreased neuroinflammation. A healthy and balanced diet is not only established by a sufficient intake of n-3 PUFA; the n-6/n-3 ratio is most important for an efficient conversion of n-3 and n-6 PUFA. The key message in maintaining this healthy LCPUFA status is finding the proper n-6/n-3 balance, with an optimal ratio of about 1/1 – 2/1 (Hjorth and Freund-Levi, 2012; Innis, 2008; Simopoulos, 2008b, 2011; van Goor *et al*., 2010; Wall *et al*., 2010).

In **chapter 3**, we aimed to investigate the influence of dietary n-3 PUFA availability during the development of C57BL/6J mice on behavior, cognition, cerebral metabolism, and brain plasticity. Our findings indicate that dietary intake of both α-linolenic acid (ALA) and preformed DHA during early development has a significant impact on neural and brain development as well as on cognition and behavior.

N-3 PUFA availability stimulated cognition and behavior in pubertal and young adult C57Bl/6J mice. Motor coordination increased already during puberty due to n-3 PUFA supplementation, but did not improve in young adulthood. This suggests that the n-3 PUFA adequate diet induced accelerated development of motor coordination already early in life before adulthood. During development, Purkinje cells are innervated by multiple climbing fibers, but around postnatal day (PND) 21 (juvenile age) many synapses are eliminated, leading

to mono-innervation of the Purkinje cells (Bearzatto *et al*., 2005). It has been suggested that a delay in the elimination of multiple climbing fibers innervating the Purkinje cells occurs in adult C57Bl/6J mice when compared to other mouse strains (Bearzatto *et al*., 2005). This results in poor motor performance of the C57Bl/6J mice at juvenile age (Bearzatto *et al*., 2005). The findings in our studies indicate that this possible delay in C57BL/6J might be shortened by adequate dietary n-3 PUFA availability, and a plateau in the development of motor coordination may already be reached before adulthood. In young adulthood, we observed increased sensorimotor integration, improved spatial memory, and exploratory behavior in the n-3 PUFA fed mice. Sensorimotor integration increased in young adult C57Bl/6J mice on an adequate n-3 PUFA intake as well. This was reflected in the prepulse inhibition test by improved habituation to startle stimuli and therefore may indicate an improved cortical development by adequate n-3 PUFA intake (Geyer and Dulawa, 2001). LCPUFA have been shown to ameliorate the impaired prepulse inhibition in a schizophrenia rat model (Maekawa *et al*., 2009). Schizophrenia is a condition that mostly develops during early adulthood and has been associated with LCPUFA deficiency (Levant *et al*., 2004; Moriguchi *et al*., 2001; Umezawa *et al*., 1995) and it is characterized by alterations in dopaminergic neurotransmission (McNamara and Carlson, 2006). These data suggest that LCPUFA supplementation may support dopaminergic neurotransmission processes by its ability to improve membrane fluidity (Levant *et al*., 2004). It has been reported that high levels of n-3 PUFA are associated with a negative effect on neural development by prolonging auditory brain stem conduction times and a delay in auditory startle reflex in rodents (Haubner *et al*., 2007; Saste *et al*., 1998). These studies show the importance of dosage and indirectly underline the relevance of the n-6/n-3 ratio. Several studies have stated the probable relation of learning and memory functions with brain fatty acid status (Fedorova and Salem, 2006; Moriguchi *et al*., 2001). However, results on spatial learning and memory remain somewhat inconsistent (Carrié *et al*., 2000a; Carrié *et al*., 2000b; Kavraal *et al*., 2012; Moriguchi *et al*., 2000). The main distinction between the studies appear to be the starting point of supplementation or deprivation and the dosage of the diets. This underlines the importance of choosing the right starting point for supplementation and composition of the diet. No specific anxiety tests were performed in the study described in chapter 3, but we did find increased exploratory behavior in n-3 PUFA adequate fed C57Bl/6J mice in the open field. On the contrary, no differences were found in time spent in the corners, indicating that there are no signs of anxiety in this parameter. However, other studies have shown decreased anxiety after n-3 PUFA supplementation in the open field task (Clouard *et al*., 2015; Moriguchi *et al*., 2001; Perez *et al*., 2013; Vinot *et al*., 2011).

We performed phosphorus magnetic resonance spectroscopy (^{31}P MRS) in young adult C57Bl/6J mice to study phosphorylated energy and lipid metabolites in the brain. Although we found decreased neurogenesis in the hippocampus of n-3 PUFA adequate fed mice with immunohistochemistry, no changes in membrane formation could be detected with ^{31}P MRS. Membrane turnover was reflected by phosphomonoesters (building blocks for membranes) and phosphodiesters (breakdown products of membranes) and showed no changes in our experiment. We were only able to measure whole brain metabolite status with the ^{31}P MRS method, making it difficult to detect subtle changes in specific brain regions. Measurements in separate brain regions such as hippocampus and cortex may be a more sensitive method to detect metabolite changes.

To our knowledge, we are the first to show diminished neurogenesis in young adult mice as effect of increased n-3 PUFA availability during development. Our immunohistochemical data on hippocampal neurogenesis strengthen the idea that n-3 PUFA induce a plateau in neurogenesis before adulthood. In line with these results, the n-3 PUFA adequate animals showed enhanced performance on the rotarod in puberty, but not in adulthood. At young adult age, n-3 PUFA adequate fed mice still demonstrated improved spatial memory in the Morris water maze (MWM). Our data obtained by quantitative real-time PCR (qRT-PCR) support our hypothesis, as

doublecortin (DCX) mRNA expression remains unaffected during both puberty and adulthood. Several studies have shown increased neurogenesis after LCPUFA supplementation already before pubertal age (Crupi *et al*., 2012; Maekawa *et al*., 2009; Niculescu *et al*., 2011). All together, these data suggest that n-3 PUFA may promote accelerated neurogenesis, possibly even before puberty. Additional gene expression analysis (for example cyclicAMP response element binding protein (CREB)): involved in long-term potentiation, or peroxisome proliferator-activated receptor and retinoid X receptor: involved in regulating synaptic plasticity, and learning and memory) may further support our findings.

Our results obtained via qRT-PCR indicate that synaptic plasticity may be increased in n-3 PUFA fed mice at pubertal age in the cortex, while it remains unaffected at adult age. Several studies demonstrated an increased expression of pre- and postsynaptic proteins after n-3 PUFA supplementation, hinting at increased synaptogenesis either early on in life or during aging (Cao *et al*., 2009a; Crupi *et al*., 2012). Our data on synaptophysin expression led us to hypothesize that synaptogenesis is stimulated at adolescent age on a n-3 PUFA adequate diet, and reaches a plateau before adult age.

The results in chapter 3 led us to hypothesize that perinatally administered dietary n-3 PUFA have the potential to improve cortical and hippocampal development and enhance cognitive functioning. While our study focused on dietary n-3 PUFA availability in a healthy mouse model, it already yields a range of beneficial effects in brain functioning. Our findings indicate that ensuring adequate dietary n-3 PUFA levels starting early in life may support optimal neural development in healthy full term infants. Furthermore, our results implicate that it would be interesting to study the effect of a sufficient n-3 PUFA intake in disorders caused by an n-3 PUFA deficiency more extensively. Therefore, based on our findings we would advise a balanced LCPUFA intake throughout life, starting already directly after conception. For pregnant women, this translates into the intake of three LCPUFA rich (containing fatty fish) portions a week starting in the early stages of pregnancy. This may help in preventing disorders caused by LCPUFA deficiency, such as autism spectrum disorders and ADHD. Future studies may help to specify the beneficial effects for their children throughout life in more detail.

Flavanols

The effects of flavonoid supplementation have been studied in normal aging and neurodegenerative models, but little is known about their influence during development. In neurodegeneration, flavonoids have shown to exert neuroprotective properties such as the modulation of neuroinflammation and improvement of cognition (Vauzour, 2012). It has been shown that flavonoids modulate neuronal signaling pathways involved in synaptic plasticity and neurogenesis (Spencer, 2010; Vauzour, 2012). *In vivo*, flavonoids are able to affect these pathways by activation of CREB receptors and brain derived neurotrophic factor (BDNF), mechanisms that are involved in memory acquisition and consolidation (Spencer, 2010). Flavonoids possibly affect neural development, but this has not been studied yet.

In **chapter 4**, we aimed to study the impact of flavanol availability during gestation and the postnatal period on brain structure, circulation, cognition, and metabolism in C57Bl/6J mice. Our study demonstrates that supplementation with dietary flavanols early in life may exert mild beneficial effects on locomotor activity, spatial learning, cerebral blood flow, and brain structural integrity during neural development.

We observed effects of flavanol supplementation in locomotor activity during both puberty and young adulthood in C57Bl/6J mice. The flavanol supplemented mice displayed increased locomotion, which has been explained as an effect of improved motor function (Barros *et al*., 2006; Joseph *et al*., 1999; Joseph *et al*., 1998). However, we did not observe effects of flavanol supplementation on motor coordination as measured in the Rotarod. Sensorimotor integration was not affected by flavanol supplementation at either pubertal or young adult

age. Surprisingly, we observed a decreased habituation response on both diets in the prepulse inhibition test during adulthood. Since the C57Bl/6J mice are not prone to hearing loss at young adult age (Johnson *et al*., 1997; Yu *et al*., 2011), nor to schizophrenia (Dashti *et al*., 2013; Singer *et al*., 2013), our finding needs further research to find a logical explanation. Spatial learning and memory may be affected by flavanol supplementation. However, flavanols did not influence spatial learning or memory in our experiment.

In this study, the flavanol supplemented diet did not affect cerebral metabolism in the hippocampus of young adult C57Bl/6J mice as investigated with proton magnetic resonance spectroscopy (^{1}H MRS). These results are supported by our data collected with immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and qRT-PCR. flavanol supplementation did not affect markers for neurogenesis, neurotrophin, and neuroinflammation.

Surprisingly, flavanol supplemented C57Bl/6J demonstrated decreased cerebral blood flow (CBF) in the cortex and thalamus, but not the hippocampus in adulthood. There is little supporting literature for this effect, since most studies describe increased CBF after flavanol supplementation (Francis *et al*., 2006; Nehlig, 2013; Shah *et al*., 2010; Sorond *et al*., 2008). An explanation for our results may be that alterations in blood pressure occur, ultimately leading to a decrease in CBF. Flavanols are mainly known to have blood pressure lowering properties, but epigallocatechine gallate (EGCG) for example, demonstrated both vasorelaxant (Alvarez *et al*., 2006; Chen *et al*., 2000; Lorenz *et al*., 2009) and vasoconstrictive properties (Sanae *et al*., 2002; Shen *et al*., 2003) in *ex vivo* studies. This effect appears to be dose-dependent, as the vasoconstrictive properties were only apparent at low doses (Shen *et al*., 2003). Wightman *et al*. also found a reduced CBF in healthy volunteers after a single dose of 135 mg EGCG (Wightman *et al*., 2012). This low dose of EGCG resulted in decreased CBF in the frontal cortex during cognitive task performance (Wightman *et al*., 2012). Further research is necessary to explore these dose-dependent effects and underlying mechanisms.

In the flavanol supplemented young adult mice, we demonstrated a trend for increased fractional anisotropy in the corpus callosum as measured with diffusion tensor imaging (DTI). Increased white matter integrity could lead to improved cognition and motor function, but currently we only observed mild changes in spatial learning in the MWM of the flavanol supplemented mice. Moreover, we found increased fractional anisotropy in the visual cortex. Though, we did not find any changes in synaptic plasticity in the visual cortex measured with immunohistochemistry. Mean diffusivity, as a measure for grey matter integrity, remained unaffected by flavanol supplementation, a finding that corresponds to our immunohistochemical data that showed no effect on neurogenesis, synaptic plasticity, and capillary density. We would expect that the changes found in spatial learning, would be reflected by structural changes in the hippocampus. Flavanols have been shown to support synaptic plasticity during aging, which leads us to hypothesize that while flavanols do not have an impact during development, they show minor indications of a protective effect during aging (Gomez-Pinilla and Nguyen, 2012; Hu *et al*., 2008; Rendeiro *et al*., 2009; Zeng *et al*., 2012). These results also support the findings in the behavioral tests, suggesting that flavanols do not have a major effect on cognition or motor function.

Last, our qRT-PCR data suggest that flavanols only have a minor influence on inflammatory processes in a healthy young mouse model. Studies on aging (AD) and flavanols have shown anti-inflammatory effects of flavanols (Esposito *et al*., 2014; Lee *et al*., 2013b; Nishizawa *et al*., 2011; Vauzour, 2013). Synaptic plasticity was not affected during neurodevelopment as measured with qRT-PCR and immunohistochemistry.

In chapter 4, we were not able to distinguish if any of the found effects can be accounted to flavanols or to their metabolites. Additionally, the dose of flavanols appears to be very important. As mentioned before, low doses may exert opposite effects compared to high doses

of flavanols (Lorenz *et al*., 2009; Shen *et al*., 2003). We should keep in mind that supplementation of a mixture of flavanols as used in this study may exert different effects compared to single component suplementation, due to antagonistic effects.

All in all, our study demonstrates that supplementation with dietary flavanols early in life may exert mild effects on activity, spatial learning, cerebral blood flow, and brain structural integrity during neural development. The changes observed appear to be very subtle in the healthy mouse model used. Comparison of published findings with our data suggests that benefits of dietary flavanols may increase with advancing age and might be more pronounced in a challenged animal model. Based on these findings, we would advise a sufficient intake of flavonoid containing fruits in pregnant women. Future studies may help to further specify the beneficial effects in their children throughout life.

Cholesterol & ApoE

In **chapter 5**, we hypothesized that ApoE4 and ApoE deficient mice are vulnerable to high-fat diet induced neurodegeneration and cognitive impairment. From 12 months of age, female mice (ApoE4 and ApoE$^{-/-}$) were fed either a standard diet or a high-fat diet throughout life. At 15 months of age, short-term memory and spatial learning and memory was assessed. Our experiments show that short term memory was not affected by a high-fat diet. Furthermore, all groups displayed spatial learning in the MWM. ApoE$^{-/-}$ mice performed the task better than WT mice. High-fat fed mice demonstrated improved spatial learning compared to standard diet. ApoE4 and ApoE$^{-/-}$ mice showed a tendency for increased neurogenesis, while neurogenesis remained unaffected by the high-fat diet. Synaptic plasticity and capillary density were not significantly affected by the high-fat diet. From these results, it appears that HF intake induces a different effect in WT versus ApoE4 and ApoE$^{-/-}$ mice with respect to markers for neurodegeneration, and behavior. A high-fat diet did not lead to signs of neurodegeneration, but rather appears to protect WT mice from neurodegeneration. Furhtermore, high-fat intake inhibits the observed compensatory mechanism of neuroinflammation and neurogenesis in aged female ApoE4 and ApoE$^{-/-}$ mice.

ApoE$^{-/-}$ have been shown to perform better in the MWM under certain (stressful) circumstances (Champagne *et al*., 2002; Grootendorst *et al*., 2001; Grootendorst *et al*., 2002; Oitzl *et al*., 1997). However, results from a study by Champagne *et al*. indicate that male ApoE$^{-/-}$ mice are not able to learn the procedural component of how to get to the platform as opposed to knowing where the platform is located. This deficit was much larger in older (12 months old) than in younger (3 months old) ApoE$^{-/-}$ mice. These older male mice also displayed deficits in their ability of knowing where the platform is located. Yin *et al*. have shown similar age-related deficits for male ApoE4 mice (Yin *et al*., 2014). In our experiment, female ApoE$^{-/-}$ mice displayed an impaired ability for spatial memory. They show an impairment in knowing where the platform is located. However, the females in our study have already reached (post-)menopausal stages where androgen levels are comparable to those of male mice (Rijpma *et al*., 2013; Suckow *et al*., 2001). In addition, Andrews-Zwilling *et al*. have shown that female ApoE4 mice display an age-dependent decrease in hilar GABAergic interneurons, correlating with the extent of ApoE4-induced learning and memory deficits in aged mice (Andrews-Zwilling *et al*., 2010). As stress was shown to affect performance in the MWM, the results may be complicated by the stress induced by the forced swimming. Additionally, differences in the temperature of the water may affect performance. Therefore, the Barnes maze may be a less stressful alternative test for ApoE$^{-/-}$ mice for assessment of spatial learning and memory.

CD68 expression is used as a measure for neuroinflammation as it reflects the expression of microglia in the brain. ApoE is known to exert anti-inflammatory effects. It was shown by Dorey *et al*. that addition of exogenous ApoE and ApoE derived peptides down-regulate activation of microglia and peripheral macrophages *in vitro* (Dorey *et al*., 2014). The absence of

ApoE and also the ApoE4 isoform are therefore associated with secretion of inflammatory factors and increased neurinflammation (Dorey *et al*., 2014). ApoE4 enhances neuroinflammation either via dysregulation of the nuclear factor-κB signaling cascade or caused by an increase in cytokine levels (Dorey *et al*., 2014; Ophir *et al*., 2005). The high-fat diet increases the number of microglia in WT mice exclusively. Strikingly, the ApoE$^{-/-}$ animals showed a tendency for increased neuroinflammation when fed standard rodent chow, but not when fed a high-fat diet. Thus, we hypothesize that the high-fat diet in the ApoE$^{-/-}$ model may trigger a possible compensation mechanism.

We expected that the high-fat diet would decrease neurogenesis and synaptic plasticity in ApoE4 and ApoE$^{-/-}$ animals due to decreased levels of brain-derived neurotrophic factor (Dhungana *et al*., 2013; Molteni *et al*., 2002). Contrarily, ApoE$^{-/-}$ mice and to a lesser extend ApoE4 mice already displayed a tendency for increased DCX expression on a control diet. Increased DCX levels lead to more neurons, however we do not know for sure if these neurons are actually surviving and/or functional. This may indicate that a repair mechanism for neurogenesis and synaptic plasticity is active when the mice are on control diet. Supplementation of the high fat diet challenges this repair mechanism, making it no longer effective. Jansen *et al*. have previously shown increased adult neurogenesis in aged male ApoE$^{-/-}$ mice (Jansen *et al*., 2013b). With ^{1}H MRS, it is possible to measure the concentration of NAA, a marker for healthy neurons, and this method may provide more clarity about the condition of the newly formed neurons (Jansen *et al*., 2013b). Furthermore, other MR imaging techniques such as functional connectivity and diffusion tensor imaging would give more insight into neuronal functioning.

Cholesterol is released from ApoE-containing lipoprotein particles and is used for support of synaptogenesis and maintenance of synaptic connections (Levi *et al*., 2003). Thus, we would expect that synaptic plasticity is decreased in ApoE$^{-/-}$ mice due to decreased cholesterol release. However, we did not find indications for alterations in synaptic plasticity in both ApoE4 and ApoE-/- mice. This suggests that the proposed compensation mechanism for neurodegeneration still appears to be sufficient in these aged female mice. Yet, high-fat supplementation did not affect synaptic plasticity.

Glucose transport 1 (GLUT-1) is responsible for the glucose transport across vessel walls. Hence, it is used as a marker capillary density and indication of blood vessel wall health. It was shown that ApoE4 expression leads to reduced cerebral vascularization that is accompanied by thinner vessel walls and decreased glucose uptake (Alata *et al*., 2015). We expect that in a suboptimal state (hypercholesterolemia or even neurodegeneration) the number of GLUT-1 transporters would have increased to ensure sufficient glucose transport (compensation mechanism). In contrast, the GLUT-1 staining did not demonstrate alterations in the number of GLUT-1 transporters in the hippocampus after high-fat supplementation. Additionally, ApoE$^{-/-}$ did not show any differences in the number of GLUT-1 transporters indicating that the proposed compensation mechanism was possibly no longer effective. In line with these findings, Alata *et al*. were also not able to show a difference in GLUT-1 expression in ApoE4 mice (Alata *et al*., 2015).

In short, the results of chapter 5 suggest that high-fat supplementation induces different effects in WT versus ApoE4 and ApoE$^{-/-}$ mice with respect to markers for neurodegeneration and cognition. We propose that high-fat intake inhibits the compensatory mechanisms of neuroinflammation and neurogenesis in aged female ApoE4 and ApoE$^{-/-}$ mice. Hence, we would advise to limit the intake of cholesterol and saturated fatty acids to protect from hypercholesterolemia, atherosclerosis, and subsequent neurodegeneration in APOE4 carriers. Underlying mechanisms for neurogenesis, synaptic plasticity and neuroinflammation need to be elucidated in order to develop a more detailed dietary management for APOE4 carriers.

Concluding remarks

In chapter 2, we summarized that LCPUFA have a beneficial effect on a healthy brain, wherein the n-6/n-3 ratio is essential when establishing a healthy and balanced diet. The key message in maintaining this healthy LCPUFA status is finding the proper n-6/n-3 balance, with a ratio of about 1/1 – 2/1.

In chapter 3, we postulate that perinatally administered dietary n-3 PUFA have the potential to improve cortical and hippocampal development and enhance cognitive functioning. While our study focused on dietary n-3 PUFA availability in a healthy mouse model, it already yields a range of beneficial effects in brain functioning. Overall, our findings indicate that ensuring adequate dietary n-3 PUFA levels starting early in life may support optimal neural development in healthy full term infants. Furthermore, our results implicate that it would be interesting to study the effect of a sufficient n-3 PUFA intake in n-3 PUFA deficient pregnant females and in disorders caused by underlying n-3 PUFA deficiency more extensively.

The results in chapter 4 indicate that supplementation with dietary flavanols early in life may exert mild beneficial effects on activity, spatial learning, cerebral blood flow, and brain structural integrity during neural development. The changes observed appear to be very subtle in the healthy mouse model used. Comparison of these data to published findings in aged or neurodegenerating animals suggest that benefits of dietary flavanols may increase with advancing age and might be more pronounced in a challenged animal model. Additionally, dietary flavanols may have a beneficial effect on neural development in pregnant females and infants with a deficiency.

And last, in chapter 5 we suggest that high-fat supplementation induces different effects in WT versus ApoE4 and ApoE$^{-/-}$ mice with respect to markers for neurodegeneration and cognition. We propose that high-fat intake inhibits the compensatory mechanisms of neuroinflammation and neurogenesis in aged female ApoE4 and ApoE$^{-/-}$ mice. Hence, limited intake of cholesterol and saturated fatty acids seems to protect from hypercholesterolemia, atherosclerosis, and subsequent neurodegeneration in APOE4 carriers. Underlying mechanisms for neurogenesis, synaptic plasticity and neuroinflammation need to be elucidated in order to develop a more detailed dietary management for APOE4 carriers.

