Mechanisms underlying brain programming by early-life adversity and nutrition

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General discussion
1. Introduction

The early-life period is characterized by rapid brain growth, which goes hand in hand with a high demand for energy and nutrients during this time. During this critical period of development, the brain is very sensitive to perturbations by environmental factors such as stress and nutritional intake\textsuperscript{1,2}. Numerous human and animal studies have shown a strong link between both early-life adversity (ELA) and early nutrition and later-life brain cognitive impairments and mental health disorders\textsuperscript{3–9}. Importantly, cognitive dysfunction is both a risk factor for and a common trait of several mental health disorders, greatly compromising quality of life\textsuperscript{10–13}. Even though several neurobiological mechanisms have been investigated in the context of brain programming by ELA and diet, still, many questions remain as to how exactly these environmental factors can affect later-life brain function. Understanding this is crucial in order to develop effective (nutritional) strategies for the prevention of later-life disease.

The main aims of this thesis were; i) to address biological mechanisms that could mediate brain programming by ELA and by early exposure to an important class of nutrients, i.e. polyunsaturated fatty acids (PUFAs), ii) to study these not only basally but also to understand if and how ELA alters response to a second inflammatory challenge and finally iii) to understand to what extent exposure to ELA in either rodents or humans during critical developmental periods (prenatally or early postnatally) contributes to the vulnerability to mental and metabolic disorders, to their comorbidity and their sex-dependent prevalence and presentation.

To enable studying such biological mechanisms we used a mouse model of chronic early-life stress (ELS), induced by limited amounts of nesting and bedding material during the first week of life\textsuperscript{14–16}. A core feature of this model is cognitive impairments later in life. We used an early-life dietary intervention based on altering the ratio between omega (\(\omega\))6 and \(\omega\)3 PUFAs; high \(\omega\)6/\(\omega\)3 ratio = 15 and a low \(\omega\)6/\(\omega\)3 ratio = 1.1, with the low ratio leading to more \(\omega\)3 fatty acid availability in the brain\textsuperscript{15}. In addition, in several studies we used a secondary inflammatory challenge in adulthood in the form of intraperitoneal (i.p.) lipopolysaccharide (LPS) injection in order to unmask possible latent effects.

In this general discussion, I will start by introducing the rising field of ‘nutritional psychiatry’ with a focus on early-life nutrition for risk groups previously exposed to ELA. Then I will discuss properties of dietary PUFAs and their potential for nutritional interventions. Thereafter, I will elaborate on several biological mechanisms involved in brain programming by ELA and dietary PUFAs, in particular the ones that were addressed in this thesis and relate them to each other. Since most of the biological mechanisms are interrelated, some are discussed in several sections, however each from a different
angle. Lastly, I will discuss the importance of assessing patients early-life history, sex, and comorbid diseases when designing (nutritional) interventions in order to prevent mental and/or metabolic disorders.

2. Early nutritional interventions to counteract ELA induced risk for disease

2.1 Nutritional psychiatry
Nutritional psychiatry is a rapidly growing field of research that studies how nutrients might be part of underlying mechanisms driving mental disorders and how we can use them for intervention strategies. It has thus the exciting potential to contribute to the development of non-invasive, non-pharmacological interventions, aimed to prevent and manage mental disorders. In the press and on social media, word goes round that specific diets can prevent the development of ADHD, autism, epilepsy, depression, Alzheimer’s disease and more. In reality however, for many of these claims there is insufficient scientific evidence as several specific factors may affect the outcomes of such studies. While there is indeed evidence that; i) impaired nutrition can disrupt brain structure, functions and mental health, and ii) nutritional interventions can have therapeutic benefits, this obviously does not hold for every situation, nor for every individual. Thus, even though nutrition is a potentially very attractive target to counter ELS-induced deficits, there is yet much unknown as to how, for whom and which specific nutritional interventions will work best. This calls for properly powered studies with clear reporting of patient-specifics and a deep understanding of biological mechanisms involved.

While nutritional psychiatry mostly studies later-life diet and its therapeutic efficacy for adult patients with mental health disorders, we have specifically focused in this thesis on the early-life diet in relation to a specific ELA-exposed risk group. As mentioned earlier, during early development, the brain displays an extraordinary growth. In general, the higher the rate of an organ’s growth, the greater its risk to be affected by insufficient supply of nutrients. Numerous human and animal studies have shown that poor maternal nutritional status and early-life malnutrition alters behaviour and increases the risk for