Overall, in this thesis we demonstrated the influence of n-3 PUFA and flavanols on neural development. Especially n-3 PUFA were confirmed to have a beneficial effect on brain functioning starting at an early age. Flavanols have shown a potentially positive influence on brain structure and functioning, though the changes were very subtle in our healthy mouse model. Mouse models at risk for atherosclerosis and AD appear to be protected from AD-like neurodegenerative changes. High-fat intake inhibits this compensatory mechanism. On the other hand, high-fat intake provides WT mice with a compensatory mechanism for neurodegeneration and neuroinflammation.

All in all, this thesis underlines the importance of a healthy and balanced diet throughout life. We have shown that n-3 PUFA and flavanols support neural development starting during pregnancy and may even be more important in at risk pregnancies. Infants suffering from disorders caused by nutrient deficiencies may benefit from a balanced diet even more. The beneficial effects of n-3 PUFA and flavanols support a healthy brain throughout life and may protect from neurodegeneration in later life. While cholesterol is required for membrane synthesis and the formation of bile acids and steroid hormones, a high-fat diet does not support healthy aging of the brain when at risk for atherosclerosis and AD. The key message in obtaining and maintaining a healthy brain is finding a proper balance in nutrients such as n-3 PUFA, flavanols and cholesterol.

Chapter 7

Summary
Samenvatting

Summary

Nutrition plays an important role in the development and maintenance of a healthy brain. It has been shown that nutrients such as lipids and polyphenols influence the health status of the brain. Current diets are associated with large cultural differences in health. For example, a typical Western diet is characterized by a high-fat intake and increases the risk of obesity and cognitive impairment. On the contrary, a Mediterranean diet is based on fish, olive oil, fruits, nuts, vegetables and whole grains and decreases the risk of cardiovascular disease and contributes to a healthy brain. Studies suggest that nutrients supporting healthy cardiovasculature are also responsible for a healthy brain. Nutrients related to atherosclerosis have also been associated with neurodegeneration and cognitive impairment. In this thesis, we focused on the influence of n-3 PUFA and flavonoids on neural development, and the influence of cholesterol in neurodegeneration in genetic risk factors for cardiovascular impairments and Alzheimer's disease.

In **chapter 2**, we have reviewed the involvement of LCPUFA from early development onto aging of the brain. We showed that LCPUFA have beneficial effects on a healthy brain by increasing the formation of neurons and decreasing inflammation of the brain. A healthy and balanced diet is not only established by a sufficient intake of n-3 PUFA. The n-6/n-3 ratio is most important for an efficient conversion of n-3 and n-6 PUFA. The key message in maintaining this healthy LCPUFA status is finding the proper n-6/n-3 balance, with an optimal ratio of about 1/1 – 2/1.

In **chapter 3**, we aimed to obtain detailed insights into the mechanisms underlying the long-term beneficial effects of n-3 PUFA availability during gestation and throughout life in mice. A broad combination of parameters was determined to assess effects on behavior, brain structure and function. Our findings indicate that dietary intake of both ALA and preformed DHA during early development has a significant impact on neural and brain development as well as on cognition and behavior. N-3 PUFA availability stimulated cognition and behavior in pubertal (PND 30) and young adult (PND 60) C57Bl/6J mice. Motor coordination increased already during puberty due to n-3 PUFA supplementation, but did not improve in young adulthood. These results suggest that n-3 PUFA induced an acceleration in the development of motor coordination. In young adulthood, we observed increased sensorimotor integration, improved spatial memory, and signs of exploratory behavior in the n-3 PUFA fed mice. No specific anxiety tests were performed in the study described in chapter 3, but we did find increased exploratory behavior in n-3 PUFA adequate fed C57Bl/6J mice in the open field. On the contrary, no signs of anxiety were demonstrated in the open field. 31P MRS was performed in young adult C57Bl/6J mice to study energy metabolism and lipid metabolites in the brain. Although we found decreased neurogenesis in the hippocampus of n-3 PUFA adequate fed mice with immunohistochemistry, no changes in membrane formation could be detected with ^{31}P MRS. Membrane turnover was reflected by phosphomonoesters (building blocks for membranes) and phosphodiesters (breakdown products of membranes) and showed no changes in our experiment. To our knowledge, we are the first to show diminished neurogenesis in young adult mice as an effect of increased n-3 PUFA availability during development. Our immunohistochemical data on hippocampal neurogenesis strengthen the idea that n-3 PUFA induce a plateau in neurogenesis before adulthood. In line with these results, the n-3 PUFA adequate animals showed enhanced performance on the rotarod in puberty, but not in adulthood. At young adult age, n-3 PUFA adequate fed mice still demonstrated improved spatial memory in the MWM. Data obtained by qRT-PCR support our hypothesis, as DCX mRNA expression remains unaffected during both puberty and adulthood. Furthermore, these data indicate that synaptic plasticity may be increased in n-3 PUFA fed mice at pubertal age in the cortex, while it remains unaffected at adult age. This has led us to hypothesize that synaptogenesis is stimulated at adolescent age on a n-3 PUFA adequate diet,

and reaches a plateau before adult age. Based on the results in chapter 3, we postulate that perinatally administered dietary n-3 PUFA may support optimal development of the brain in healthy full term infants. Additionally, n-3 PUFA may also provide beneficial effects in disorders caused by an n-3 PUFA deficiency. We would advise a balanced LCPUFA intake throughout life. For pregnant women, this translates into the intake of three LCPUFA rich (containing fatty fish) portions a week starting in the early stages of pregnancy. This may help in preventing disorders caused by LCPUFA deficiency, such as autism spectrum disorders and ADHD.

In **chapter 4**, we evaluated the impact of flavanol availability during gestation and the postnatal period in mice to understand the mechanisms underlying potential long-term beneficial effects throughout life. The effects of flavonoid supplementation have been studied in normal aging and neurodegenerative models, but little is known about their influence during development. In neurodegeneration, flavonoids have shown to exert neuroprotective properties. A broad spectrum of parameters was investigated in order to assess effects on brain structure and function. Flavanol supplemented mice displayed increased locomotion, but motor coordination remained unaffected. The flavanol diet did not affect cerebral metabolism as measured with ^{1}H MRS. Markers for neurogenesis, neurotrophin, and neuroinflammation also showed no effect of flavanol supplementation. Surprisingly, flavanol supplemented C57Bl/6J demonstrated decreased CBF in the cortex and thalamus, but not the hippocampus in adulthood. There is little supporting literature for this effect, since most studies describe increased CBF after flavanol supplementation. An explanation to our results may be that alterations in blood pressure occur, ultimately leading to a decrease in CBF. Additionally, some flavanols, such as epigallocatechine gallate, have demonstrated both vasorelaxant and vasoconstrictive properties. Further research is necessary to explore dose-dependent effects and underlying mechanisms. We found no major structural changes in the brain as measured with DTI. In this experiment, we were not able to distinguish if any of the found effects can be accounted to flavanols or to their metabolites. We should keep in mind that supplementation of a mixture of flavanols as used in this study may act differently than a single component, due to antagonistic effects. All in all, our study demonstrates that supplementation with dietary flavanols early in life may exert mild effects on activity, spatial learning, cerebral blood flow, and brain structural integrity during neural development. The changes observed appear to be very subtle in the healthy mouse model used. Comparison of these data to published findings in aged or neurodegenerating animals suggests that benefits of dietary flavanols may increase with advancing age and might be more pronounced in a challenged animal model. Based on these findings, we would advise a sufficient intake of flavonoid containing fruits in pregnant women. Future studies may help to further specify the beneficial effects in their children throughout life.

In **chapter 5**, we hypothesized that ApoE4 and ApoE$^{-/-}$ mice are vulnerable to high-fat diet induced neurodegeneration and cognitive impairment. Our experiments show that short term memory was not affected by a high-fat diet in 15 months old female ApoE4, ApoE$^{-/-}$ and WT mice. All mice demonstrated a spatial learning effect in the MWM task, where ApoE$^{-/-}$ mice performed better than WT mice. The high-fat diet displayed a beneficial effect on spatial learning in WT mice, while no effect of the diet was observed in ApoE4 and ApoE$^{-/-}$ mice. In the current study, it appears that female ApoE$^{-/-}$ are impaired in the procedural component of knowing where the platform is located. It is not likely that these findings can be attributed to estrogen, as the female mice in our study have already reached (post-)menopausal stages where androgen levels are comparable to those of male mice. It is known that stress can affect the performance of the mice in the MWM, thus the results may be influenced by stress from forced swimming. In addition, differences in the temperature of the water may affect performance. Therefore, the Barnes maze may be a less stressful alternative test for ApoE$^{-/-}$ mice for assessment of spatial learning

and memory. Strikingly, the ApoE$^{-/-}$ animals showed increased neuroinflammation when fed standard rodent chow, but a decrease when fed a HF diet. The same was found in ApoE4 mice. We hypothesized that the HF diet would decrease neurogenesis and synaptic plasticity in ApoE4 and ApoE$^{-/-}$ animals due to decreased levels of brain-derived neurotrophic factor. However, on a control diet the ApoE$^{-/-}$ mice and to a lesser extent the ApoE4 mice already displayed a tendency for increased DCX expression. Increased DCX levels result in more neurons, but these neurons may not survive and/or be functional. This may indicate that a repair mechanism for neurogenesis and synaptic plasticity is active in the control diet, but that this effect is no longer effective on HF diet. Cholesterol is released from ApoE-containing lipoprotein particles and is used for support of synaptogenesis and maintenance of synaptic connections. From this, we expected that synaptic plasticity is decreased in ApoE$^{-/-}$ mice due to decreased cholesterol release. Indeed, we did not find alterations in synaptic plasticity in neither ApoE4 nor ApoE$^{-/-}$ mice. This suggests that the proposed compensation mechanism for neurodegeneration appears to be sufficient at this age. We expected that in a suboptimal state (hypercholesterolemia or even neurodegeneration) the number of GLUT-1 transporters would increase to ensure sufficient glucose transport (compensation mechanism). However, we found no effects in the number of GLUT-1 transporters in the hippocampus of both ApoE4 and WT mice after HF supplementation. Similarly, ApoE$^{-/-}$ did not show any differences in the number of GLUT-1 transporters compared to WT mice. In short, these data suggest that a high-fat diet induces different effects in WT mice compared to ApoE4 and ApoE$^{-/-}$ mice. Underlying mechanisms for neurogenesis, synaptic plasticity and neuroinflammation need to be elucidated in order to develop the dietary management for APOE4-carriers.

Overall, this thesis has demonstrated the influence of n-3 PUFA and flavanols on neural development. Especially n-3 PUFA were confirmed to have a beneficial effect on brain functioning starting at an early age. Flavanols have shown a potentially positive influence on brain structure and functioning, though the changes were very subtle in our healthy mouse model. We were not able to elucidate the effect of dietary cholesterol in neurodegeneration and cognitive impairment in mouse models at risk for atherosclerosis and Alzheimer's disease.

This thesis underlines the importance of a healthy and balanced diet throughout life. We have shown that n-3 PUFA and flavanols support neural development starting during pregnancy and may even be more important in at risk pregnancies. Infants suffering from disorders caused by nutrient deficiencies may benefit from a balanced diet even more. The beneficial effects of n-3 PUFA and flavanols support a healthy brain throughout life and may protect from neurodegeneration in later life. As cholesterol is required for membrane synthesis and the formation of bile acids and steroid hormones, a high-fat diet may inhibit the compensatory mechanisms of neuroinflammation and neurogenesis in aged female mice at risk for AD. The key message in obtaining and maintaining a healthy brain is finding a proper balance in nutrients such as n-3 PUFA, flavanols and cholesterol.

Samenvatting

Voeding speelt een belangrijke rol in de ontwikkeling en het onderhouden van een gezond brein. Onderzoek heeft aangetoond dat voedingsstoffen zoals vetten en polyfenolen invloed hebben op de gezondheid van de hersenen. Moderne voedingspatronen leiden tot wisselende effecten op gezondheid door grote culturele verschillen. Een voorbeeld hiervan is het Westerse dieet dat wordt gekenmerkt door een hoog-vetgehalte en het risico op obesitas en cognitieve beperkingen verhoogt. Een Mediterraan dieet daarentegen is gebaseerd op vis, olijfolie, vruchten, noten, fruit en volkoren en vermindert het risico op cardiovasculaire ziekten en draagt bij aan een gezond brein. Onderzoekers veronderstellen dat voedingsstoffen die een gezond hart- en vaatstelsel ondersteunen ook verantwoordelijk zijn voor een gezond brein. Zo zijn voedingsstoffen die atherosclerose veroorzaken ook geassocieerd met neurodegeneratie en cognitieve beperkingen. In dit proefschrift werd de invloed van omega-3 vetzuren en flavonoïden op neurale ontwikkeling, en op de invloed van cholesterol in combinatie met genetische risicofactoren voor hart- en waardeverminderingen en de ziekte van Alzheimer op neurodegeneratie bestudeerd.

In **hoofdstuk 2** werd de invloed van meervoudig onverzadigde vetzuren op de vroege ontwikkeling en op veroudering van de hersenen geëvalueerd. Literatuuronderzoek toonde aan dat meervoudig onverzadigde vetzuren gunstige effecten hebben op de gezondheid van het brein door een toegenomen vorming van neuronen en een afname in ontsteking van de hersenen. Een gezonde en evenwichtige voeding wordt ni*et al*leen bereikt door voldoende inname van omega-3 vetzuren. De verhouding tussen omega-6 en -3 vetzuren is zeer belangrijk voor een efficiënte omzetting van deze vetzuren. Het belangrijkst voor het onderhouden van een gezonde vetzuurstatus is het vinden van de juiste balans tussen omega-6 en -3 vetzuren, met een optimale verhouding van ongeveer 1/1 - 2/1.

In **hoofdstuk 3** werd getracht inzicht in de werkingsmechanismen van omega-3 vetzuren te verkrijgen. De beschikbaarheid van omega-3 vetzuren tijdens de dracht en gedurende het hele leven van muizen geven op de lange termijn positieve effecten. Een breed scala aan parameters werd gebruikt om de effecten op het gedrag, de structuur en functie van de hersenen te beoordelen. Ons onderzoek heeft aangetoond dat de inname van zowel ALA als DHA tijdens de vroege ontwikkeling een gunstige invloed heeft op neurale ontwikkeling en op cognitie en gedrag. De beschikbaarheid van omega-3 vetzuren verbeterde cognitie en gedrag in zowel puberale als jong volwassenen C57BL/6J muizen. Motorisch coördinatievermogen was tijdens de puberteit al verbeterd onder invloed van omega-3 vetzuren, maar liet geen verdere verbetering zien tijdens volwassenheid. Deze resultaten duiden erop dat voldoende voedselinname van omega-3 meervoudig onverzadigde vetzuren leidt tot een versnelde ontwikkeling van motorisch coördinatievermogen. In jong volwassen muizen werd een verhoogde sensorimotorische integratie, verbeterd ruimtelijk geheugen, en tekenen van exploratief gedrag waargenomen in de met omega-3 vetzuren gevoede muizen. Er werden geen specifieke testen voor angst uitgevoerd, er werd echter wel een toename in exploratief gedrag in met omega-3 vetzuren gevoede C57BL/6J muizen waargenomen. Met ^{31}P MRS in jong volwassen C57BL/6J muizen werden het energiemetabolisme en vetmetabolieten in de hersenen bestudeerd. Hoewel we in hoofdstuk 3 een afname van neurogenese in de hippocampus van met omega-3 vetzuren gevoede muizen aantoonden, konden met immunohistochemie geen veranderingen in de membraanvorming worden gedetecteerd. De omzet van membranen wordt weerspiegeld door phosphomonoesters (bouwstenen voor membranen) en fosfodiesters (afbraakproducten van membranen), maar toonde geen veranderingen in ons experiment. Voor zover ons bekend, zijn wij de eersten die een afname van neurogenese in jong volwassen muizen onder invloed van omega-3 vetzuren tijdens de ontwikkeling laten zien. De immunohistochemische

data betreffende neurogenese in de hippocampus versterken onze hypothese dat omega-3 vetzuren een plateaufase in neurogenese veroorzaken vóór het bereiken van volwassenheid. In overeenstemming met deze resultaten vertoonden de met omega-3 vetzuren gevoede dieren een verbetering in motorisch coördinatievermogen op de Rotarod tijdens puberteit, maar niet tijdens volwassenheid. Op jong volwassen leeftijd vertoonden de muizen een versterkt ruimtelijk geheugen onder invloed van omega-3 vetzuren. Resultaten verkregen met qRT-PCR ondersteunen deze hypothese, want de mRNA expressie van het eiwit doublecortin blijft onveranderd in zowel de puberteit als volwassenheid. Bovendien geven deze resultaten aan dat synaptische plasticiteit in de cortex kan worden verhoogd met omega-3 vetzuren tijdens de puberteit, terwijl deze niet verandert tijdens volwassenheid. Op basis van de resultaten uit hoofdstuk 3 veronderstellen we dat synaptogenese door omega-3 vetzuren wordt gestimuleerd tijdens de adolescentie en dat er een plateau wordt bereikt vóórdat volwassenheid bereikt is. Dit betekent dat perinataal toegediende omega-3 vetzuren in gezonde voldragen zuigelingen een optimale ontwikkeling van de hersenen te ondersteunen. Bovendien kunnen omega-3 vetzuren ook gunstige effecten bij aandoeningen veroorzaakt door een tekort in omega-3 vetzuren. Daarom adviseren we om gedurende het hele leven voldoende omega-3 vetzuren in het voedingspatroon op te nemen. Bij zwangere vrouwen vertaalt zich dit in de inname van drie omega-3 vetzuurrijke (met vette vis) porties per week vanaf de vroege zwangerschap. Dit kan bijdragen aan het voorkomen van aandoeningen die kunnen worden veroorzaakt door een tekort aan omega-3 vetzuren, zoals autisme spectrum stoornissen en ADHD.

In **hoofdstuk 4** hebben we het effect van de inname van flavanolen tijdens de dracht en gedurende het hele leven van muizen bestudeerd. De effecten van diëten verrijkt met flavonoïden zijn reeds onderzocht in normale veroudering en modellen voor neurodegeneratie, maar er is nog weinig bekend over hun invloed tijdens de ontwikkeling. Onderzoekers hebben aangetoond dat flavonoïden bescherming kunnen bieden tegen neurodegeneratie. Een breed spectrum van parameters werd onderzocht om de effecten op de hersenen structuur en functie te bestuderen. Een met flavanolen verrijkt dieet veroorzaakte toegenomen algehele activiteit in muizen, maar had geen effect op het motorisch coördinatievermogen. Flavanolen hadden geen invloed op het breinmetabolisme, zoals werd gemeten met ^{1}H MRS. Ook markers voor neurogenese, neurotrofine en neuroinflammatie vertoonden geen effect van flavanolen. Opmerkelijk was de waarneming dat onder invloed van flavanolen de hersendoorbloeding CBF in de cortex en thalamus daalde in volwassen muizen. De hersendoorbloeding in de hippocampus van volwassen muizen veranderde echter niet. De meeste wetenschappelijke studies beschrijven een verhoging van de hersendoorbloeding onder invloed van flavanolen. Een verklaring voor onze bevindingen kan zijn dat er veranderingen in de bloeddruk optreden die leiden tot een afname van de hersendoorbloeding. Daarnaast is voor een aantal flavanolen, zoals epigallocatechine gallaat, aangetoond dat ze zowel vaatverwijdende en vasoconstrictieve eigenschappen bezitten. Verder onderzoek is dan ook nodig om de dosis-afhankelijke effecten en de onderliggende mechanismen te bestuderen. We vonden in dit hoofdstuk geen grote structurele veranderingen in de hersenen zoals gemeten met DTI. We konden echter niet onderscheiden of de gevonden effecten verklaard kunnen worden door flavanolen of door hun metabolieten. In deze studie is er gebruik gemaakt van een mengsel van flavanolen. Mogelijk leidt dit tot andere effecten dan bij het gebruik van de individuele componenten als gevolg van antagonistische effecten. Al m*et al*, toont deze studie aan dat een dieet verrijkt met flavanolen al op vroege leeftijd milde effecten op algehele activiteit, ruimtelijk leren, hersendoorbloeding en de structuur van de hersenen uit kunnen oefenen. Onze bevindingen in een gezonde muismodel zijn echter zeer subtiel. Onderzoekers hebben reeds aangetoond dat de potentie van flavanolen groter is in dieren met neurodegeneratie. Op basis van deze studie adviseren we zwangere vrouwen om voldoende flavanoïden in het voedingspatroon op te nemen door

dagelijks voldoende fruit zoals bijvoorbeeld appels en druiven te eten. Verder onderzoek is nodig om de mogelijk gunstige effecten in kinderen gedurende het hele leven in detail te kunnen bestuderen.

In **hoofdstuk 5** werd de invloed van een hoog-vetdieet op neurodegeneratie en cognitie in ApoE4 en ApoE$^{-/-}$ bestudeerd. Onze studie toont aan dat het korte termijngeheugen niet werd beïnvloed door een hoog-vetdieet in verouderde ApoE4 en ApoE$^{-/-}$ muizen. Alle muizen vertoonden het vermogen tot ruimtelijk leren in de MWM, maar ApoE$^{-/-}$ presteerden beter in deze test dan WT muizen. Het hoog-vetdieet liet een gunstig effect in het ruimtelijk leervermogen van WT muizen zien, maar had geen effect op ApoE4 en ApoE$^{-/-}$ muizen. Uit deze studie blijkt dat de ApoE$^{-/-}$ muizen beperkt zijn in de procedurele component van de test: het weten waar het platform zich bevindt. Het lijkt niet waarschijnlijk dat dit effect veroorzaakt wordt door oestrogeen, omdat de vrouwelijke muizen in deze studie zich al in de (post-)menopauze bevinden. In deze fase zijn de androgeenniveaus vergelijkbaar met die van mannelijke muizen. Het is al eerder aangetoond dat stress de prestaties van muizen in de MWM test kan beïnvloeden. Mogelijk hebben de muizen in deze studie dan ook stress ondervonden doordat ze gedwongen hebben moeten zwemmen. Ook kunnen temperatuurverschillen van het water de prestaties beïnvloeden. Wellicht kan de zogenaamde Barnes maze een minder stressvol alternatief bieden om het ruimtelijk leervermogen en geheugen van de ApoE4 en ApoE$^{-/-}$ te bestuderen. Het is opmerkelijk dat de ApoE$^{-/-}$ muizen een toename in neuroinflammatie laten zien op een controledieet, maar een afname vertonen op het hoog-vetdieet. Hetzelfde patroon werd gevonden in ApoE4 muizen. We hadden verwacht dat deze muizen een afname van synaptische plasticiteit en neurogenese onder invloed van het hoog-vetdieet zouden vertonen als gevolg van een verlaging van BDNF-niveaus. De ApoE$^{-/-}$ muizen, en in mindere mate de ApoE4 muizen, laten echter een tendens voor toegenomen expressie van het eiwit doublecortin zien. Een verhoogde expressie van doublecortin leidt tot een toegenomen vorming van neuronen, maar geeft nog geen uitsluitsel of deze neuronen ook overleven en/of functioneel zijn. Deze resultaten kunnen erop duiden dat er een herstelmechanisme voor neurogenese en synaptische plasticiteit actief is onder invloed van het controledieet, en dat dit mechanisme niet langer werkzaam is onder invloed van cholesterol en verzadigde vetten. Cholesterol komt vrij uit lipoproteïnen die ApoE bevatten en wordt gebruikt ter ondersteuning van synaptogenese en onderhoud van de synaptische verbindingen. Daarom verwachtten we dat de synaptische plasticiteit in ApoE$^{-/-}$ muizen verlaagd zou zijn door de afgenomen afgifte van cholesterol. Zoals verwacht, vertoonden zowel de ApoE4 als de ApoE$^{-/-}$ muizen geen veranderingen in synaptische plasticiteit. Het lijkt erop dat het beschreven compensatiemechanisme voor neurodegeneratie op deze leeftijd nog werkzaam is in de ApoE4 en ApoE$^{-/-}$ muizen. Er wordt verondersteld dat het aantal GLUT-1 transporters in een suboptimale situatie (hypercholesterolemie of zelfs neurodegeneratie) toe zou nemen om voldoende transport van glucose te garanderen (als compensatiemechanisme). In hoofdstuk 5 werden echter geen veranderingen in het aantal GLUT-1 transporters in ApoE4 en WT muizen gevonden onder invloed van het hoog-vetdieet. Ook in de ApoE$^{-/-}$ muizen werden geen veranderingen in GLUT-1 transporters gevonden. Al m*et al* tonen de bevindingen uit hoofdstuk 5 aan dat een dieet rijk aan vetten verschillen effecten veroorzaakt in WT muizen vergeleken met ApoE4 en ApoE$^{-/-}$ muizen. De onderliggende werkingsmechanismen voor neurogenese, synaptische plasticiteit en neuroinflammatie moeten worden opgehelderd om een dieetadvies voor dragers van het APOE4-gen op te kunnen stellen.

Samengevat toont dit proefschrift de invloed van de omega-3 vetzuren en flavanolen op neurale ontwikkeling aan. Vooral omega-3 vetzuren vertonen al vanaf jonge leeftijd een gunstige invloed op het functioneren van de hersenen. Flavanolen hebben mogelijk een positieve

invloed op de structuur en het functioneren van de hersenen, maar de veranderingen waren erg subtiel in gezonde muizen. De invloed van een hoog-vetdieet op neurodegeneratie en cognitie in muismodellen met een risico op atherosclerose en de ziekte van Alzheimer is niet geheel ontrafeld.

De bevindingen in dit proefschrift benadrukken het belang van een gezonde en evenwichtige voeding gedurende het hele leven. We hebben aangetoond dat omega-3 vetzuren en flavanolen de neurale ontwikkeling al vanaf het begin van de zwangerschap en gedurende het hele leven beïnvloeden. Deze voedingsstoffen zijn mogelijk nog meer van belang in risicovolle zwangerschappen waarbij sprake is van een tekort aan voedingsstoffen. Zuigelingen die een risico hebben op aandoeningen veroorzaakt door tekorten aan voedingsstoffen kunnen wellicht meer profiteren van een gebalanceerd voedingspatroon van de moeder. De positieve effecten van omega-3 vetzuren en flavanolen ondersteunen gezonde hersenen gedurende het hele leven en kunnen beschermen bieden tegen neurodegeneratie op latere leeftijd. Hoewel cholesterol essentieel is voor membraansynthese en de vorming van galzuren en steroïde hormonen, biedt een vetrijk dieet geen ondersteuning voor een gezonde veroudering van de hersenen. De belangrijkste boodschap van dit proefschrift voor het ontwikkelen en behouden van een gezond brein is het vinden van de juiste balans in voedingsstoffen zoals omega-3 vetzuren, flavanolen en cholesterol.

Appendices

References

2003. Diet, nutrition and the prevention of chronic diseases. World Health Organ Tech Rep Ser 916, i-viii, 1-149, backcover.

Aggett, P.J., Haschke, F., Heine, W., Hernell, O., Koletzko, B., Launiala, K., Rey, J., Rubino, A., Schoch, Senterre, J., *et al*., 1991. Comment on the content and composition of lipids in infant formulas. ESPGAN Committee on Nutrition. Acta Paediatr Scand 80, 887-896.

Agostoni, C., 2008. Role of Long-chain Polyunsaturated Fatty Acids in the First Year of Life. 47, S41-S44 10.1097/1001.mpg.0000338811.0000352062.b0000338812.

Agrawal, R., Gomez-Pinilla, F., 2012. 'Metabolic syndrome' in the brain: deficiency in omega-3 fatty acid exacerbates dysfunctions in insulin receptor signalling and cognition. J Physiol 590, 2485-2499.

Aid, S., Vancassel, S., Poumes-Ballihaut, C., Chalon, S., Guesnet, P., Lavialle, M., 2003. Effect of a diet-induced n-3 PUFA depletion on cholinergic parameters in the rat hippocampus. J Lipid Res 44, 1545-1551.

Ailhaud, G., Massiera, F., Weill, P., Legrand, P., Alessandri, J.M., Guesnet, P., 2006. Temporal changes in dietary fats: role of n-6 polyunsaturated fatty acids in excessive adipose tissue development and relationship to obesity. Prog Lipid Res 45, 203-236.

Alata, W., Ye, Y., St-Amour, I., Vandal, M., Calon, F., 2015. Human apolipoprotein E varepsilon4 expression impairs cerebral vascularization and blood-brain barrier function in mice. J Cereb Blood Flow Metab 35, 86-94.

Alvarez, E., Campos-Toimil, M., Justiniano-Basaran, H., Lugnier, C., Orallo, F., 2006. Study of the mechanisms involved in the vasorelaxation induced by (-)-epigallocatechin-3-gallate in rat aorta. Br J Pharmacol 147, 269-280.

Amminger, G.P., Berger, G.E., Schafer, M.R., Klier, C., Friedrich, M.H., Feucht, M., 2007. Omega-3 fatty acids supplementation in children with autism: a double-blind randomized, placebo-controlled pilot study. Biol Psychiatry 61, 551-553.

Andreeva, V.A., Kesse-Guyot, E., Barberger-Gateau, P., Fezeu, L., Hercberg, S., Galan, P., 2011. Cognitive function after supplementation with B vitamins and long-chain omega-3 fatty acids: ancillary findings from the SU.FOL.OM3 randomized trial. Am J Clin Nutr 94, 278-286.

Andrews-Zwilling, Y., Bien-Ly, N., Xu, Q., Li, G., Bernardo, A., Yoon, S.Y., Zwilling, D., Yan, T.X., Chen, L., Huang, Y., 2010. Apolipoprotein E4 causes age- and Tau-dependent impairment of GABAergic interneurons, leading to learning and memory deficits in mice. J Neurosci 30, 13707-13717.

Arendash, G.W., Jensen, M.T., Salem, N., Jr., Hussein, N., Cracchiolo, J., Dickson, A., Leighty, R., Potter, H., 2007. A diet high in omega-3 fatty acids does not improve or protect cognitive performance in Alzheimer's transgenic mice. Neuroscience 149, 286-302.

Arola-Arnal, A., Oms-Oliu, G., Crescenti, A., del Bas, J.M., Ras, M.R., Arola, L., Caimari, A., 2013. Distribution of grape seed flavanols and their metabolites in pregnant rats and their fetuses. Mol Nutr Food Res 57, 1741-1752.

Arsenault, D., Julien, C., Tremblay, C., Calon, F., 2011. DHA improves cognition and prevents dysfunction of entorhinal cortex neurons in 3xTg-AD mice. PLoS ONE 6, e17397.

Attwell, D., Buchan, A.M., Charpak, S., Lauritzen, M., Macvicar, B.A., Newman, E.A., 2010. Glial and neuronal control of brain blood flow. Nature 468, 232-243.

Auestad, N., Halter, R., Hall, R.T., Blatter, M., Bogle, M.L., Burks, W., Erickson, J.R., Fitzgerald, K.M., Dobson, V., Innis, S.M., Singer, L.T., Montalto, M.B., Jacobs, J.R., Qiu, W., Bornstein, M.H., 2001. Growth and development in term infants fed long-chain polyunsaturated fatty acids: a double-masked, randomized, parallel, prospective, multivariate study. Pediatrics 108, 372-381.

Auestad, N., Scott, D.T., Janowsky, J.S., Jacobsen, C., Carroll, R.E., Montalto, M.B., Halter, R., Qiu, W., Jacobs, J.R., Connor, W.E., Connor, S.L., Taylor, J.A., Neuringer, M., Fitzgerald, K.M., Hall, R.T., 2003. Visual, cognitive, and language assessments at 39 months: a follow-up study of children fed formulas containing long-chain polyunsaturated fatty acids to 1 year of age. Pediatrics 112, e177-183.

Bang, H.O., Dyerberg, J., Hjoorne, N., 1976. The composition of food consumed by Greenland Eskimos. Acta

Med Scand 200, 69-73.
Bang, H.O., Dyerberg, J., Nielsen, A.B., 1971. Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos. Lancet 1, 1143-1145.
Bang, H.O., Dyerberg, J., Sinclair, H.M., 1980. The composition of the Eskimo food in north western Greenland. Am J Clin Nutr 33, 2657-2661.
Barros, D., Amaral, O.B., Izquierdo, I., Geracitano, L., do Carmo Bassols Raseira, M., Henriques, A.T., Ramirez, M.R., 2006. Behavioral and genoprotective effects of Vaccinium berries intake in mice. Pharmacol Biochem Behav 84, 229-234.
Bazan, N.G., Molina, M.F., Gordon, W.C., 2011. Docosahexaenoic acid signalolipidomics in nutrition: significance in aging, neuroinflammation, macular degeneration, Alzheimer's, and other neurodegenerative diseases. Annu Rev Nutr 31, 321-351.
Bearzatto, B., Servais, L., Cheron, G., Schiffmann, S.N., 2005. Age dependence of strain determinant on mice motor coordination. Brain Res 1039, 37-42.
Belanger, S.A., Vanasse, M., Spahis, S., Sylvestre, M.P., Lippe, S., L'Heureux, F., Ghadirian, P., Vanasse, C.M., Levy, E., 2009. Omega-3 fatty acid treatment of children with attention-deficit hyperactivity disorder: A randomized, double-blind, placebo-controlled study. Paediatr Child Health 14, 89-98.
Belkind-Gerson, J., Carreon-Rodriguez, A., Contreras-Ochoa, C.O., Estrada-Mondaca, S., Parra-Cabrera, M.S., 2008. Fatty Acids and Neurodevelopment. 47, S7-S9 10.1097/MPG.1090b1013e3181818e3181813f.
Bent, S., Bertoglio, K., Ashwood, P., Bostrom, A., Hendren, R.L., 2011. A pilot randomized controlled trial of omega-3 fatty acids for autism spectrum disorder. J Autism Dev Disord 41, 545-554.
Berger, G.E., Wood, S.J., Wellard, R.M., Proffitt, T.M., McConchie, M., Amminger, G.P., Jackson, G.D., Velakoulis, D., Pantelis, C., McGorry, P.D., 2008. Ethyl-eicosapentaenoic acid in first-episode psychosis. A 1H-MRS study. Neuropsychopharmacology 33, 2467-2473.
Birch, E.E., Carlson, S.E., Hoffman, D.R., Fitzgerald-Gustafson, K.M., Fu, V.L., Drover, J.R., Castaneda, Y.S., Minns, L., Wheaton, D.K., Mundy, D., Marunycz, J., Diersen-Schade, D.A., 2010. The DIAMOND (DHA Intake And Measurement Of Neural Development) Study: a double-masked, randomized controlled clinical trial of the maturation of infant visual acuity as a function of the dietary level of docosahexaenoic acid. Am J Clin Nutr 91, 848-859.
Birch, E.E., Garfield, S., Castaneda, Y., Hughbanks-Wheaton, D., Uauy, R., Hoffman, D., 2007. Visual acuity and cognitive outcomes at 4 years of age in a double-blind, randomized trial of long-chain polyunsaturated fatty acid-supplemented infant formula. Early Hum Dev 83, 279-284.
Birch, E.E., Garfield, S., Hoffman, D.R., Uauy, R., Birch, D.G., 2000. A randomized controlled trial of early dietary supply of long-chain polyunsaturated fatty acids and mental development in term infants. Dev Med Child Neurol 42, 174-181.
Birdsill, A.C., Carlsson, C.M., Willette, A.A., Okonkwo, O.C., Johnson, S.C., Xu, G., Oh, J.M., Gallagher, C.L., Koscik, R.L., Jonaitis, E.M., Hermann, B.P., LaRue, A., Rowley, H.A., Asthana, S., Sager, M.A., Bendlin, B.B., 2013. Low cerebral blood flow is associated with lower memory function in metabolic syndrome. Obesity (Silver Spring) 21, 1313-1320.
Bolca, S., Van de Wiele, T., Possemiers, S., 2013. Gut metabotypes govern health effects of dietary polyphenols. Curr Opin Biotechnol 24, 220-225.
Boston, P.F., Bennett, A., Horrobin, D.F., Bennett, C.N., 2004. Ethyl-EPA in Alzheimer's disease--a pilot study. Prostaglandins Leukot Essent Fatty Acids 71, 341-346.
Bour, A., Grootendorst, J., Vogel, E., Kelche, C., Dodart, J.C., Bales, K., Moreau, P.H., Sullivan, P.M., Mathis, C., 2008. Middle-aged human apoE4 targeted-replacement mice show retention deficits on a wide range of spatial memory tasks. Behav Brain Res 193, 174-182.
Bourre, J.M., 2007. Dietary omega-3 fatty acids for women. Biomed Pharmacother 61, 105-112.
Bourre, J.M., Pascal, G., Durand, G., Masson, M., Dumont, O., Piciotti, M., 1984. Alterations in the Fatty-Acid Composition of Rat-Brain Cells (Neurons, Astrocytes, and Oligodendrocytes) and of Subcellular-Fractions (Myelin and Synaptosomes) Induced by a Diet Devoid of N-3 Fatty-Acids. Journal of Neurochemistry 43, 342-348.

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

Bousquet, M., Gue, K., Emond, V., Julien, P., Kang, J.X., Cicchetti, F., Calon, F., 2011. Transgenic conversion of omega-6 into omega-3 fatty acids in a mouse model of Parkinson's disease. J Lipid Res 52, 263-271.

Bouwstra, H., Dijck-Brouwer, D.J., Decsi, T., Boehm, G., Boersma, E.R., Muskiet, F.A., Hadders-Algra, M., 2006a. Relationship between umbilical cord essential fatty acid content and the quality of general movements of healthy term infants at 3 months. Pediatr Res 59, 717-722.

Bouwstra, H., Dijck-Brouwer, J., Decsi, T., Boehm, G., Boersma, E.R., Muskiet, F.A., Hadders-Algra, M., 2006b. Neurologic condition of healthy term infants at 18 months: positive association with venous umbilical DHA status and negative association with umbilical trans-fatty acids. Pediatr Res 60, 334-339.

Brenna, J.T., 2011. Animal studies of the functional consequences of suboptimal polyunsaturated fatty acid status during pregnancy, lactation and early post-natal life. Matern Child Nutr 7 Suppl 2, 59-79.

Brenna, J.T., Salem, N., Jr., Sinclair, A.J., Cunnane, S.C., 2009. alpha-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. Prostaglandins Leukot Essent Fatty Acids 80, 85-91.

Breslow, J.L., 2006. n-3 fatty acids and cardiovascular disease. Am J Clin Nutr 83, 1477S-1482S.

Brown, D.R., 2009. Role of Microglia in Age-Related Changes to the Nervous System. Thescientificworldjournal 9, 1061-1071.

Calder, P.C., Yaqoob, P., 2010. Omega-3 (n-3) fatty acids, cardiovascular disease and stability of atherosclerotic plaques. Cell Mol Biol (Noisy-le-grand) 56, 28-37.

Calon, F., 2011. Omega-3 polyunsaturated fatty acids in Alzheimer's disease: key questions and partial answers. Curr Alzheimer Res 8, 470-478.

Calon, F., Cole, G., 2007. Neuroprotective action of omega-3 polyunsaturated fatty acids against neurodegenerative diseases: evidence from animal studies. Prostaglandins Leukot Essent Fatty Acids 77, 287-293.

Calon, F., Lim, G.P., Morihara, T., Yang, F., Ubeda, O., Salem, N., Jr., Frautschy, S.A., Cole, G.M., 2005. Dietary n-3 polyunsaturated fatty acid depletion activates caspases and decreases NMDA receptors in the brain of a transgenic mouse model of Alzheimer's disease. Eur J Neurosci 22, 617-626.

Calon, F., Lim, G.P., Yang, F., Morihara, T., Teter, B., Ubeda, O., Rostaing, P., Triller, A., Salem, N., Jr., Ashe, K.H., Frautschy, S.A., Cole, G.M., 2004. Docosahexaenoic acid protects from dendritic pathology in an Alzheimer's disease mouse model. Neuron 43, 633-645.

Cao, D., Kevala, K., Kim, J., Moon, H.S., Jun, S.B., Lovinger, D., Kim, H.Y., 2009a. Docosahexaenoic acid promotes hippocampal neuronal development and synaptic function. J Neurochem 111, 510-521.

Cao, D.H., Kevala, K., Kim, J., Moon, H.S., Jun, S.B., Lovinger, D., Kim, H.Y., 2009b. Docosahexaenoic acid promotes hippocampal neuronal development and synaptic function. Journal of Neurochemistry 111, 510-521.

Cardoso, H.D., Passos, P.P., Lagranha, C.J., Ferraz, A.C., Santos Junior, E.F., Oliveira, R.S., Oliveira, P.E., Santos Rde, C., Santana, D.F., Borba, J.M., Rocha-de-Melo, A.P., Guedes, R.C., Navarro, D.M., Santos, G.K., Borner, R., Picanco-Diniz, C.W., Beltrao, E.I., Silva, J.F., Rodrigues, M.C., Andrade da Costa, B.L., 2012. Differential vulnerability of substantia nigra and corpus striatum to oxidative insult induced by reduced dietary levels of essential fatty acids. Front Hum Neurosci 6, 249.

Carlson, S.E., 1996. Arachidonic acid status of human infants: influence of gestational age at birth and diets with very long chain n-3 and n-6 fatty acids. J Nutr 126, 1092S-1098S.

Carlson, S.E., Cooke, R.J., Rhodes, P.G., Peeples, J.M., Werkman, S.H., Tolley, E.A., 1991. Long-term feeding of formulas high in linolenic acid and marine oil to very low birth weight infants: phospholipid fatty acids. Pediatr Res 30, 404-412.

Carlson, S.E., Rhodes, P.G., Rao, V.S., Goldgar, D.E., 1987. Effect of fish oil supplementation on the n-3 fatty acid content of red blood cell membranes in preterm infants. Pediatr Res 21, 507-510.

Carlson, S.J., Fallon, E.M., Kalish, B.T., Gura, K.M., Puder, M., 2013. The role of the omega-3 fatty acid DHA in

the human life cycle. JPEN J Parenter Enteral Nutr 37, 15-22.
Carrié, I., Clement, M., de Javel, D., Frances, H., Bourre, J.M., 2000a. Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. J Lipid Res 41, 473-480.
Carrié, I., Guesnet, P., Bourre, J.M., Frances, H., 2000b. Diets containing long-chain n-3 polyunsaturated fatty acids affect behaviour differently during development than ageing in mice. Br J Nutr 83, 439-447.
Cavassila, S., Deval, S., Huegen, C., van Ormondt, D., Graveron-Demilly, D., 2001. Cramer-Rao bounds: an evaluation tool for quantitation. NMR Biomed 14, 278-283.
Cederholm, T., Palmblad, J., 2010. Are omega-3 fatty acids options for prevention and treatment of cognitive decline and dementia? Curr Opin Clin Nutr Metab Care 13, 150-155.
Chalon, S., 2006. Omega-3 fatty acids and monoamine neurotransmission. Prostaglandins Leukot Essent Fatty Acids 75, 259-269.
Chalon, S., 2009. The role of fatty acids in the treatment of ADHD. Neuropharmacology 57, 636-639.
Champagne, D., Dupuy, J.B., Rochford, J., Poirier, J., 2002. Apolipoprotein E knockout mice display procedural deficits in the Morris water maze: analysis of learning strategies in three versions of the task. Neuroscience 114, 641-654.
Chen, Z.Y., Law, W.I., Yao, X.Q., Lau, C.W., Ho, W.K., Huang, Y., 2000. Inhibitory effects of purified green tea epicatechins on contraction and proliferation of arterial smooth muscle cells. Acta Pharmacol Sin 21, 835-840.
Cherniack, E.P., 2012. A berry thought-provoking idea: the potential role of plant polyphenols in the treatment of age-related cognitive disorders. Br J Nutr 108, 794-800.
Chiu, C.C., Su, K.P., Cheng, T.C., Liu, H.C., Chang, C.J., Dewey, M.E., Stewart, R., Huang, S.Y., 2008. The effects of omega-3 fatty acids monotherapy in Alzheimer's disease and mild cognitive impairment: a preliminary randomized double-blind placebo-controlled study. Prog Neuropsychopharmacol Biol Psychiatry 32, 1538-1544.
Clandinin, M.T., Parrott, A., Van Aerde, J.E., Hervada, A.R., Lien, E., 1992. Feeding preterm infants a formula containing C20 and C22 fatty acids simulates plasma phospholipid fatty acid composition of infants fed human milk. Early Hum Dev 31, 41-51.
Clouard, C., Gerrits, W.J., van Kerkhof, I., Smink, W., Bolhuis, J.E., 2015. Dietary linoleic and alpha-linolenic acids affect anxiety-related responses and exploratory activity in growing pigs. J Nutr 145, 358-364.
Cole, G.M., Frautschy, S.A., 2006. Docosahexaenoic acid protects from amyloid and dendritic pathology in an Alzheimer's disease mouse model. Nutr Health 18, 249-259.
Cole, G.M., Ma, Q.L., Frautschy, S.A., 2009. Omega-3 fatty acids and dementia. Prostaglandins Leukot Essent Fatty Acids 81, 213-221.
Coluccia, A., Borracci, P., Renna, G., Giustino, A., Latronico, T., Riccio, P., Carratu, M.R., 2009. Developmental omega-3 supplementation improves motor skills in juvenile-adult rats. Int J Dev Neurosci 27, 599-605.
Connor, W.E., 2000. Importance of n-3 fatty acids in health and disease. Am J Clin Nutr 71, 171S-175S.
Conquer, J.A., Tierney, M.C., Zecevic, J., Bettger, W.J., Fisher, R.H., 2000. Fatty acid analysis of blood plasma of patients with Alzheimer's disease, other types of dementia, and cognitive impairment. Lipids 35, 1305-1312.
Corcoran, M.P., McKay, D.L., Blumberg, J.B., Flavonoid Basics: Chemistry, Sources, Mechanisms of Action, and Safety. Journal of Nutrition in Gerontology and Geriatrics 31, 176-189.
Crupi, R., Cambiaghi, M., Deckelbaum, R., Hansen, I., Mindes, J., Spina, E., Battaglia, F., 2012. n-3 fatty acids prevent impairment of neurogenesis and synaptic plasticity in B-cell activating factor (BAFF) transgenic mice. Prev Med 54 Suppl, S103-108.
da Silva, T.M., Munhoz, R.P., Alvarez, C., Naliwaiko, K., Kiss, A., Andreatini, R., Ferraz, A.C., 2008. Depression in Parkinson's disease: a double-blind, randomized, placebo-controlled pilot study of omega-3 fatty-acid supplementation. J Affect Disord 111, 351-359.
Dagai, L., Peri-Naor, R., Birk, R.Z., 2009. Docosahexaenoic acid significantly stimulates immediate early response genes and neurite outgrowth. Neurochem Res 34, 867-875.

Dangour, A.D., Allen, E., Elbourne, D., Fasey, N., Fletcher, A.E., Hardy, P., Holder, G.E., Knight, R., Letley, L., Richards, M., Uauy, R., 2010. Effect of 2-y n-3 long-chain polyunsaturated fatty acid supplementation on cognitive function in older people: a randomized, double-blind, controlled trial. Am J Clin Nutr 91, 1725-1732.

Danthiir, V., Burns, N.R., Nettelbeck, T., Wilson, C., Wittert, G., 2011. The older people, omega-3, and cognitive health (EPOCH) trial design and methodology: a randomised, double-blind, controlled trial investigating the effect of long-chain omega-3 fatty acids on cognitive ageing and wellbeing in cognitively healthy older adults. Nutr J 10, 117.

Das, U.N., 2008. Folic acid and polyunsaturated fatty acids improve cognitive function and prevent depression, dementia, and Alzheimer's disease--but how and why? Prostaglandins Leukot Essent Fatty Acids 78, 11-19.

Dashti, S., Aboutaleb, N., Shahbazi, A., 2013. The effect of leptin on prepulse inhibition in a developmental model of schizophrenia. Neurosci Lett 555, 57-61.

Davidson, T.L., Kanoski, S.E., Walls, E.K., Jarrard, L.E., 2005. Memory inhibition and energy regulation. Physiol Behav 86, 731-746.

de Boer, V.C., Dihal, A.A., van der Woude, H., Arts, I.C., Wolffram, S., Alink, G.M., Rietjens, I.M., Keijer, J., Hollman, P.C., 2005. Tissue distribution of quercetin in rats and pigs. J Nutr 135, 1718-1725.

de Jong, C., Kikkert, H.K., Fidler, V., Hadders-Algra, M., 2010. The Groningen LCPUFA study: no effect of postnatal long-chain polyunsaturated fatty acids in healthy term infants on neurological condition at 9 years. Br J Nutr 104, 566-572.

de la Torre, J.C., 2004. Is Alzheimer's disease a neurodegenerative or a vascular disorder? Data, dogma, and dialectics. Lancet Neurol 3, 184-190.

de la Torre, J.C., 2012. A turning point for Alzheimer's disease? Biofactors 38, 78-83.

de la Torre, J.C., Stefano, G.B., 2000. Evidence that Alzheimer's disease is a microvascular disorder: the role of constitutive nitric oxide. Brain Res Brain Res Rev 34, 119-136.

Desouza, R., Moro, E., Lang, A., Schapira, A., 2013. Timing of deep brain stimulation in Parkinson's disease: A need for reappraisal? Ann Neurol 8, 23890.

Dhungana, H., Rolova, T., Savchenko, E., Wojciechowski, S., Savolainen, K., Ruotsalainen, A.K., Sullivan, P.M., Koistinaho, J., Malm, T., 2013. Western-type diet modulates inflammatory responses and impairs functional outcome following permanent middle cerebral artery occlusion in aged mice expressing the human apolipoprotein E4 allele. J Neuroinflammation 10, 102.

Donahue, J.E., Johanson, C.E., 2008. Apolipoprotein E, amyloid-beta, and blood-brain barrier permeability in Alzheimer disease. J Neuropathol Exp Neurol 67, 261-270.

Dorey, E., Chang, N., Liu, Q.Y., Yang, Z., Zhang, W., 2014. Apolipoprotein E, amyloid-beta, and neuroinflammation in Alzheimer's disease. Neurosci Bull 30, 317-330.

Dorsey, E.R., Constantinescu, R., Thompson, J.P., Biglan, K.M., Holloway, R.G., Kieburtz, K., Marshall, F.J., Ravina, B.M., Schifitto, G., Siderowf, A., Tanner, C.M., 2007. Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. Neurology 68, 384-386.

Dunstan, J.A., Simmer, K., Dixon, G., Prescott, S.L., 2008. Cognitive assessment of children at age 2(1/2) years after maternal fish oil supplementation in pregnancy: a randomised controlled trial. Arch Dis Child Fetal Neonatal Ed 93, F45-50.

Dyall, S.C., Michael-Titus, A.T., 2008. Neurological Benefits of Omega-3 Fatty Acids. Neuromolecular Med 10, 219-235.

Dyall, S.C., Michael, G.J., Michael-Titus, A.T., 2010. Omega-3 fatty acids reverse age-related decreases in nuclear receptors and increase neurogenesis in old rats. J Neurosci Res 88, 2091-2102.

Dyerberg, J., Bang, H.O., 1979. Lipid metabolism, atherogenesis, and haemostasis in Eskimos: the role of the prostaglandin-3 family. Haemostasis 8, 227-233.

Eilander, A., Hundscheid, D.C., Osendarp, S.J., Transler, C., Zock, P.L., 2007. Effects of n-3 long chain polyunsaturated fatty acid supplementation on visual and cognitive development throughout childhood: a review of human studies. Prostaglandins Leukot Essent Fatty Acids 76, 189-203.

Enslen, M., Milon, H., Malnoe, A., 1991. Effect of low intake of n-3 fatty acids during development on brain phospholipid fatty acid composition and exploratory behavior in rats. Lipids 26, 203-208.

Esposito, D., Chen, A., Grace, M.H., Komarnytsky, S., Lila, M.A., 2014. Inhibitory Effects of Wild Blueberry Anthocyanins and Other Flavonoids on Biomarkers of Acute and Chronic Inflammation *in vitro*. J Agric Food Chem.

Estruch, R., Ros, E., Salas-Salvado, J., Covas, M.I., Corella, D., Aros, F., Gomez-Gracia, E., Ruiz-Gutierrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R.M., Serra-Majem, L., Pinto, X., Basora, J., Munoz, M.A., Sorli, J.V., Martinez, J.A., Martinez-Gonzalez, M.A., Investigators, P.S., 2013. Primary prevention of cardiovascular disease with a Mediterranean diet. N Engl J Med 368, 1279-1290.

Fang, P.C., Kuo, H.K., Huang, C.B., Ko, T.Y., Chen, C.C., Chung, M.Y., 2005. The effect of supplementation of docosahexaenoic acid and arachidonic acid on visual acuity and neurodevelopment in larger preterm infants. Chang Gung Med J 28, 708-715.

Faria, A., Meireles, M., Fernandes, I., Santos-Buelga, C., Gonzalez-Manzano, S., Duenas, M., de Freitas, V., Mateus, N., Calhau, C., 2014. Flavonoid metabolites transport across a human BBB model. Food Chem 149, 190-196.

Feart, C., Samieri, C., Rondeau, V., Amieva, H., Portet, F., Dartigues, J.F., Scarmeas, N., Barberger-Gateau, P., 2009. Adherence to a Mediterranean diet, cognitive decline, and risk of dementia. Jama 302, 638-648.

Fedorova, I., Alvheim, A.R., Hussein, N., Salem, N., Jr., 2009a. Deficit in prepulse inhibition in mice caused by dietary n-3 fatty acid deficiency. Behav Neurosci 123, 1218-1225.

Fedorova, I., Hussein, N., Baumann, M.H., Di Martino, C., Salem, N., Jr., 2009b. An n-3 fatty acid deficiency impairs rat spatial learning in the Barnes maze. Behav Neurosci 123, 196-205.

Fedorova, I., Salem, N., Jr., 2006. Omega-3 fatty acids and rodent behavior. Prostaglandins Leukot Essent Fatty Acids 75, 271-289.

Fewtrell, M.S., Abbott, R.A., Kennedy, K., Singhal, A., Morley, R., Caine, E., Jamieson, C., Cockburn, F., Lucas, A., 2004. Randomized, double-blind trial of long-chain polyunsaturated fatty acid supplementation with fish oil and borage oil in preterm infants. J Pediatr 144, 471-479.

Fewtrell, M.S., Morley, R., Abbott, R.A., Singhal, A., Isaacs, E.B., Stephenson, T., MacFadyen, U., Lucas, A., 2002. Double-blind, randomized trial of long-chain polyunsaturated fatty acid supplementation in formula fed to preterm infants. Pediatrics 110, 73-82.

Firmansyah, A., Dwipoerwantoro, P.G., Kadim, M., Alatas, S., Conus, N., Lestarina, L., Bouisset, F., Steenhout, P., 2011. Improved growth of toddlers fed a milk containing synbiotics. Asia Pac J Clin Nutr 20, 69-76.

Fitzgerald, M.J.T., Jean Folan-Curran, 2002. Clinical neuroanatomy and related neuroscience, fourth ed. WB Saunders, Edinburgh.

Foldi, C.J., Eyles, D.W., McGrath, J.J., Burne, T.H., 2011. The effects of breeding protocol in C57BL/6J mice on adult offspring behaviour. PLoS ONE 6, e18152.

Foreman-van Drongelen, M.M., Houwelingen, A.C., Kester, A.D., de Jong, A.E., Blanco, C.E., Hasaart, T.H., Hornstra, G., 1995. Long-chain polyene status of preterm infants with regard to the fatty acid composition of their diet: comparison between absolute and relative fatty acid levels in plasma and erythrocyte phospholipids. Br J Nutr 73, 405-422.

Francis, S.T., Head, K., Morris, P.G., Macdonald, I.A., 2006. The effect of flavanol-rich cocoa on the fMRI response to a cognitive task in healthy young people. J Cardiovasc Pharmacol 47 Suppl 2, S215-220.

Franklin, K.B.J., Paxinos, G., 2008. The mouse brain in stereotaxic coordinates. , 3 ed. Academic Press, San Diego.

Freemantle, E., Vandal, M., Tremblay-Mercier, J., Tremblay, S., Blachere, J.C., Begin, M.E., Brenna, J.T., Windust, A., Cunnane, S.C., 2006. Omega-3 fatty acids, energy substrates, and brain function during aging. Prostaglandins Leukot Essent Fatty Acids 75, 213-220.

Freund-Levi, Y., Basun, H., Cederholm, T., Faxen-Irving, G., Garlind, A., Grut, M., Vedin, I., Palmblad, J., Wahlund, L.O., Eriksdotter-Jonhagen, M., 2008. Omega-3 supplementation in mild to moderate Alzheimer's disease: effects on neuropsychiatric symptoms. Int J Geriatr Psychiatry 23, 161-169.

Freund-Levi, Y., Eriksdotter-Jonhagen, M., Cederholm, T., Basun, H., Faxen-Irving, G., Garlind, A., Vedin, I.,

Vessby, B., Wahlund, L.O., Palmblad, J., 2006. Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial. Arch Neurol 63, 1402-1408.

Frisardi, V., Panza, F., Seripa, D., Imbimbo, B.P., Vendemiale, G., Pilotto, A., Solfrizzi, V., 2010. Nutraceutical properties of Mediterranean diet and cognitive decline: possible underlying mechanisms. J Alzheimers Dis 22, 715-740.

Garcia, C., Millet, V., Coste, T.C., Mimoun, M., Ridet, A., Antona, C., Simeoni, U., Armand, M., 2011. French mothers' milk deficient in DHA contains phospholipid species of potential interest for infant development. J Pediatr Gastroenterol Nutr 53, 206-212.

Gardener, H., Wright, C.B., Gu, Y., Demmer, R.T., Boden-Albala, B., Elkind, M.S., Sacco, R.L., Scarmeas, N., 2011. Mediterranean-style diet and risk of ischemic stroke, myocardial infarction, and vascular death: the Northern Manhattan Study. Am J Clin Nutr 94, 1458-1464.

Geleijnse, J.M., Giltay, E.J., Grobbee, D.E., Donders, A.R., Kok, F.J., 2002. Blood pressure response to fish oil supplementation: metaregression analysis of randomized trials. J Hypertens 20, 1493-1499.

Geyer, M.A., Dulawa, S.C., 2001. Assessment of Murine Startle Reactivity, Prepulse Inhibition, and Habituation. John Wiley & Sons, Inc.

Ghosh, D., Scheepens, A., 2009. Vascular action of polyphenols. Mol Nutr Food Res 53, 322-331.

Gibson, R.A., Muhlhausler, B., Makrides, M., 2011. Conversion of linoleic acid and alpha-linolenic acid to long-chain polyunsaturated fatty acids (LCPUFAs), with a focus on pregnancy, lactation and the first 2 years of life. Matern Child Nutr 7 Suppl 2, 17-26.

Gillies, D., Sinn, J., Lad, S.S., Leach, M.J., Ross, M.J., 2012. Polyunsaturated fatty acids (PUFA) for attention deficit hyperactivity disorder (ADHD) in children and adolescents. Cochrane Database Syst Rev 7, CD007986.

Gomez-Pinilla, F., Nguyen, T.T., 2012. Natural mood foods: the actions of polyphenols against psychiatric and cognitive disorders. Nutr Neurosci 15, 127-133.

Goodfellow, J., Bellamy, M.F., Ramsey, M.W., Jones, C.J., Lewis, M.J., 2000. Dietary supplementation with marine omega-3 fatty acids improve systemic large artery endothelial function in subjects with hypercholesterolemia. J Am Coll Cardiol 35, 265-270.

Govindaraju, V., Young, K., Maudsley, A.A., 2000. Proton NMR chemical shifts and coupling constants for brain metabolites. NMR Biomed 13, 129-153.

Grammas, P., Yamada, M., Zlokovic, B., 2002. The cerebromicrovasculature: a key player in the pathogenesis of Alzheimer's disease. J Alzheimers Dis 4, 217-223.

Green, K.N., Martinez-Coria, H., Khashwji, H., Hall, E.B., Yurko-Mauro, K.A., Ellis, L., LaFerla, F.M., 2007a. Dietary docosahexaenoic acid and docosapentaenoic acid ameliorate amyloid-beta and tau pathology via a mechanism involving presenilin 1 levels. J Neurosci 27, 4385-4395.

Green, K.N., Smith, I.F., Laferla, F.M., 2007b. Role of calcium in the pathogenesis of Alzheimer's disease and transgenic models. Subcell Biochem 45, 507-521.

Greiner, R.S., Catalan, J.N., Moriguchi, T., Salem, N., Jr., 2003. Docosapentaenoic acid does not completely replace DHA in n-3 FA-deficient rats during early development. Lipids 38, 431-435.

Grootendorst, J., de Kloet, E.R., Dalm, S., Oitzl, M.S., 2001. Reversal of cognitive deficit of apolipoprotein E knockout mice after repeated exposure to a common environmental experience. Neuroscience 108, 237-247.

Grootendorst, J., Enthoven, L., Dalm, S., de Kloet, E.R., Oitzl, M.S., 2004. Increased corticosterone secretion and early-onset of cognitive decline in female apolipoprotein E-knockout mice. Behav Brain Res 148, 167-177.

Grootendorst, J., Kempes, M.M., Lucassen, P.J., Dalm, S., de Kloet, E.R., Oitzl, M.S., 2002. Differential effect of corticosterone on spatial learning abilities in apolipoprotein E knockout and C57BL/6J mice. Brain Res 953, 281-285.

Gruetter, R., 1993. Automatic, localized *in vivo* adjustment of all first- and second-order shim coils. Magn Reson Med 29, 804-811.

Gu, Y., Luchsinger, J.A., Stern, Y., Scarmeas, N., 2010. Mediterranean diet, inflammatory and metabolic biomarkers, and risk of Alzheimer's disease. J Alzheimers Dis 22, 483-492.

Guasch-Ferre, M., Bullo, M., Martinez-Gonzalez, M.A., Ros, E., Corella, D., Estruch, R., Fito, M., Aros, F., Warnberg, J., Fiol, M., Lapetra, J., Vinyoles, E., Lamuela-Raventos, R.M., Serra-Majem, L., Pinto, X., Ruiz-Gutierrez, V., Basora, J., Salas-Salvado, J., group, P.s., 2013. Frequency of nut consumption and mortality risk in the PREDIMED nutrition intervention trial. BMC Med 11, 164.

Gustafsson, P.A., Birberg-Thornberg, U., Duchen, K., Landgren, M., Malmberg, K., Pelling, H., Strandvik, B., Karlsson, T., 2010. EPA supplementation improves teacher-rated behaviour and oppositional symptoms in children with ADHD. Acta Paediatr 99, 1540-1549.

Haan, M.N., Wallace, R., 2004. Can dementia be prevented? Brain aging in a population-based context. Annu Rev Public Health 25, 1-24.

Hacioglu, G., Seval-Celik, Y., Tanriover, G., Ozsoy, O., Saka-Topcuoglu, E., Balkan, S., Agar, A., 2012. Docosahexaenoic acid provides protective mechanism in bilaterally MPTP-lesioned rat model of Parkinson's disease. Folia Histochem Cytobiol 50, 228-238.

Hadders-Algra, M., 2005. The role of long-chain polyunsaturated fatty acids (LCPUFA) in growth and development. Adv Exp Med Biol 569, 80-94.

Hadders-Algra, M., 2011. Prenatal and early postnatal supplementation with long-chain polyunsaturated fatty acids: neurodevelopmental considerations. Am J Clin Nutr 94, 1874S-1879S.

Harris, W.S., Lu, G., Rambjor, G.S., Walen, A.I., Ontko, J.A., Cheng, Q., Windsor, S.L., 1997. Influence of n-3 fatty acid supplementation on the endogenous activities of plasma lipases. Am J Clin Nutr 66, 254-260.

Harsan, L.A., Paul, D., Schnell, S., Kreher, B.W., Hennig, J., Staiger, J.F., von Elverfeldt, D., 2010. *In vivo* diffusion tensor magnetic resonance imaging and fiber tracking of the mouse brain. NMR Biomed 23, 884-896.

Hashimoto, M., Hossain, S., Shimada, T., Shido, O., 2006. Docosahexaenoic acid-induced protective effect against impaired learning in amyloid beta-infused rats is associated with increased synaptosomal membrane fluidity. Clin Exp Pharmacol Physiol 33, 934-939.

Haubner, L., Sullivan, J., Ashmeade, T., Saste, M., Wiener, D., Carver, J., 2007. The effects of maternal dietary docosahexaenoic acid intake on rat pup myelin and the auditory startle response. Dev Neurosci 29, 460-467.

He, K., Song, Y., Daviglus, M.L., Liu, K., Van Horn, L., Dyer, A.R., Greenland, P., 2004. Accumulated evidence on fish consumption and coronary heart disease mortality: a meta-analysis of cohort studies. Circulation 109, 2705-2711.

Helland, I.B., Smith, L., Saarem, K., Saugstad, O.D., Drevon, C.A., 2003a. Maternal Supplementation With Very-Long-Chain n-3 Fatty Acids During Pregnancy and Lactation Augments Children's IQ at 4 Years of Age. 111, e39-44.

Helland, I.B., Smith, L., Saarem, K., Saugstad, O.D., Drevon, C.A., 2003b. Maternal Supplementation With Very-Long-Chain n-3 Fatty Acids During Pregnancy and Lactation Augments Children's IQ at 4 Years of Age. Pediatrics 111, e39-44.

Henriksen, C., Haugholt, K., Lindgren, M., Aurvag, A.K., Ronnestad, A., Gronn, M., Solberg, R., Moen, A., Nakstad, B., Berge, R.K., Smith, L., Iversen, P.O., Drevon, C.A., 2008. Improved cognitive development among preterm infants attributable to early supplementation of human milk with docosahexaenoic acid and arachidonic acid. Pediatrics 121, 1137-1145.

Hirayama, S., Hamazaki, T., Terasawa, K., 2004. Effect of docosahexaenoic acid-containing food administration on symptoms of attention-deficit/hyperactivity disorder - a placebo-controlled double-blind study. Eur J Clin Nutr 58, 467-473.

Hjorth, E., Freund-Levi, Y., 2012. Immunomodulation of microglia by docosahexaenoic acid and eicosapentaenoic acid. Curr Opin Clin Nutr Metab Care 15, 134-143.

Hoevenaar-Blom, M.P., Nooyens, A.C., Kromhout, D., Spijkerman, A.M., Beulens, J.W., van der Schouw, Y.T., Bueno-de-Mesquita, B., Verschuren, W.M., 2012. Mediterranean style diet and 12-year incidence of cardiovascular diseases: the EPIC-NL cohort study. PLoS One 7, e45458.

Hoffman, D.R., Birch, E.E., Castaneda, Y.S., Fawcett, S.L., Wheaton, D.H., Birch, D.G., Uauy, R., 2003. Visual

function in breast-fed term infants weaned to formula with or without long-chain polyunsaturates at 4 to 6 months: a randomized clinical trial. J Pediatr 142, 669-677.

Hoffman, D.R., Boettcher, J.A., Diersen-Schade, D.A., 2009. Toward optimizing vision and cognition in term infants by dietary docosahexaenoic and arachidonic acid supplementation: a review of randomized controlled trials. Prostaglandins Leukot Essent Fatty Acids 81, 151-158.

Hollenberg, N.K., Fisher, N.D., McCullough, M.L., 2009. Flavanols, the Kuna, cocoa consumption, and nitric oxide. J Am Soc Hypertens 3, 105-112.

Hooijmans, C.R., Rutters, F., Dederen, P.J., Gambarota, G., Veltien, A., van Groen, T., Broersen, L.M., Lutjohann, D., Heerschap, A., Tanila, H., Kiliaan, A.J., 2007. Changes in cerebral blood volume and amyloid pathology in aged Alzheimer APP/PS1 mice on a docosahexaenoic acid (DHA) diet or cholesterol enriched Typical Western Diet (TWD). Neurobiol Dis 28, 16-29.

Hooijmans, C.R., Van der Zee, C.E., Dederen, P.J., Brouwer, K.M., Reijmer, Y.D., van Groen, T., Broersen, L.M., Lutjohann, D., Heerschap, A., Kiliaan, A.J., 2009. DHA and cholesterol containing diets influence Alzheimer-like pathology, cognition and cerebral vasculature in APPswe/PS1dE9 mice. Neurobiol Dis 33, 482-498.

Horby Jorgensen, M., Holmer, G., Lund, P., Hernell, O., Michaelsen, K.F., 1998. Effect of formula supplemented with docosahexaenoic acid and gamma-linolenic acid on fatty acid status and visual acuity in term infants. J Pediatr Gastroenterol Nutr 26, 412-421.

Hu, F.B., Bronner, L., Willett, W.C., Stampfer, M.J., Rexrode, K.M., Albert, C.M., Hunter, D., Manson, J.E., 2002. Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. JAMA 287, 1815-1821.

Hu, P., Wang, M., Chen, W.H., Liu, J., Chen, L., Yin, S.T., Yong, W., Chen, J.T., Wang, H.L., Ruan, D.Y., 2008. Quercetin relieves chronic lead exposure-induced impairment of synaptic plasticity in rat dentate gyrus *in vivo*. Naunyn Schmiedebergs Arch Pharmacol 378, 43-51.

Ikemoto, A., Ohishi, M., Sato, Y., Hata, N., Misawa, Y., Fujii, Y., Okuyama, H., 2001. Reversibility of n-3 fatty acid deficiency-induced alterations of learning behavior in the rat: level of n-6 fatty acids as another critical factor. J Lipid Res 42, 1655-1663.

Innis, S.M., 2008. Dietary omega 3 fatty acids and the developing brain. Brain Res 1237, 35-43.

Innis, S.M., 2009. Omega-3 Fatty acids and neural development to 2 years of age: do we know enough for dietary recommendations? J Pediatr Gastroenterol Nutr 48 Suppl 1, S16-24.

Innis, S.M., Friesen, R.W., 2008. Essential n-3 fatty acids in pregnant women and early visual acuity maturation in term infants. Am J Clin Nutr 87, 548-557.

Isaacs, E.B., Ross, S., Kennedy, K., Weaver, L.T., Lucas, A., Fewtrell, M.S., 2011. 10-year cognition in preterms after random assignment to fatty acid supplementation in infancy. Pediatrics 128, e890-898.

James, S., Montgomery, P., Williams, K., 2011. Omega-3 fatty acids supplementation for autism spectrum disorders (ASD). Cochrane Database Syst Rev, CD007992.

Jankowsky, J.L., Slunt, H.H., Gonzales, V., Jenkins, N.A., Copeland, N.G., Borchelt, D.R., 2004. APP processing and amyloid deposition in mice haplo-insufficient for presenilin 1. Neurobiol Aging 25, 885-892.

Jankowsky, J.L., Slunt, H.H., Ratovitski, T., Jenkins, N.A., Copeland, N.G., Borchelt, D.R., 2001. Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. Biomol Eng 17, 157-165.

Jansen, D., Janssen, C.I., Vanmierlo, T., Dederen, P.J., van Rooij, D., Zinnhardt, B., Nobelen, C.L., Janssen, A.L., Hafkemeijer, A., Mutsaers, M.P., Doedee, A.M., Kuipers, A.A., Broersen, L.M., Mulder, M., Kiliaan, A.J., 2012. Cholesterol and synaptic compensatory mechanisms in Alzheimer's disease mice brain during aging. J Alzheimers Dis 31, 813-826.

Jansen, D., Zerbi, V., Arnoldussen, I.A., Wiesmann, M., Rijpma, A., Fang, X.T., Dederen, P.J., Mutsaers, M.P., Broersen, L.M., Lutjohann, D., Miller, M., Joosten, L.A., Heerschap, A., Kiliaan, A.J., 2013a. Effects of specific multi-nutrient enriched diets on cerebral metabolism, cognition and neuropathology in AbetaPPswe-PS1dE9 mice. PLoS ONE 8, e75393.

Jansen, D., Zerbi, V., Janssen, C.I., van Rooij, D., Zinnhardt, B., Dederen, P.J., Wright, A.J., Broersen, L.M., Lutjohann, D., Heerschap, A., Kiliaan, A.J., 2013b. Impact of a multi-nutrient diet on cognition, brain metabolism, hemodynamics, and plasticity in apoE4 carrier and apoE knockout mice. Brain Struct Funct.

Jansen, D., Zerbi, V., Janssen, C.I., van Rooij, D., Zinnhardt, B., Dederen, P.J., Wright, A.J., Broersen, L.M.,

Lutjohann, D., Heerschap, A., Kiliaan, A.J., 2014. Impact of a multi-nutrient diet on cognition, brain metabolism, hemodynamics, and plasticity in apoE4 carrier and apoE knockout mice. Brain Struct Funct 219, 1841-1868.

Jansen, P.J., Lutjohann, D., Thelen, K.M., von Bergmann, K., van Leuven, F., Ramaekers, F.C., Monique, M., 2009. Absence of ApoE upregulates murine brain ApoD and ABCA1 levels, but does not affect brain sterol levels, while human ApoE3 and human ApoE4 upregulate brain cholesterol precursor levels. J Alzheimers Dis 18, 319-329.

Janssen, C.I., Zerbi, V., Mutsaers, M.P., de Jong, B.S., Wiesmann, M., Arnoldussen, I.A., Geenen, B., Heerschap, A., Muskiet, F.A., Jouni, Z.E., van Tol, E.A., Gross, G., Homberg, J.R., Berg, B.M., Kiliaan, A.J., 2015. Impact of dietary n-3 polyunsaturated fatty acids on cognition, motor skills and hippocampal neurogenesis in developing C57BL/6J mice. J Nutr Biochem 26, 24-35.

Janssen, C.I.F., Kiliaan, A.J., 2014. Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: The influence of LCPUFA on neural development, aging, and neurodegeneration. Prog Lipid Res 53, 1-17.

Jenner, A.M., Lim, W.L., Ng, M.P., Wenk, M.R., Shui, G., Sharman, M.J., Gandy, S.E., Martins, R.N., 2010. The effect of APOE genotype on brain levels of oxysterols in young and old human APOE epsilon2, epsilon3 and epsilon4 knock-in mice. Neuroscience 169, 109-115.

Jensen, C.L., Voigt, R.G., Prager, T.C., Zou, Y.L., Fraley, J.K., Rozelle, J.C., Turcich, M.R., Llorente, A.M., Anderson, R.E., Heird, W.C., 2005. Effects of maternal docosahexaenoic acid intake on visual function and neurodevelopment in breastfed term infants. Am J Clin Nutr 82, 125-132.

Ji, A., Diao, H., Wang, X., Yang, R., Zhang, J., Luo, W., Cao, R., Cao, Z., Wang, F., Cai, T., 2012. n-3 polyunsaturated fatty acids inhibit lipopolysaccharide-induced microglial activation and dopaminergic injury in rats. Neurotoxicology 33, 780-788.

Jicha, G.A., Markesbery, W.R., 2010. Omega-3 fatty acids: potential role in the management of early Alzheimer's disease. Clin Interv Aging 5, 45-61.

Johnson, K.R., Erway, L.C., Cook, S.A., Willott, J.F., Zheng, Q.Y., 1997. A major gene affecting age-related hearing loss in C57BL/6J mice. Hear Res 114, 83-92.

Johnson, M., Ostlund, S., Fransson, G., Kadesjo, B., Gillberg, C., 2009. Omega-3/omega-6 fatty acids for attention deficit hyperactivity disorder: a randomized placebo-controlled trial in children and adolescents. J Atten Disord 12, 394-401.

Joseph, J., Shukitt-Hale, B., Denisova, N.A., Martin, A., Perry, G., Smith, M.A., 2001. Copernicus revisited: amyloid beta in Alzheimer's disease. Neurobiol Aging 22, 131-146.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEwen, J.J., Bickford, P.C., 1999. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J Neurosci 19, 8114-8121.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Prior, R.L., Cao, G., Martin, A., Taglialatela, G., Bickford, P.C., 1998. Long-term dietary strawberry, spinach, or vitamin E supplementation retards the onset of age-related neuronal signal-transduction and cognitive behavioral deficits. J Neurosci 18, 8047-8055.

Judge, M.P., Harel, O., Lammi-Keefe, C.J., 2007a. A docosahexaenoic acid-functional food during pregnancy benefits infant visual acuity at four but not six months of age. Lipids 42, 117-122.

Judge, M.P., Harel, O., Lammi-Keefe, C.J., 2007b. Maternal consumption of a docosahexaenoic acid-containing functional food during pregnancy: benefit for infant performance on problem-solving but not on recognition memory tasks at age 9 mo. Am J Clin Nutr 85, 1572-1577.

Kalaria, R.N., 2010. Vascular basis for brain degeneration: faltering controls and risk factors for dementia. Nutr Rev 68, S74-S87.

Kalaria, R.N., Akinyemi, R., Ihara, M., 2012. Does vascular pathology contribute to Alzheimer changes? J Neurol Sci 322, 141-147.

Kamphuis, P.J., Verhey, F.R., Olde Rikkert, M.G., Twisk, J.W., Swinkels, S.H., Scheltens, P., 2011. Efficacy of a medical food on cognition in Alzheimer's disease: results from secondary analyses of a randomized, controlled trial. J Nutr Health Aging 15, 720-724.

Kanoski, S.E., Davidson, T.L., 2011. Western diet consumption and cognitive impairment: links to hippocampal dysfunction and obesity. Physiol Behav 103, 59-68.

Kariv-Inbal, Z., Yacobson, S., Berkecz, R., Peter, M., Janaky, T., Lutjohann, D., Broersen, L.M., Hartmann, T., Michaelson, D.M., 2012. The isoform-specific pathological effects of apoE4 *in vivo* are prevented by a fish oil (DHA) diet and are modified by cholesterol. J Alzheimers Dis 28, 667-683.

Kavraal, S., Oncu, S.K., Bitiktas, S., Artis, A.S., Dolu, N., Gunes, T., Suer, C., 2012. Maternal intake of Omega-3 essential fatty acids improves long term potentiation in the dentate gyrus and Morris water maze performance in rats. Brain Res 1482, 32-39.

Kennedy, E.P., Weiss, S.B., 1956. The function of cytidine coenzymes in the biosynthesis of phospholipides. J Biol Chem 222, 193-214.

Kim, J., Basak, J.M., Holtzman, D.M., 2009. The role of apolipoprotein E in Alzheimer's disease. Neuron 63, 287-303.

Kim, S.G., 1995. Quantification of relative cerebral blood flow change by flow-sensitive alternating inversion recovery (FAIR) technique: application to functional mapping. Magn Reson Med 34, 293-301.

Kiso, Y., 2011. Pharmacology in health foods: effects of arachidonic acid and docosahexaenoic acid on the age-related decline in brain and cardiovascular system function. J Pharmacol Sci 115, 471-475.

Kitajka, K., Sinclair, A.J., Weisinger, R.S., Weisinger, H.S., Mathai, M., Jayasooriya, A.P., Halver, J.E., Puskas, L.G., 2004. Effects of dietary omega-3 polyunsaturated fatty acids on brain gene expression. Proc Natl Acad Sci U S A 101, 10931-10936.

Klann, E., Dever, T.E., 2004. Biochemical mechanisms for translational regulation in synaptic plasticity. Nat Rev Neurosci 5, 931-942.

Knouff, C., Hinsdale, M.E., Mezdour, H., Altenburg, M.K., Watanabe, M., Quarfordt, S.H., Sullivan, P.M., Maeda, N., 1999. Apo E structure determines VLDL clearance and atherosclerosis risk in mice. J Clin Invest 103, 1579-1586.

Koletzko, B., Agostoni, C., Carlson, S.E., Clandinin, T., Hornstra, G., Neuringer, M., Uauy, R., Yamashiro, Y., Willatts, P., 2001. Long chain polyunsaturated fatty acids (LC-PUFA) and perinatal development. Acta Paediatr 90, 460-464.

Koletzko, B., Lien, E., Agostoni, C., Böhles, H., Campoy, C., Cetin, I., Decsi, T., Dudenhausen, J.W., Dupont, C., Forsyth, S., Hoesli, I., Holzgreve, W., Lapillonne, A., Putet, G., Secher, N.J., Symonds, M., Szajewska, H., Willatts, P., Uauy, R., 2008. The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. Journal of Perinatal Medicine 36, 5-14.

Koletzko, B., Schmidt, E., Bremer, H.J., Haug, M., Harzer, G., 1989. Effects of dietary long-chain polyunsaturated fatty acids on the essential fatty acid status of premature infants. Eur J Pediatr 148, 669-675.

Kooij, J.J., Buitelaar, J.K., van den Oord, E.J., Furer, J.W., Rijnders, C.A., Hodiamont, P.P., 2005. Internal and external validity of attention-deficit hyperactivity disorder in a population-based sample of adults. Psychol Med 35, 817-827.

Kotani, S., Sakaguchi, E., Warashina, S., Matsukawa, N., Ishikura, Y., Kiso, Y., Sakakibara, M., Yoshimoto, T., Guo, J., Yamashima, T., 2006. Dietary supplementation of arachidonic and docosahexaenoic acids improves cognitive dysfunction. Neurosci Res 56, 159-164.

Kris-Etherton, P.M., Harris, W.S., Appel, L.J., Committee, f.t.A.N., 2003. Omega-3 Fatty Acids and Cardiovascular Disease: New Recommendations From the American Heart Association. Arteriosclerosis, Thrombosis, and Vascular Biology 23, 151-152.

Kwong, K.K., McKinstry, R.C., Chien, D., Crawley, A.P., Pearlman, J.D., Rosen, B.R., 1991. CSF-suppressed quantitative single-shot diffusion imaging. Magn Reson Med 21, 157-163.

Kyle, D.J., Schaefer, E., Patton, G., Beiser, A., 1999. Low serum docosahexaenoic acid is a significant risk factor for Alzheimer's dementia. Lipids 34 Suppl, S245.

Labrousse, V.F., Nadjar, A., Joffre, C., Costes, L., Aubert, A., Gregoire, S., Bretillon, L., Laye, S., 2012. Short-term long chain omega3 diet protects from neuroinflammatory processes and memory impairment in aged mice. PLoS ONE 7, e36861.

Lapillonne, A., Groh-Wargo, S., Lozano Gonzalez, C.H., Uauy, R., 2013. Lipid needs of preterm infants: updated recommendations. J Pediatr 162, 052.

Larsen, R., Eilertsen, K.E., Elvevoll, E.O., 2011. Health benefits of marine foods and ingredients. Biotechnol Adv 29, 508-518.

Launer, L.J., Hofman, A., 2000. Frequency and impact of neurologic diseases in the elderly of Europe: A collaborative study of population-based cohorts. Neurology 54, S1-8.

Lauritzen, L., Jorgensen, M.H., Olsen, S.F., Straarup, E.M., Michaelsen, K.F., 2005. Maternal fish oil supplementation in lactation: effect on developmental outcome in breast-fed infants. Reprod Nutr Dev 45, 535-547.

Leaf, A., Kang, J.X., Xiao, Y.F., Billman, G.E., 2003. Clinical prevention of sudden cardiac death by n-3 polyunsaturated fatty acids and mechanism of prevention of arrhythmias by n-3 fish oils. Circulation 107, 2646-2652.

Lee, L.K., Shahar, S., Chin, A.V., Yusoff, N.A., 2013a. Docosahexaenoic acid-concentrated fish oil supplementation in subjects with mild cognitive impairment (MCI): a 12-month randomised, double-blind, placebo-controlled trial. Psychopharmacology (Berl) 225, 605-612.

Lee, Y.J., Choi, D.Y., Yun, Y.P., Han, S.B., Oh, K.W., Hong, J.T., 2013b. Epigallocatechin-3-gallate prevents systemic inflammation-induced memory deficiency and amyloidogenesis via its anti-neuroinflammatory properties. J Nutr Biochem 24, 298-310.

Levant, B., Radel, J.D., Carlson, S.E., 2004. Decreased brain docosahexaenoic acid during development alters dopamine-related behaviors in adult rats that are differentially affected by dietary remediation. Behav Brain Res 152, 49-57.

Levi, O., Jongen-Relo, A.L., Feldon, J., Roses, A.D., Michaelson, D.M., 2003. ApoE4 impairs hippocampal plasticity isoform-specifically and blocks the environmental stimulation of synaptogenesis and memory. Neurobiol Dis 13, 273-282.

Levi, O., Michaelson, D.M., 2007. Environmental enrichment stimulates neurogenesis in apolipoprotein E3 and neuronal apoptosis in apolipoprotein E4 transgenic mice. J Neurochem 100, 202-210.

Libby, P., Lichtman, A.H., Hansson, G.K., 2013. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. Immunity 38, 1092-1104.

Lorenz, M., Urban, J., Engelhardt, U., Baumann, G., Stangl, K., Stangl, V., 2009. Green and black tea are equally potent stimuli of NO production and vasodilation: new insights into tea ingredients involved. Basic Res Cardiol 104, 100-110.

Lucas, A., Stafford, M., Morley, R., Abbott, R., Stephenson, T., MacFadyen, U., Elias-Jones, A., Clements, H., 1999. Efficacy and safety of long-chain polyunsaturated fatty acid supplementation of infant-formula milk: a randomised trial. Lancet 354, 1948-1954.

Luchtman, D.W., Song, C., 2013. Cognitive enhancement by omega-3 fatty acids from child-hood to old age: findings from animal and clinical studies. Neuropharmacology 64, 550-565.

Lyall, K., Munger, K.L., O'Reilly, E.J., Santangelo, S.L., Ascherio, A., 2013. Maternal dietary fat intake in association with autism spectrum disorders. Am J Epidemiol 178, 209-220.

Maekawa, M., Takashima, N., Matsumata, M., Ikegami, S., Kontani, M., Hara, Y., Kawashima, H., Owada, Y., Kiso, Y., Yoshikawa, T., Inokuchi, K., Osumi, N., 2009. Arachidonic acid drives postnatal neurogenesis and elicits a beneficial effect on prepulse inhibition, a biological trait of psychiatric illnesses. PLoS ONE 4, e5085.

Mahley, R.W., Huang, Y., Weisgraber, K.H., 2006. Putting cholesterol in its place: apoE and reverse cholesterol transport. J Clin Invest 116, 1226-1229.

Mahmoudi, S., Samadi, P., Gilbert, F., Ouattara, B., Morissette, M., Gregoire, L., Rouillard, C., Di Paolo, T., Levesque, D., 2009. Nur77 mRNA levels and L-Dopa-induced dyskinesias in MPTP monkeys treated with docosahexaenoic acid. Neurobiol Dis 36, 213-222.

Makrides, M., Gibson, R.A., McPhee, A.J., Yelland, L., Quinlivan, J., Ryan, P., Team, D.O.I., 2010. Effect of DHA supplementation during pregnancy on maternal depression and neurodevelopment of young children: a randomized controlled trial. JAMA 304, 1675-1683.

Makrides, M., Neumann, M.A., Gibson, R.A., 1996. Effect of maternal docosahexaenoic acid (DHA)

supplementation on breast milk composition. Eur J Clin Nutr 50, 352-357.
Malcolm, C.A., McCulloch, D.L., Montgomery, C., Shepherd, A., Weaver, L.T., 2003. Maternal docosahexaenoic acid supplementation during pregnancy and visual evoked potential development in term infants: a double blind, prospective, randomised trial. Arch Dis Child Fetal Neonatal Ed 88, F383-390.
Manach, C., Williamson, G., Morand, C., Scalbert, A., Remesy, C., 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 81, 230S-242S.
Masliah, E., Crews, L., Hansen, L., 2006. Synaptic remodeling during aging and in Alzheimer's disease. J Alzheimers Dis 9, 91-99.
Masliah, E., Mallory, M., Hansen, L., Alford, M., Deteresa, R., Terry, R., 1993. An Antibody against Phosphorylated Neurofilaments Identifies a Subset of Damaged Association Axons in Alzheimers-Disease. American Journal of Pathology 142, 871-882.
Mathieu, G., Oualian, C., Denis, I., Lavialle, M., Gisquet-Verrier, P., Vancassel, S., 2011. Dietary n-3 polyunsaturated fatty acid deprivation together with early maternal separation increases anxiety and vulnerability to stress in adult rats. Prostaglandins Leukot Essent Fatty Acids 85, 129-136.
Matsuo, N., Takao, K., Nakanishi, K., Yamasaki, N., Tanda, K., Miyakawa, T., 2010. Behavioral profiles of three C57BL/6 substrains. Front Behav Neurosci 4, 29.
McNamara, R.K., Able, J., Jandacek, R., Rider, T., Tso, P., Lindquist, D.M., 2009. Perinatal n-3 fatty acid deficiency selectively reduces myo-inositol levels in the adult rat PFC: an *in vivo* (1)H-MRS study. J Lipid Res 50, 405-411.
McNamara, R.K., Carlson, S.E., 2006. Role of omega-3 fatty acids in brain development and function: potential implications for the pathogenesis and prevention of psychopathology. Prostaglandins Leukot Essent Fatty Acids 75, 329-349.
McNamara, R.K., Jandacek, R., Rider, T., Tso, P., Dwivedi, Y., Pandey, G.N., 2013a. Adult medication-free schizophrenic patients exhibit long-chain omega-3 Fatty Acid deficiency: implications for cardiovascular disease risk. Cardiovasc Psychiatry Neurol 2013, 796462.
McNamara, R.K., Jandacek, R., Tso, P., Weber, W., Chu, W.J., Strakowski, S.M., Adler, C.M., Delbello, M.P., 2013b. Low docosahexaenoic acid status is associated with reduced indices in cortical integrity in the anterior cingulate of healthy male children: a 1H MRS Study. Nutr Neurosci 16, 183-190.
Meguid, N.A., Atta, H.M., Gouda, A.S., Khalil, R.O., 2008. Role of polyunsaturated fatty acids in the management of Egyptian children with autism. Clin Biochem 41, 1044-1048.
Meldrum, S.J., Smith, M.A., Prescott, S.L., Hird, K., Simmer, K., 2011. Achieving definitive results in long-chain polyunsaturated fatty acid supplementation trials of term infants: factors for consideration. Nutr Rev 69, 205-214.
Michikawa, M., Fan, Q.W., Isobe, I., Yanagisawa, K., 2000. Apolipoprotein E exhibits isoform-specific promotion of lipid efflux from astrocytes and neurons in culture. J Neurochem 74, 1008-1016.
Milte, C.M., Parletta, N., Buckley, J.D., Coates, A.M., Young, R.M., Howe, P.R., 2012. Eicosapentaenoic and docosahexaenoic acids, cognition, and behavior in children with attention-deficit/hyperactivity disorder: a randomized controlled trial. Nutrition 28, 670-677.
Miyazawa, D., Yasui, Y., Yamada, K., Ohara, N., Okuyama, H., 2010. Regional differences of the mouse brain in response to an alpha-linolenic acid-restricted diet: Neurotrophin content and protein kinase activity. Life Sci 87, 490-494.
Molteni, R., Barnard, R.J., Ying, Z., Roberts, C.K., Gomez-Pinilla, F., 2002. A high-fat, refined sugar diet reduces hippocampal brain-derived neurotrophic factor, neuronal plasticity, and learning. Neuroscience 112, 803-814.
Moretti, L., Cristofori, I., Weaver, S.M., Chau, A., Portelli, J.N., Grafman, J., 2012. Cognitive decline in older adults with a history of traumatic brain injury. Lancet Neurol 11, 1103-1112.
Mori, T.A., Bao, D.Q., Burke, V., Puddey, I.B., Beilin, L.J., 1999. Docosahexaenoic acid but not eicosapentaenoic acid lowers ambulatory blood pressure and heart rate in humans. Hypertension 34, 253-260.
Moriguchi, T., Greiner, R.S., Salem, N., Jr., 2000. Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. J Neurochem 75, 2563-2573.

Moriguchi, T., Loewke, J., Garrison, M., Catalan, J.N., Salem, N., Jr., 2001. Reversal of docosahexaenoic acid deficiency in the rat brain, retina, liver, and serum. J Lipid Res 42, 419-427.

Moriguchi, T., Salem, N., 2003. Recovery of brain docosahexaenoate leads to recovery of spatial task performance. J Neurochem 87, 297-309.

Mostofsky, D.I., Schlomo Yehuda, Norman Salem Jr., 2001. Fatty acids: Physiological and behavioral functions, In: Bendich, A. (Ed.), Nutr Health,Humana Press Inc., Totowa.

Mozaffarian, D., Wu, J.H., 2012. (n-3) fatty acids and cardiovascular health: are effects of EPA and DHA shared or complementary? J Nutr 142, 614S-625S.

Mulder, M., Blokland, A., van den Berg, D.J., Schulten, H., Bakker, A.H., Terwel, D., Honig, W., de Kloet, E.R., Havekes, L.M., Steinbusch, H.W., de Lange, E.C., 2001. Apolipoprotein E protects against neuropathology induced by a high-fat diet and maintains the integrity of the blood-brain barrier during aging. Lab Invest 81, 953-960.

Mulder, M., Koopmans, G., Wassink, G., Al Mansouri, G., Simard, M.L., Havekes, L.M., Prickaerts, J., Blokland, A., 2007. LDL receptor deficiency results in decreased cell proliferation and presynaptic bouton density in the murine hippocampus. Neurosci Res 59, 251-256.

Muller, M., Tang, M.X., Schupf, N., Manly, J.J., Mayeux, R., Luchsinger, J.A., 2007. Metabolic syndrome and dementia risk in a multiethnic elderly cohort. Dement Geriatr Cogn Disord 24, 185-192.

Muskiet, F.A.J., 2010. Pathophysiology and Evolutionary Aspects of Dietary Fats and Long-Chain Polyunsaturated Fatty Acids across the Life Cycle, In: Montmayeur, J.l.C., J (Ed.), Fat Detection: Taste, Texture, and Post Ingestive Effects.,CRC Press, Boca Raton (FL).

Nakashima, Y., Yuasa, S., Hukamizu, Y., Okuyama, H., Ohhara, T., Kameyama, T., Nabeshima, T., 1993. Effect of a high linoleate and a high alpha-linolenate diet on general behavior and drug sensitivity in mice. J Lipid Res 34, 239-247.

Nehlig, A., 2013. The neuroprotective effects of cocoa flavanol and its influence on cognitive performance. Br J Clin Pharmacol 75, 716-727.

Niculescu, M.D., Lupu, D.S., Craciunescu, C.N., 2011. Maternal alpha-linolenic acid availability during gestation and lactation alters the postnatal hippocampal development in the mouse offspring. Int J Dev Neurosci 29, 795-802.

Nilsson, A., Radeborg, K., Salo, I., Bjorck, I., 2012. Effects of supplementation with n-3 polyunsaturated fatty acids on cognitive performance and cardiometabolic risk markers in healthy 51 to 72 years old subjects: a randomized controlled cross-over study. Nutr J 11, 99.

Nishimura, K., Takahashi, J., 2013. Therapeutic Application of Stem Cell Technology toward the Treatment of Parkinson’s Disease. Biological and Pharmaceutical Bulletin 36, 171-175.

Nishizawa, M., Hara, T., Miura, T., Fujita, S., Yoshigai, E., Ue, H., Hayashi, Y., Kwon, A.H., Okumura, T., Isaka, T., 2011. Supplementation with a flavanol-rich lychee fruit extract influences the inflammatory status of young athletes. Phytother Res 25, 1486-1493.

O'Connor, D.L., Hall, R., Adamkin, D., Auestad, N., Castillo, M., Connor, W.E., Connor, S.L., Fitzgerald, K., Groh-Wargo, S., Hartmann, E.E., Jacobs, J., Janowsky, J., Lucas, A., Margeson, D., Mena, P., Neuringer, M., Nesin, M., Singer, L., Stephenson, T., Szabo, J., Zemon, V., Ross Preterm Lipid, S., 2001. Growth and development in preterm infants fed long-chain polyunsaturated fatty acids: a prospective, randomized controlled trial. Pediatrics 108, 359-371.

O'Leary, T.P., Gunn, R.K., Brown, R.E., 2013. What are we measuring when we test strain differences in anxiety in mice? Behav Genet 43, 34-50.

Oitzl, M.S., Mulder, M., Lucassen, P.J., Havekes, L.M., Grootendorst, J., de Kloet, E.R., 1997. Severe learning deficits in apolipoprotein E-knockout mice in a water maze task. Brain Res 752, 189-196.

Oksman, M., Iivonen, H., Hogyes, E., Amtul, Z., Penke, B., Leenders, I., Broersen, L., Lutjohann, D., Hartmann, T., Tanila, H., 2006. Impact of different saturated fatty acid, polyunsaturated fatty acid and cholesterol containing diets on beta-amyloid accumulation in APP/PS1 transgenic mice. Neurobiol Dis 23, 563-572.

Okuda, N., Ueshima, H., Okayama, A., Saitoh, S., Nakagawa, H., Rodriguez, B.L., Sakata, K., Choudhury, S.R., Curb, J.D., Stamler, J., Group, I.R., 2005. Relation of long chain n-3 polyunsaturated fatty acid intake to

serum high density lipoprotein cholesterol among Japanese men in Japan and Japanese-American men in Hawaii: the INTERLIPID study. Atherosclerosis 178, 371-379.

Ophir, G., Amariglio, N., Jacob-Hirsch, J., Elkon, R., Rechavi, G., Michaelson, D.M., 2005. Apolipoprotein E4 enhances brain inflammation by modulation of the NF-kappaB signaling cascade. Neurobiol Dis 20, 709-718.

Ozsoy, O., Seval-Celik, Y., Hacioglu, G., Yargicoglu, P., Demir, R., Agar, A., Aslan, M., 2011. The influence and the mechanism of docosahexaenoic acid on a mouse model of Parkinson's disease. Neurochem Int 59, 664-670.

Parain, K., Murer, M.G., Yan, Q., Faucheux, B., Agid, Y., Hirsch, E., Raisman-Vozari, R., 1999. Reduced expression of brain-derived neurotrophic factor protein in Parkinson's disease substantia nigra. Neuroreport 10, 557-561.

Pauwels, E.K., Volterrani, D., Mariani, G., Kairemo, K., 2009. Fatty acid facts, Part IV: docosahexaenoic acid and Alzheimer's disease. A story of mice, men and fish. Drug News Perspect 22, 205-213.

Pearce, J.M., Komoroski, R.A., Mrak, R.E., 2009. Phospholipid composition of postmortem schizophrenic brain by 31P NMR spectroscopy. Magn Reson Med 61, 28-34.

Perera, H., Jeewandara, K.C., Seneviratne, S., Guruge, C., 2012. Combined omega3 and omega6 supplementation in children with attention-deficit hyperactivity disorder (ADHD) refractory to methylphenidate treatment: a double-blind, placebo-controlled study. J Child Neurol 27, 747-753.

Perez, M.A., Terreros, G., Dagnino-Subiabre, A., 2013. Long-term omega-3 fatty acid supplementation induces anti-stress effects and improves learning in rats. Behav Brain Funct 9, 25.

Piedrahita, J.A., Zhang, S.H., Hagaman, J.R., Oliver, P.M., Maeda, N., 1992. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc Natl Acad Sci U S A 89, 4471-4475.

Pivik, R.T., Dykman, R.A., Jing, H., Gilchrist, J.M., Badger, T.M., 2009. Early infant diet and the omega 3 fatty acid DHA: effects on resting cardiovascular activity and behavioral development during the first half-year of life. Dev Neuropsychol 34, 139-158.

Politi, P., Cena, H., Comelli, M., Marrone, G., Allegri, C., Emanuele, E., Ucelli di Nemi, S., 2008. Behavioral effects of omega-3 fatty acid supplementation in young adults with severe autism: an open label study. Arch Med Res 39, 682-685.

Puri, B.K., Koepp, M.J., Holmes, J., Hamilton, G., Yuen, A.W., 2007. A 31-phosphorus neurospectroscopy study of omega-3 long-chain polyunsaturated fatty acid intervention with eicosapentaenoic acid and docosahexaenoic acid in patients with chronic refractory epilepsy. Prostaglandins Leukot Essent Fatty Acids 77, 105-107.

Qiu, C., 2012. Preventing Alzheimer's disease by targeting vascular risk factors: hope and gap. J Alzheimers Dis 32, 721-731.

Quinn, J.F., Raman, R., Thomas, R.G., Yurko-Mauro, K., Nelson, E.B., Van Dyck, C., Galvin, J.E., Emond, J., Jack, C.R., Jr., Weiner, M., Shinto, L., Aisen, P.S., 2010. Docosahexaenoic acid supplementation and cognitive decline in Alzheimer disease: a randomized trial. JAMA 304, 1903-1911.

Raber, J., Wong, D., Buttini, M., Orth, M., Bellosta, S., Pitas, R.E., Mahley, R.W., Mucke, L., 1998. Isoform-specific effects of human apolipoprotein E on brain function revealed in ApoE knockout mice: increased susceptibility of females. Proc Natl Acad Sci U S A 95, 10914-10919.

Rao, J.S., Ertley, R.N., Lee, H.J., DeMar, J.C., Jr., Arnold, J.T., Rapoport, S.I., Bazinet, R.P., 2007. n-3 polyunsaturated fatty acid deprivation in rats decreases frontal cortex BDNF via a p38 MAPK-dependent mechanism. Mol Psychiatry 12, 36-46.

Rapp, A., Gmeiner, B., Huttinger, M., 2006. Implication of apoE isoforms in cholesterol metabolism by primary rat hippocampal neurons and astrocytes. Biochimie 88, 473-483.

Raz, N., Gunning, F.M., Head, D., Dupuis, J.H., McQuain, J., Briggs, S.D., Loken, W.J., Thornton, A.E., Acker, J.D., 1997. Selective aging of the human cerebral cortex observed *in vivo*: Differential vulnerability of the prefrontal gray matter. Cerebral Cortex 7, 268-282.

Raz, R., Carasso, R.L., Yehuda, S., 2009. The influence of short-chain essential fatty acids on children with

attention-deficit/hyperactivity disorder: a double-blind placebo-controlled study. J Child Adolesc Psychopharmacol 19, 167-177.

Reeves, P.G., Nielsen, F.H., Fahey, G.C., Jr., 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123, 1939-1951.

Renaud, S., de Lorgeril, M., Delaye, J., Guidollet, J., Jacquard, F., Mamelle, N., Martin, J.L., Monjaud, I., Salen, P., Toubol, P., 1995. Cretan Mediterranean diet for prevention of coronary heart disease. Am J Clin Nutr 61, 1360S-1367S.

Rendeiro, C., Guerreiro, J.D., Williams, C.M., Spencer, J.P., 2012. Flavonoids as modulators of memory and learning: molecular interactions resulting in behavioural effects. Proc Nutr Soc 71, 246-262.

Rendeiro, C., Spencer, J.P., Vauzour, D., Butler, L.T., Ellis, J.A., Williams, C.M., 2009. The impact of flavonoids on spatial memory in rodents: from behaviour to underlying hippocampal mechanisms. Genes Nutr 4, 251-270.

Rendeiro, C., Vauzour, D., Rattray, M., Waffo-Teguo, P., Merillon, J.M., Butler, L.T., Williams, C.M., Spencer, J.P., 2013. Dietary levels of pure flavonoids improve spatial memory performance and increase hippocampal brain-derived neurotrophic factor. PLoS ONE 8, e63535.

Richardson, A.J., 2006. Omega-3 fatty acids in ADHD and related neurodevelopmental disorders. Int Rev Psychiatry 18, 155-172.

Rijpma, A., Jansen, D., Arnoldussen, I.A.C., Fang, X.T., Wiesmann, M., Mutsaers, M.P.C., Dederen, P.J., Janssen, C.I.F., Kiliaan , A.J., 2013. Sex Differences in Presynaptic Density and Neurogenesis in Middle-Aged ApoE4 and ApoE Knockout Mice. Journal of Neurodegenerative Diseases, 9.

Rodrigues, J., Assuncao, M., Lukoyanov, N., Cardoso, A., Carvalho, F., Andrade, J.P., 2013. Protective effects of a catechin-rich extract on the hippocampal formation and spatial memory in aging rats. Behav Brain Res 246, 94-102.

Ruitenberg, A., Skoog, I., Ott, A., Aevarsson, O., Witteman, J.C., Lernfelt, B., van Harskamp, F., Hofman, A., Breteler, M.M., 2001. Blood pressure and risk of dementia: results from the Rotterdam study and the Gothenburg H-70 Study. Dement Geriatr Cogn Disord 12, 33-39.

Rutten, B.P., Van der Kolk, N.M., Schafer, S., van Zandvoort, M.A., Bayer, T.A., Steinbusch, H.W., Schmitz, C., 2005. Age-related loss of synaptophysin immunoreactive presynaptic boutons within the hippocampus of APP751SL, PS1M146L, and APP751SL/PS1M146L transgenic mice. American Journal of Pathology 167, 161-173.

Rzehak, P., Heinrich, J., Klopp, N., Schaeffer, L., Hoff, S., Wolfram, G., Illig, T., Linseisen, J., 2009. Evidence for an association between genetic variants of the fatty acid desaturase 1 fatty acid desaturase 2 (FADS1 FADS2) gene cluster and the fatty acid composition of erythrocyte membranes. Br J Nutr 101, 20-26.

Salat, D.H., Kaye, J.A., Janowsky, J.S., 1999. Prefrontal gray and white matter volumes in healthy aging and Alzheimer disease. Arch Neurol 56, 338-344.

Samii, A., Nutt, J.G., Ransom, B.R., 2004. Parkinson's disease. The Lancet 363, 1783-1793.

Sanae, F., Miyaichi, Y., Kizu, H., Hayashi, H., 2002. Effects of catechins on vascular tone in rat thoracic aorta with endothelium. Life Sci 71, 2553-2562.

Sasaki, K., Han, J., Shimozono, H., Villareal, M.O., Isoda, H., 2013. Caffeoylquinic acid-rich purple sweet potato extract, with or without anthocyanin, imparts neuroprotection and contributes to the improvement of spatial learning and memory of SAMP8 mouse. J Agric Food Chem 61, 5037-5045.

Saste, M.D., Carver, J.D., Stockard, J.E., Benford, V.J., Chen, L.T., Phelps, C.P., 1998. Maternal diet fatty acid composition affects neurodevelopment in rat pups. J Nutr 128, 740-743.

Sastry, P.S., 1985. Lipids of nervous tissue: composition and metabolism. Prog Lipid Res 24, 69-176.

Scarmeas, N., Luchsinger, J.A., Mayeux, R., Stern, Y., 2007. Mediterranean diet and Alzheimer disease mortality. Neurology 69, 1084-1093.

Scarmeas, N., Stern, Y., Tang, M.X., Mayeux, R., Luchsinger, J.A., 2006. Mediterranean diet and risk for Alzheimer's disease. Ann Neurol 59, 912-921.

Schaeffer, L., Gohlke, H., Muller, M., Heid, I.M., Palmer, L.J., Kompauer, I., Demmelmair, H., Illig, T., Koletzko,

B., Heinrich, J., 2006. Common genetic variants of the FADS1 FADS2 gene cluster and their reconstructed haplotypes are associated with the fatty acid composition in phospholipids. Hum Mol Genet 15, 1745-1756.

Scheltens, P., Kamphuis, P.J., Verhey, F.R., Olde Rikkert, M.G., Wurtman, R.J., Wilkinson, D., Twisk, J.W., Kurz, A., 2010. Efficacy of a medical food in mild Alzheimer's disease: A randomized, controlled trial. Alzheimers Dement 6, 1-10 e11.

Scheltens, P., Twisk, J.W., Blesa, R., Scarpini, E., von Arnim, C.A., Bongers, A., Harrison, J., Swinkels, S.H., Stam, C.J., de Waal, H., Wurtman, R.J., Wieggers, R.L., Vellas, B., Kamphuis, P.J., 2012. Efficacy of Souvenaid in mild Alzheimer's disease: results from a randomized, controlled trial. J Alzheimers Dis 31, 225-236.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3, 1101-1108.

Schmitz, G., Ecker, J., 2008. The opposing effects of n-3 and n-6 fatty acids. Prog Lipid Res 47, 147-155.

Schupbach, W.M., Maltete, D., Houeto, J.L., du Montcel, S.T., Mallet, L., Welter, M.L., Gargiulo, M., Behar, C., Bonnet, A.M., Czernecki, V., Pidoux, B., Navarro, S., Dormont, D., Cornu, P., Agid, Y., 2007. Neurosurgery at an earlier stage of Parkinson disease: a randomized, controlled trial. Neurology 68, 267-271.

Scott, D.T., Janowsky, J.S., Carroll, R.E., Taylor, J.A., Auestad, N., Montalto, M.B., 1998. Formula supplementation with long-chain polyunsaturated fatty acids: are there developmental benefits? Pediatrics 102, E59.

Sethom, M.M., Fares, S., Bouaziz, N., Melki, W., Jemaa, R., Feki, M., Hechmi, Z., Kaabachi, N., 2010. Polyunsaturated fatty acids deficits are associated with psychotic state and negative symptoms in patients with schizophrenia. Prostaglandins Leukot Essent Fatty Acids 83, 131-136.

Shah, R.C., 2011. Medical foods for Alzheimer's disease. Drugs Aging 28, 421-428.

Shah, Z.A., Li, R.C., Ahmad, A.S., Kensler, T.W., Yamamoto, M., Biswal, S., Dore, S., 2010. The flavanol (-)-epicatechin prevents stroke damage through the Nrf2/HO1 pathway. J Cereb Blood Flow Metab 30, 1951-1961.

Shen, J.Z., Zheng, X.F., Wei, E.Q., Kwan, C.Y., 2003. Green tea catechins evoke a phasic contraction in rat aorta via H2O2-mediated multiple-signalling pathways. Clin Exp Pharmacol Physiol 30, 88-95.

Sidhu, V.K., Huang, B.X., Kim, H.Y., 2011. Effects of Docosahexaenoic Acid on Mouse Brain Synaptic Plasma Membrane Proteome Analyzed by Mass Spectrometry and O-16/O-18 Labeling. Journal of Proteome Research 10, 5472-5480.

Siegel, G., Ermilov, E., 2012. Omega-3 fatty acids: benefits for cardio-cerebro-vascular diseases. Atherosclerosis 225, 291-295.

Simmer, K., Patole, S.K., Rao, S.C., 2011. Long-chain polyunsaturated fatty acid supplementation in infants born at term. Cochrane Database Syst Rev, CD000376.

Simopoulos, A.P., 2008a. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med (Maywood) 233, 674-688.

Simopoulos, A.P., 2008b. The omega-6/omega-3 fatty acid ratio, genetic variation, and cardiovascular disease. Asia Pac J Clin Nutr 17 Suppl 1, 131-134.

Simopoulos, A.P., 2010. Genetic variants in the metabolism of omega-6 and omega-3 fatty acids: their role in the determination of nutritional requirements and chronic disease risk. Exp Biol Med (Maywood) 235, 785-795.

Simopoulos, A.P., 2011. Evolutionary aspects of diet: the omega-6/omega-3 ratio and the brain. Mol Neurobiol 44, 203-215.

Singer, P., Hauser, J., Llano Lopez, L., Peleg-Raibstein, D., Feldon, J., Gargiulo, P.A., Yee, B.K., 2013. Prepulse inhibition predicts working memory performance whilst startle habituation predicts spatial reference memory retention in C57BL/6 mice. Behav Brain Res 242, 166-177.

Singh, R.B., Gupta, S., Dherange, P., De Meester, F., Wilczynska, A., Alam, S.E., Pella, D., Wilson, D.W., 2012. Metabolic syndrome: a brain disease. Can J Physiol Pharmacol 90, 1171-1183.

Sinn, N., Milte, C.M., Street, S.J., Buckley, J.D., Coates, A.M., Petkov, J., Howe, P.R., 2012. Effects of n-3 fatty acids, EPA v. DHA, on depressive symptoms, quality of life, memory and executive function in older adults with mild cognitive impairment: a 6-month randomised controlled trial. Br J Nutr 107, 1682-1693.

Skoog, I., Gustafson, D., 2006. Update on hypertension and Alzheimer's disease. Neurol Res 28, 605-611.

Smithers, L.G., Collins, C.T., Simmonds, L.A., Gibson, R.A., McPhee, A., Makrides, M., 2010. Feeding preterm infants milk with a higher dose of docosahexaenoic acid than that used in current practice does not influence language or behavior in early childhood: a follow-up study of a randomized controlled trial. Am J Clin Nutr 91, 628-634.

Smithers, L.G., Gibson, R.A., Makrides, M., 2011. Maternal supplementation with docosahexaenoic acid during pregnancy does not affect early visual development in the infant: a randomized controlled trial. Am J Clin Nutr 93, 1293-1299.

Smithers, L.G., Gibson, R.A., McPhee, A., Makrides, M., 2008. Higher dose of docosahexaenoic acid in the neonatal period improves visual acuity of preterm infants: results of a randomized controlled trial. Am J Clin Nutr 88, 1049-1056.

Sofi, F., Giorgi, G., Cesari, F., Gori, A.M., Mannini, L., Parisi, G., Casini, A., Abbate, R., Gensini, G.F., Poli, B.M., 2013. The atherosclerotic risk profile is affected differently by fish flesh with a similar EPA and DHA content but different n-6/n-3 ratio. Asia Pac J Clin Nutr 22, 32-40.

Sorgi, P.J., Hallowell, E.M., Hutchins, H.L., Sears, B., 2007. Effects of an open-label pilot study with high-dose EPA/DHA concentrates on plasma phospholipids and behavior in children with attention deficit hyperactivity disorder. Nutr J 6, 16.

Sorond, F.A., Lipsitz, L.A., Hollenberg, N.K., Fisher, N.D., 2008. Cerebral blood flow response to flavanol-rich cocoa in healthy elderly humans. Neuropsychiatr Dis Treat 4, 433-440.

Spencer, J.P., The impact of fruit flavonoids on memory and cognition. Br J Nutr 104 Suppl 3, S40-47.

Spencer, J.P., 2009. Flavonoids and brain health: multiple effects underpinned by common mechanisms. Genes Nutr 4, 243-250.

Spencer, J.P., 2010. The impact of fruit flavonoids on memory and cognition. Br J Nutr 104 Suppl 3, S40-47.

Spieles-Engemann, A.L., Behbehani, M.M., Collier, T.J., Wohlgenant, S.L., Steece-Collier, K., Paumier, K., Daley, B.F., Gombash, S., Madhavan, L., Mandybur, G.T., Lipton, J.W., Terpstra, B.T., Sortwell, C.E., 2010. Stimulation of the rat subthalamic nucleus is neuroprotective following significant nigral dopamine neuron loss. Neurobiol Dis 39, 105-115.

Spinney, L., 2014. Alzheimer's disease: The forgetting gene. Nature 510, 26-28.

Stojakovic, T., Scharnagl, H., Marz, W., 2004. ApoE: crossroads between Alzheimer's disease and atherosclerosis. Semin Vasc Med 4, 279-285.

Storck Lindholm, E., Strandvik, B., Altman, D., Moller, A., Palme Kilander, C., 2013. Different fatty acid pattern in breast milk of obese compared to normal-weight mothers. Prostaglandins Leukot Essent Fatty Acids 88, 211-217.

Stough, C., Downey, L., Silber, B., Lloyd, J., Kure, C., Wesnes, K., Camfield, D., 2012. The effects of 90-day supplementation with the omega-3 essential fatty acid docosahexaenoic acid (DHA) on cognitive function and visual acuity in a healthy aging population. Neurobiol Aging 33, 824 e821-823.

Streijger, F., Oerlemans, F., Ellenbroek, B.A., Jost, C.R., Wieringa, B., Van der Zee, C.E., 2005. Structural and behavioural consequences of double deficiency for creatine kinases BCK and UbCKmit. Behav Brain Res 157, 219-234.

Suchy, J., Chan, A., Shea, T.B., 2009. Dietary supplementation with a combination of alpha-lipoic acid, acetyl-L-carnitine, glycerophosphocoline, docosahexaenoic acid, and phosphatidylserine reduces oxidative damage to murine brain and improves cognitive performance. Nutr Res 29, 70-74.

Suckow, M.A., Danneman, P., Brayton, C., 2001. The Laboratory Mouse. CRC Press LLC.

Takizawa, S., Fukuyama, N., Hirabayashi, H., Kohara, S., Kazahari, S., Shinohara, Y., Nakazawa, H., 2003. Quercetin, a natural flavonoid, attenuates vacuolar formation in the optic tract in rat chronic cerebral hypoperfusion model. Brain Res 980, 156-160.

Tanriover, G., Seval-Celik, Y., Ozsoy, O., Akkoyunlu, G., Savcioglu, F., Hacioglu, G., Demir, N., Agar, A., 2010. The effects of docosahexaenoic acid on glial derived neurotrophic factor and neurturin in bilateral rat model of Parkinson's disease. Folia Histochem Cytobiol 48, 434-441.

Tanzi, R.E., Bertram, L., 2001. New frontiers in Alzheimer's disease genetics. Neuron 32, 181-184.

Tapiero, H., Ba, G.N., Couvreur, P., Tew, K.D., 2002. Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. Biomed Pharmacother 56, 215-222.

Tassoni, D., Kaur, G., Weisinger, R.S., Sinclair, A.J., 2008. The role of eicosanoids in the brain. Asia Pac J Clin Nutr 17, 220-228.

Terano, T., Fujishiro, S., Ban, T., Yamamoto, K., Tanaka, T., Noguchi, Y., Tamura, Y., Yazawa, K., Hirayama, T., 1999. Docosahexaenoic acid supplementation improves the moderately severe dementia from thrombotic cerebrovascular diseases. Lipids 34 Suppl, S345-346.

Thambisetty, M., Beason-Held, L., An, Y., Kraut, M.A., Resnick, S.M., APOE epsilon4 genotype and longitudinal changes in cerebral blood flow in normal aging. Arch Neurol 67, 93-98.

Thies, F., Garry, J.M., Yaqoob, P., Rerkasem, K., Williams, J., Shearman, C.P., Gallagher, P.J., Calder, P.C., Grimble, R.F., 2003. Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. Lancet 361, 477-485.

Tian, S.S., Jiang, F.S., Zhang, K., Zhu, X.X., Jin, B., Lu, J.J., Ding, Z.S., 2014. Flavonoids from the leaves of Carya cathayensis Sarg. inhibit vascular endothelial growth factor-induced angiogenesis. Fitoterapia 92, 34-40.

Tisserand, D.J., Pruessner, J.C., Arigita, E.J.S., van Boxtel, M.P.J., Evans, A.C., Jolles, J., Uylings, H.B.M., 2002. Regional frontal cortical volumes decrease differentially in aging: An MRI study to compare volumetric approaches and voxel-based morphometry. Neuroimage 17, 657-669.

Umezawa, M., Ohta, A., Tojo, H., Yagi, H., Hosokawa, M., Takeda, T., 1995. Dietary alpha-linolenate/linoleate balance influences learning and memory in the senescence-accelerated mouse (SAM). Brain Res 669, 225-233.

van de Rest, O., Geleijnse, J.M., Kok, F.J., van Staveren, W.A., Dullemeijer, C., Olderikkert, M.G., Beekman, A.T., de Groot, C.P., 2008. Effect of fish oil on cognitive performance in older subjects: a randomized, controlled trial. Neurology 71, 430-438.

van Duynhoven, J., Vaughan, E.E., Jacobs, D.M., Kemperman, R.A., van Velzen, E.J., Gross, G., Roger, L.C., Possemiers, S., Smilde, A.K., Dore, J., Westerhuis, J.A., Van de Wiele, T., 2011. Metabolic fate of polyphenols in the human superorganism. Proc Natl Acad Sci U S A 108 Suppl 1, 4531-4538.

van Goor, S.A., Dijck-Brouwer, D.A., Doornbos, B., Erwich, J.J., Schaafsma, A., Muskiet, F.A., Hadders-Algra, M., 2010. Supplementation of DHA but not DHA with arachidonic acid during pregnancy and lactation influences general movement quality in 12-week-old term infants. Br J Nutr 103, 235-242.

van Goor, S.A., Dijck-Brouwer, D.A.J., Erwich, J.J.H.M., Schaafsma, A., Hadders-Algra, M., 2011a. The influence of supplemental docosahexaenoic and arachidonic acids during pregnancy and lactation on neurodevelopment at eighteen months. Prostaglandins, Leukotrienes and Essential Fatty Acids 84, 139-146.

van Goor, S.A., Janneke Dijck-Brouwer, D.A., Erwich, J.J.H.M., Schaafsma, A., Hadders-Algra, M., 2011b. The influence of supplemental docosahexaenoic and arachidonic acids during pregnancy and lactation on neurodevelopment at eighteen months. Prostaglandins, Leukotrienes and Essential Fatty Acids 84, 139-146.

Vauzour, D., 2012. Dietary polyphenols as modulators of brain functions: biological actions and molecular mechanisms underpinning their beneficial effects. Oxid Med Cell Longev 2012, 914273.

Vauzour, D., 2013. Effect of flavonoids on learning, memory and neurocognitive performance: relevance and potential implications for Alzheimer's disease pathophysiology. J Sci Food Agric.

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

Voigt, R.G., Llorente, A.M., Jensen, C.L., Fraley, J.K., Berretta, M.C., Heird, W.C., 2001. A randomized, double-blind, placebo-controlled trial of docosahexaenoic acid supplementation in children with attention-deficit/hyperactivity disorder. J Pediatr 139, 189-196.

von Schacky, C., Harris, W.S., 2007. Cardiovascular risk and the omega-3 index. J Cardiovasc Med (Hagerstown) 8 Suppl 1, S46-49.

Wall, R., Ross, R.P., Fitzgerald, G.F., Stanton, C., 2010. Fatty acids from fish: the anti-inflammatory potential of

long-chain omega-3 fatty acids. Nutr Rev 68, 280-289.
Warner, T.T., Schapira, A.H., 2003. Genetic and environmental factors in the cause of Parkinson's disease. Ann Neurol 53 Suppl 3, S16-23; discussion S23-15.
Wendell, C.R., Waldstein, S.R., Ferrucci, L., O'Brien, R.J., Strait, J.B., Zonderman, A.B., 2012. Carotid atherosclerosis and prospective risk of dementia. Stroke 43, 3319-3324.
Westerberg, A.C., Schei, R., Henriksen, C., Smith, L., Veierod, M.B., Drevon, C.A., Iversen, P.O., 2011. Attention among very low birth weight infants following early supplementation with docosahexaenoic and arachidonic acid. Acta Paediatr 100, 47-52.
Wightman, E.L., Haskell, C.F., Forster, J.S., Veasey, R.C., Kennedy, D.O., 2012. Epigallocatechin gallate, cerebral blood flow parameters, cognitive performance and mood in healthy humans: a double-blind, placebo-controlled, crossover investigation. Hum Psychopharmacol 27, 177-186.
Willatts, P., Forsyth, S., Agostoni, C., Casaer, P., Riva, E., Boehm, G., 2013. Effects of long-chain PUFA supplementation in infant formula on cognitive function in later childhood. Am J Clin Nutr.
Witte, A.V., Kerti, L., Hermannstadter, H.M., Fiebach, J.B., Schreiber, S.J., Schuchardt, J.P., Hahn, A., Floel, A., 2013. Long-Chain Omega-3 Fatty Acids Improve Brain Function and Structure in Older Adults. Cerebral Cortex.
Wu, A., Ying, Z., Gomez-Pinilla, F., 2008. Docosahexaenoic acid dietary supplementation enhances the effects of exercise on synaptic plasticity and cognition. Neuroscience 155, 751-759.
Wurtman, R.J., 2008. Synapse formation and cognitive brain development: effect of docosahexaenoic acid and other dietary constituents. Metabolism 57 Suppl 2, S6-10.
Wurtman, R.J., Cansev, M., Sakamoto, T., Ulus, I.H., 2009. Use of phosphatide precursors to promote synaptogenesis. Annu Rev Nutr 29, 59-87.
Xie, L., Innis, S.M., 2008. Genetic variants of the FADS1 FADS2 gene cluster are associated with altered (n-6) and (n-3) essential fatty acids in plasma and erythrocyte phospholipids in women during pregnancy and in breast milk during lactation. J Nutr 138, 2222-2228.
Yarchoan, M., Xie, S.X., Kling, M.A., Toledo, J.B., Wolk, D.A., Lee, E.B., Van Deerlin, V., Lee, V.M., Trojanowski, J.Q., Arnold, S.E., 2012. Cerebrovascular atherosclerosis correlates with Alzheimer pathology in neurodegenerative dementias. Brain 135, 3749-3756.
Yehuda, S., Rabinovtz, S., Carasso, R.L., Mostofsky, D.I., 1996. Essential fatty acids preparation (SR-3) improves Alzheimer's patients quality of life. Int J Neurosci 87, 141-149.
Yin, J., Turner, G.H., Coons, S.W., Maalouf, M., Reiman, E.M., Shi, J., 2014. Association of amyloid burden, brain atrophy and memory deficits in aged apolipoprotein epsilon4 mice. Curr Alzheimer Res 11, 283-290.
Youdim, K.A., Dobbie, M.S., Kuhnle, G., Proteggente, A.R., Abbott, N.J., Rice-Evans, C., 2003. Interaction between flavonoids and the blood-brain barrier: *in vitro* studies. J Neurochem 85, 180-192.
Yu, Y.F., Zhai, F., Dai, C.F., Hu, J.J., 2011. The relationship between age-related hearing loss and synaptic changes in the hippocampus of C57BL/6J mice. Exp Gerontol 46, 716-722.
Yui, K., Koshiba, M., Nakamura, S., Kobayashi, Y., 2012. Effects of large doses of arachidonic acid added to docosahexaenoic acid on social impairment in individuals with autism spectrum disorders: a double-blind, placebo-controlled, randomized trial. J Clin Psychopharmacol 32, 200-206.
Yurko-Mauro, K., 2010. Cognitive and cardiovascular benefits of docosahexaenoic acid in aging and cognitive decline. Curr Alzheimer Res 7, 190-196.
Yurko-Mauro, K., McCarthy, D., Rom, D., Nelson, E.B., Ryan, A.S., Blackwell, A., Salem, N., Jr., Stedman, M., 2010. Beneficial effects of docosahexaenoic acid on cognition in age-related cognitive decline. Alzheimers Dement 6, 456-464.
Zeng, Y., Lv, F., Li, L., Yu, H., Dong, M., Fu, Q., 2012. 7,8-dihydroxyflavone rescues spatial memory and synaptic plasticity in cognitively impaired aged rats. J Neurochem 122, 800-811.
Zerbi, V., Jansen, D., Wiesmann, M., Fang, X., Broersen, L.M., Veltien, A., Heerschap, A., Kiliaan, A.J., 2014a. Multinutrient diets improve cerebral perfusion and neuroprotection in a murine model of Alzheimer's disease. Neurobiol Aging 35, 600-613.
Zerbi, V., Kleinnijenhuis, M., Fang, X., Jansen, D., Veltien, A., Van Asten, J., Timmer, N., Dederen, P.J., Kiliaan,

A.J., Heerschap, A., 2013. Gray and white matter degeneration revealed by diffusion in an Alzheimer mouse model. Neurobiol Aging 34, 1440-1450.

Zerbi, V., Wiesmann, M., Emmerzaal, T.L., Jansen, D., Van Beek, M., Mutsaers, M.P., Beckmann, C.F., Heerschap, A., Kiliaan, A.J., 2014b. Resting-State Functional Connectivity Changes in Aging apoE4 and apoE-KO Mice. J Neurosci 34, 13963-13975.

Zhao, D., Qin, C., Fan, X., Li, Y., Gu, B., 2013. Inhibitory effects of quercetin on angiogenesis in larval zebrafish and human umbilical vein endothelial cells. Eur J Pharmacol.

Zhao, Y., Calon, F., Julien, C., Winkler, J.W., Petasis, N.A., Lukiw, W.J., Bazan, N.G., 2011. Docosahexaenoic acid-derived neuroprotectin D1 induces neuronal survival via secretase- and PPARgamma-mediated mechanisms in Alzheimer's disease models. PLoS ONE 6, e15816.

Zhou, Y., Nijland, M., Miller, M., Ford, S., Nathanielsz, P.W., Brenna, J.T., 2008. The influence of maternal early to mid-gestation nutrient restriction on long chain polyunsaturated fatty acids in fetal sheep. Lipids 43, 525-531.

List of abbreviations

^{1}H MRS	proton MR spectroscopy
^{31}P MRS	phosphorus MR spectroscopy
5-LOX	5-lipoxygenase
Aβ	β-amyloid
AD	Alzheimer's disease
ADAS-cog	cognitive subscale of the Alzheimer's disease assessment scale
ADHD	attention deficit hyperactivity disorder
adq	adequate
ADP	adenosine diphosphate
ALA	α-linolenic acid
AMI	acute myocardial infarction
APOE	apolipoprotein E
apple+GSE	apple + grape seed extract
ARA	arachidonic acid
ASL	arterial spin labeling
ATP	adenosine triphosphate
AUC	auditory cortex
B12	vitamin B12
B6	vitamin B6
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BSID	Bayley Scales of Infant Development
CA1	cornu ammonis 1
CA3	cornu ammonis 3
cAMP	cyclic adenosine monophosphate
CBF	cerebral blood flow
CC	corpus callosum
CD36	cluster of differentiation 36
CD68	cluster of differentiation 68
CDP-choline	cytidine diphosphate choline
CDR	clinical dementia rating
CIBIC-plus	clinician's interview-based impression of change scale which included caregiver-supplied information
CK	choline kinase
COX	cyclooxygenase
CPT	1,2 diacylglycerol choline phosphotransferase
CREB	cAMP response element binding protein
CRLB	Cramér-Rao lower bounds
CT	cytidine triphosphate-phosphocholine cytidyl transferase
CTP	cytidine triphosphate
CTRL	control (standard rodent chow)
DAG	diacylglycerol
DAB-Ni	diaminobenzidine-nickel
DBS	deep brain stimulation
DCX	doublecortin
def	deficient
DEPC	diethylpyrocarbonate
DG	dentate gyrus

DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DHASCO	docosahexaenoic acid single cell oil
DMII	diabetes mellitus type 2
DTI	diffusion tensor imaging
EGCG	epigallocatechine gallate
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
F	fornix
FA	fractional anisotropy
FADS	fatty acid desaturase
FAIR	flow-sensitive alternating inversion recovery
FAME	fatty acid methyl esters
FOV	field of view
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GD	gestational day
GDNF	glial cell-derived neurotrophic factor
GE	gradient echo
GLA	γ linolenic acid
Gln	glutamine
Glu	glutamate
Glu+Gln	glutamate + glutamine
GLUT-1	glucose transporter 1
GPC	glycerophosphocholine
HC	hippocampus
HF	high-fat
Iba1	ionized calcium-binding adapter molecule 1
l-DOPA	l-dihydroxyphenylalanine
IL-1β	interleukin-1β
IL-6	interleukin-6
IML	inner molecular layer
IQ	intelligence quotient
LA	linoleic acid
LCPUFA	long-chain polyunsaturated fatty acids
LDL	low density lipoprotein
LT	leukotrienes
MC	motor cortex
MCI	mild cognitive impairment
MCP-1	monocyte chemoattractant protein-1
MD	mean diffusivity
MDI	mental development index
MetS	metabolic syndrome
*m*I	*myo*-Inositol
*m*I+Gly	*myo*-Inositol + glycine
MMSE	mini-mental state examination
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
MR	magnetic resonance
MWM	Morris water maze
n-3 PUFA	omega-3 polyunsaturated fatty acids
n-6 PUFA	omega-6 polyunsaturated fatty acids

n.a.	not applicable
NAA	N-acetylaspartate
NAAG	N-acetylaspartate + N-acetylaspartateglutamate
NPD1	neuroprotectin D1
NPI	neuropsychiatric inventory
NS	not significant
OML	outer molecular layer
OT	optic tract
p.p.	post-partum
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCr	phosphocreatine
PD	Parkinson's disease
PDE	phosphodiesters
PDI	psychomotor development index
PE	phosphatidylethanolamine
PF	paraformaldehyde
PG	prostaglandins
Pi	inorganic phosphate
PME	phosphomonoesters
PND	postnatal day
PPAR	peroxisome proliferator-activated receptor
PRESS	point-resolved spectroscopy sequence
PS	phosphatidylserine
PSD95	postsynaptic density 95
qRT-PCR	quantitative real-time polymerase chain reaction
RAR	retinoic acid receptor
RBANS	repeatable battery for the assessment of neuropsychological status
rCBF	relative cerebral blood flow
rdbpc	randomized double blind placebo controlled
ROI	region of interest
ROS	reactive oxygen species
RT	room temperature
RXR	retinoid X receptor
SC	sensory cortex
SE-EPI	spin-echo planar imaging protocol
SEM	standard error of the mean
SEN	sensory cortex
SFA	saturated fatty acids
SIPBs	synaptophysin immunoreactive presynaptic boutons
SL	stratum lucidum
SNpc	substantia nigra pars compacta
SR	stratum radiatum
SSC	somatosensory cortex
Tau	taurine
TBHQ	tertiary butylhydroquinone
tCho	choline + glycerophosphocholine + phosphocholine
TE	echo time
TNF-α	tumor necrosis factor-α

TR	repetition time
Trk B	tyrosine kinase B
TX	thromboxanes
UTP	uridine triphosphate
VC	visual cortex
VEGF	vascular endothelial growth factor
VEP	visual evoked potential
VIS	visual cortex
Vit free	vitamin free
VLDL	very low density lipoprotein
VOI	volume of interest
WT	wild-type

Dankwoord | Acknowledgements

Na bijna vijf jaar van experimenten, analyses en artikelen schrijven ligt hier dan eindelijk het eindproduct. Dit was natuurlijk niet tot stand gekomen zonder de hulp van een heleboel mensen (en muizen!). Daarom maak ik van de gelegenheid gebruik om iedereen die heeft bijgedragen aan dit proefschrift te bedanken. Een aantal mensen wil ik graag persoonlijk bedanken.

Allereerst mijn begeleiders, jullie hebben mij de kans geboden om dit avontuur aan te gaan en daar ben ik jullie erg dankbaar voor.
Dr. Amanda Kiliaan: lieve Amanda, ik heb ontzettend veel van je geleerd in al die jaren dat we samenwerkten, zowel op professioneel als persoonlijk vlak. Jouw passie en enthousiasme voor onderzoek hebben mijn ambities alleen maar versterkt. Je bent betrokken en bood mij een klankbord wanneer alles tegen leek te zitten, maar was ook van de partij om van de successen te genieten. Daarnaast delen we een aantal voorliefdes, zoals onder andere Swarovski (heb je nog naar de nieuwsbrief gekeken?) ;-) en niet te vergeten cocktails en lekker eten. Natuurlijk wil ik je ook hartelijk bedanken voor alle heerlijke en gezellige borrels en lunches bij jullie thuis.
Prof. dr. Tamas Kozicz: beste Tamas, bedankt dat je mijn promotor wilde zijn. Als afdelingshoofd heb je op de afdeling Anatomie het onderzoeksvuur steeds meer aan weten te wakkeren.
Prof. dr. Arend Heerschap: beste Arend, bedankt dat je mijn tweede promotor wilde zijn. Dank voor alle hulp bij de imaging en de interpretatie van resultaten en dat we gebruik mochten maken van de ClinScan en de Biospec. Dit heeft mijn project naar een hoger niveau weten te tillen.
Dr. Judith Homberg: beste Judith, jij bent ontzettend gedreven in je werk, iets wat erg aanstekelijk is. Bedankt voor je enthousiasme en het razendsnel, maar vooral ook efficiënt corrigeren van manuscripten.

Geachte leden van de manuscriptcommissie, prof. dr. Olde Rikkert, prof. dr. Lafeber en prof. dr. Hadders-Algra, hartelijk dank dat u bereid was om mijn proefschrift te beoordelen.

Maartje, samen stonden we aan de wieg van de uitvoering van dit project. Met vallen en opstaan leerden we de fijne kneepjes van het fokken van muizen. We hebben aardig wat tijd in onze 'kraamkamer' door mogen brengen. Ik kijk sindsdien dan ook nooit meer op dezelfde manier naar een roombroodje! ;-) We hebben samen veel beleefd en ook steun aan elkaar gehad, vaak letterlijk en figuurlijk, haha. Zonder jou had ik dit project nooit zo succesvol af kunnen ronden en daarom wil ik je ontzettend bedanken voor al je werk en onze leuke tijd samen!
Jos, al vanaf het moment dat ik aan mijn stage bij Anatomie begon, sta jij klaar om hulp en ondersteuning te bieden bij het uitvoeren van kleuringen. Je denkt mee bij het opzetten van experimenten en komt met praktische oplossingen als het even niet lukt. Je blijft altijd bescheiden op de achtergrond, maar dat maakt je niet minder belangrijk in de groep. Bedankt voor de fijne samenwerking!
Diane, onze samenwerking begon toen jij mijn stagebegeleidster was en ik vond het erg fijn om later ook je collega te mogen worden. Zo heb ik de fijne kneepjes van het vak van jou mogen leren. Een experiment is niets zonder goede voorbereiding, bedankt voor al je hulp! In onze tijd als kamergenootjes hebben we veel aan elkaar gehad, we konden ni*et al*leen brainstormen over de interpretatie van resultaten, maar ook over alle bijzaken die bij een promotie komen kijken.
Valerio, first of all thank you for all the imaging lessons throughout the years (and especially for all your patience during these lessons!). Your help and support added valuable parameters to my data. Besides from helping me out with imaging, I would like to thank you for your ability to remain calm in times when I was totally stressing out. Your relaxed attitude prevented me from several meltdowns.

Ilse, samen met die andere twee musketiers begon je aan je stage en daar begon ook onze samenwerking. Toen je aan je eigen promotie begon werden we kamergenootjes. Je staat altijd klaar om een helpende hand te bieden bij het opzetten en uitvoeren van experimenten. Ik heb erg veel bewondering voor jouw doorzettingsvermogen en veerkracht. Samen konden we uitgebreid kletsen over een breed spectrum aan onderwerpen, zowel wat betreft werk als privé. Jij weet in elke situatie de juiste snaar te raken. Lieve Ilse, heel erg bedankt dat je ook vandaag weer een helpende hand biedt en mijn paranimf wilt zijn!
Max, Herr Wiesmann, jouw enthousiasme voor onderzoek is ontzettend aanstekelijk, en ik wil je bedanken voor je hulpvaardigheid en niet te vergeten vindingrijkheid. Ook ben je werkzaam als DJ van de oio-kamer. Je hebt voor elke situatie een playlist klaar staan en weet daar een geheel eigen wending aan te geven. Het is alleen jammer dat je ni*et al*tijd naar verzoeknummers luistert... Zielig? Wiesmann vindt van niet. :-P
Rick, in 2013 kwam jij de oio-kamer versterken. Na verloop van tijd bleek je onze eigen wandelende zoekmachine te zijn en de term 'Rickipedia' werd geïntroduceerd. Bedankt voor de gezellige tijd en veel succes in ut stedje van lol en plezeer!
Tim, you've made it! Eindelijk sta je dan in mijn acknowledgements! :-) Ook jij begon in onze groep als student en bent nu aan je eigen promotie begonnen. Je bent een gezellige en waardevolle toevoeging aan de oio-kamer. En ni*et al*leen vanwege al je 'nieuwsgroepen', Adobe-lesjes en GoT!
Bram, jij kwam de afdeling versterken als analist, maar ook als onze ICT-man (tegen wil en dank?). Jouw no-nonsense instelling en efficiëntie zijn een onmisbare toevoeging aan het onderzoek op de afdeling. Ook helpen die twee kwaliteiten ontzettend om wat orde in het oio-kippenhok te brengen!
Prof. dr. Dirk Ruiter: beste Dirk, bedankt voor de begeleiding aan het begin van mijn promotietraject. Ik ben erg blij dat je vandaag als fungerend rector bij mijn verdediging aanwezig bent.
Daarnaast wil ik natuurlijk iedereen van de afdeling Anatomie bedanken voor een leuke tijd en alle gesprekken in de koffiepauze over de zin en onzin van het leven.

Van de afdeling Radiologie wil ik Andor en Sjaak bedanken voor hun hulp en ondersteuning bij het uitvoeren en analyseren van de MR experimenten. Ook wil ik een paar mensen in PRIME en het CDL bedanken. Met name Janneke, Karin, Henk, Bianca en Nicole: bedankt voor de goede zorg voor mijn muizen! Ik heb ontzettend fijn met jullie samengewerkt. Jullie stonden altijd klaar om mee te helpen bij het opzetten en uitvoeren van de dierstudies. Voor mij zijn jullie dan ook het bewijs dat je van een proefdierexperiment alleen een succes kunt maken als je het belang van de dieren voorop stelt.

I would like to thank a few people at Mead Johnson Pediatric Nutrition Institute with whom I worked closely during my PhD project. Brian, Gabriele, Zeina and Ric, thank you for your input in our experiments. Your valuable ideas and comments have been a great contribution to our work. It has been a pleasure working with you! Special thanks to you Brian, for taking place in the corona today.

Monique, bedankt voor de prettige samenwerking bij de experimenten met de ApoE muizen. Ik vind het dan ook erg fijn dat je vandaag onderdeel van de corona bent.

Studenten zijn ontzettend belangrijk bij de uitvoering van onze uitgebreide experimenten. Hierbij wil ik mijn voormalige studenten bedanken: Sarita, Elina, Bas, Maarten, Liën, Inge, Annelies, Julle, Claudia en Mieke, zonder jullie had ik dit boekje niet kunnen voltooien. Jullie hebben allemaal je eigen inbreng in de experimenten gehad. Ik vond het erg leuk om jullie stages te begeleiden!

Echte vrienden heb je niet voor even, die heb je voor het leven.
Lieve Joke, al sinds de brugklas zijn we vriendinnen door dik en dun. In onze studententijd werden we ook nog eens huisgenootjes. Na deze tijd in Nijmegen zijn we ieder een andere (onderzoeks) richting op gegaan, maar we zijn elkaar nooit uit het oog verloren. Ik kan altijd op je bouwen, in goede en in slechte tijden. Als h*et al*lemaal even niet mee zat, kon ik bij jou terecht voor een kop thee en een luisterend oor. Maar gelukkig maken we ook een heleboel leuke dingen mee en liggen we samen in een deuk om de meest onbenullige dingen. Nadat ik vorig jaar aan jouw zijde mocht staan als paranimf zijn de rollen dit keer omgedraaid. Bedankt dat je de lat zo hoog hebt gelegd, dr. Konings… ;-) Ik ben erg blij dat je ook vandaag weer mijn steunpilaar wilt zijn!
Ingrid, al sinds de middelbare school zijn we bevriend met elkaar. De laatste jaren met een flinke geografische afstand, maar zelfs als we elkaar pas na een paar maanden weer spreken, pikken we de draad zo op waar we de vorige keer zijn gebleven.
En daar is dan mijn BMW-clubje! Het is alweer ruim 11 jaar geleden dat we bevriend raakten in ons eerste jaar Biomedische Wetenschappen. Hoewel er bij BMW slechts 3 van ons overbleven, bleef de vriendschap bestaan. We delen lief en leed met elkaar en het is elke keer weer gezellig als we elkaar zien. Hierbij wil ik jullie één voor één bedanken. Dat er nog vele BMW-uitjes mogen volgen!
Anouk, samen met Floor waren we de die-hard BMW-ers. Na flink zwoegen en gedeelde frustraties, volg ik nu je voorbeeld en ben ik ook aan de beurt om mijn proefschrift te verdedigen. Regelmatig verzorgde je een BBQ in het zonnige zuiden om even bij te komen en bij te kletsen. Tevens zorg je op zijn tijd voor een vitamine-boost uit Tims groentetuin (zelfs die enorme pompoen ging gelukkig toch op!).
Frances, na het eerste jaar BMW ben je naar Utrecht gegaan om diergeneeskunde te studeren, maar dat betekende niet dat we elkaar uit het oog verloren. Inmiddels ben jij alsnog een promotietraject gestart en ik kijk met spanning uit naar jouw boekje (doe rustig aan, je hebt nog even). Je weet precies op de juiste momenten een hart onder de riem te steken. Dankjewel daarvoor!
Floor, de andere die-hard BMW-er. Ook jij zat in een promotietraject en wist daarom als geen ander van de struikelblokken waar we samen tegenaan liepen. Ik kan nog altijd veel leren van jouw positieve instelling en jij hebt ook al zo'n goed voorbeeld neergezet met je eigen verdediging. PS: het is weer hoog tijd voor sushi!
Lian, jij maakt het BMW-clubje compleet. Jij maakte de overstap naar geneeskunde en zorgt voor een verfrissende blik in een vriendengroep vol promovendi. Jarenlang waren we ni*et al*leen vriendinnen, maar ook huisgenootjes. Daar begon ook de filmavond. Ook al zijn we inmiddels geen huisgenootjes meer, de filmavond met pizza helpt nog altijd om te ontspannen en gezellig bij te kletsen.

Natuurlijk mag ik mijn familie niet vergeten. Allererst soll ich mich jetzt schon entschuldigen an die Wedershovens. Leider wird das Thema meiner Doktorarbeit für euch noch immer nicht ganz klar werden weil meine Präsentation auf Niederländisch ist. Aber für 'ne Party ist Sprache überhaupt keine Barriere, oder? Das versteht ihr als die Besten! ;-)
Na jaren van vage verhalen over voeding en muizen hoop ik vandaag wat duidelijkheid te scheppen. Eindelijk kan ik jullie laten zien wat ik nu in hemelsnaam heb uitgevreten de afgelopen jaren. Ik hoop dat jullie er allemaal bij kunnen zijn om na mijn verdediging een feestje te vieren!
Oma, ugge mondharmonica meug thoes blieve huer! Veur de muziek wuert al gezurg. :-)
Ik wil ook even stilstaan bij mijn familieleden die er vandaag helaas niet meer bij kunnen zijn. Mijn lieve opa's en oma, al van kleins af aan lieten jullie me merken dat jullie trots op me waren. Ik weet zeker dat jullie dat vandaag ook zouden zijn. Brigitte en Frits, mijn lieve tante en peetoom, jullie hadden als geen ander van dit feestje genoten. Ik denk nog vaak aan het compliment van Frits bij mijn afstuderen. Het was er eentje zoals alleen hij dat kon: "Wat busse toch un sjlum wief!"

Dan ben ik nu eindelijk aangekomen bij degenen aan wie ik dit proefschrift heb opgedragen, mijn lieve ouders! Pap en mam, ich wil ug hieël erg bedanke veur alle steun daet ge mich door de jaore haer gebaoje heb. Al van kleins af aan heb ge mich gelierd um altied ut beste oet michzelf te haole en door te zetten tot ich mien doel bereik had. Nag altied kin ich beej ug terech um efkes beej te komme van alle rompslomp. Zonger uch zoel ich heej vandaag neet staon en ich haop dan auch det ge vandaag gruuëts op mich kint zien. Dit bukske is beej lange nao neet diek genóg um mienen dank voloet onger weurt te bringe... Daorum haaj ich ut simpel: **Bedangk veur alles!!!**

Curriculum vitae

Carola Janssen werd op 10 juni 1985 geboren in Venlo, waar zij in 2004 haar gymnasium diploma behaalde aan het Valuascollege. In datzelfde jaar begon zij haar studie Biomedische Wetenschappen aan de Radboud Universiteit te Nijmegen. Vanwege haar interesse in het ontstaan van ziekten en de invloed van externe factoren hierop, koos zij na het behalen van haar bachelordiploma voor een masteropleiding m*et al*s hoofdvak pathobiologie en de bijvakken toxicologie en geneesmiddelenonderzoek.
Tijdens haar eerste masterstage onderzocht zij de potentie van atorvastatine om ischemie-reperfusieschade te voorkomen in gezonde vrijwilligers (onder begeleiding van dr. C.W. Wouters en prof. dr. G.A. Rongen, afdeling Farmacologie-Toxicologie, Radboudumc, Nijmegen). Gedurende haar opleiding werd tevens haar interesse voor proefdieronderzoek gewekt. Dit kwam goed van pas in haar tweede masterstage, waarin zij het effect van met DHA-verrijkte diëten op neurodegeneratie in een muismodel voor de ziekte van Alzheimer onderzocht (onder begeleiding van dr. D. Jansen, dr. V. Zerbi en dr. A.J. Kiliaan, afdeling Anatomie, Radboudumc, Nijmegen).

In januari 2010 begon Carola aan haar promotie-onderzoek op de afdeling Anatomie van het Radboudumc in Nijmegen onder begeleiding van copromotoren dr. A.J. Kiliaan (afdeling Anatomie) en dr. J.R. Homberg (afdeling Cognitive Neuroscience) en promotoren prof. dr. L.T. Kozicz (afdeling Anatomie) en prof. dr. A. Heerschap (afdeling Radiologie). Tijdens haar promotie, beschreven in dit proefschrift, begeleidde zij een tiental studenten gedurende hun stages. Tevens presenteerde zij haar bevindingen op verschillende nationale en internationale bijeenkomsten, in de vorm van posters en mondelinge presentaties. Op basis van haar CV en resultaten won Carola in 2014 een travel grant op het FENS Forum of Neuroscience. Sinds maart 2015 is zij werkzaam als Consultant Life Sciences bij Hezelburcht B.V. in Nijmegen.

List of publications

Original papers

CIF Janssen, D Jansen, MPC Mutsaers, PJWC Dederen, B Geenen, MT Mulder, AJ Kiliaan. The Effect of a High-Fat Diet on Brain Plasticity, Inflammation and Cognition in ApoE4-knockin and ApoE-knockout Mice. Submitted

CIF Janssen, V Zerbi, MPC Mutsaers, M Jochems, CA Vos, JO Vos, BM Berg, EAF van Tol, G Gross, ZE Jouni, A Heerschap, AJ Kiliaan. Effect of Perinatally Supplemented Flavonoids on Brain Structure, Circulation, Cognition, and Metabolism in C57BL/6J Mice. Neurochem Int 2015; 89: 157-169

CIF Janssen, V Zerbi, MPC Mutsaers, BSW de Jong, M Wiesmann, IAC Arnoldussen, A Heerschap, FAJ Muskiet, ZE Jouni, EAF van Tol, G Gross, JR Homberg, BM Berg & AJ Kiliaan. Impact of dietary n-3 polyunsaturated fatty acids on cognition, motor skills, and hippocampal neurogenesis in developing C57BL/6J mice. J Nutr Biochem 2015; 26 (1): 24-35

CIF Janssen & AJ Kiliaan. Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: The influence of LCPUFA on neural development, aging, and neurodegeneration. Prog Lipid Res 2014; 53 (1): 1-17

D Jansen, V Zerbi, **CIF Janssen**, D van Rooij, B Zinnhardt, PJ Dederen, AJ Wright, LM Broersen, D Lütjohann, A Heerschap & AJ Kiliaan. Impact of a multi-nutrient diet on cognition, brain metabolism, hemodynamics, and plasticity in apoE4 carrier and apoE knockout mice. Brain Struct Funct 2014; 219 (5): 1841-1868

D Jansen, V Zerbi, **CIF Janssen**, PJ Dederen, MPC Mutsaers, A Hafkemeijer, AL Janssen, CLM Nobelen, A Veltien, JJ van Asten, A Heerschap & AJ Kiliaan. A longitudinal study of cognition, proton MR spectroscopy and synaptic and neuronal pathology in aging wild-type and AβPPswe-PS1dE9 mice. PLoS One 2013 8 (5): e63643

A Rijpma, D Jansen, IAC Arnoldussen, XT Fang, M Wiesmann, MPC Mutsaers, PJ Dederen, **CIF Janssen** & AJ Kiliaan. Sex differences in synaptic density and neurogenesis in middle-aged apoE4 and apoE knockout mice. J Neurodegenerative Diseases, vol. 2013, Article ID 531326, 9 pages, 2013.

D Jansen, **CIF Janssen**, T Vanmierlo, PJ Dederen, D van Rooij, B Zinnhardt, CLM Nobelen, AL Janssen, A Hafkemeijer, MPC Mutsaers, AMCM Doedée, AAM Kuipers, LM Broersen, M Mulder & AJ Kiliaan. Cholesterol and synaptic compensatory mechanisms in Alzheimer's disease mice brain during aging. J Alzheimers Dis 2012; 31 (4): 813-826

CW Wouters, P Meijer, **CIF Janssen**, GW Frederix, WJ Oyen, OC Boerman, P Smits & GA Rongen. Atorvastatin does not affect ischaemia-induced phosphatidylserine exposition in humans in-vivo. J Atheroscler Thromb 2012; 19 (3): 285-91

Oral presentations

CIF Janssen, V Zerbi, MPC Mutsaers, BSW de Jong, M Wiesmann, IAC Arnoldussen, A Heerschap, G Gross, JR Homberg, BM Berg & AJ Kiliaan (2013). The effect of dietary long-chain polyunsaturated fatty acids during the gestational and postnatal period on cognition, brain metabolism, and neurohistology in C57BL/6J mice. In: 11th Dutch Endo-Neuro-Psycho Meeting. May 29-31 2013, Lunteren, the Netherlands. *Invited speaker*

CIF Janssen, D Jansen, MPC Mutsaers, PJ Dederen, V Zerbi, A Heerschap & AJ Kiliaan (2012). Effect of dietary intake on mouse brain from cradle to grave. In: 5th Donders Discussion. October 25-26 2012, Nijmegen, the Netherlands. *Invited speaker*

CIF Janssen, V Zerbi, MPC Mutsaers, BSW de Jong, A Heerschap, BM Berg, JR Homberg & AJ Kiliaan (2012). The effect of dietary long chain polyunsaturated fatty acids during the gestational and postnatal period on cognition, brain metabolism, and neurohistology in C57BL/6J mice. In: 42nd Society for Neuroscience Meeting. October 13-17 2012, New Orleans, LA, USA

Poster presentations

CIF Janssen, V Zerbi, MPC Mutsaers, M Jochems, CA Vos, JO Vos, A Heerschap, EAF van Tol, G Gross, JR Homberg, BM Berg & AJ Kiliaan. The effect of perinatally supplemented flavonoids on cognition, brain metabolism, and neurohistology in C57BL/6J Mice. In: 9th FENS Forum of Neuroscience. July 5-9 2014, Milan, Italy.

CIF Janssen, V Zerbi, MPC Mutsaers, M Jochems, CA Vos, JO Vos, A Heerschap, EAF van Tol, G Gross, JR Homberg, BM Berg & AJ Kiliaan. The effect of perinatal dietary flavonoid intake on cognition, brain metabolism, and neurohistology in C57BL/6J Mice. In: 8th World Congress on Polyphenols Applications. June 5-6 2014, Lisbon, Portugal.

CIF Janssen, D Jansen, C Capone, M Mulder & AJ Kiliaan (2011). The effect of a cholesterol enhanced diet on spatial memory and learning in ApoE4 and ApoE ko mice. In: 10th AD/PD Meeting. March 9-13 2011, Barcelona, Spain.

CIF Janssen, D Jansen, V Zerbi, B Zinnhardt, D van Rooij, LM Broersen, A Heerschap & AJ Kiliaan (2010). The effects of specific DHA and cholesterol containing diets on behaviour, spatial learning, and brain metabolism in APP/PS1 Alzheimer mice. In: 7th FENS Forum of European Neuroscience. July 3-7 2010, Amsterdam, the Netherlands.

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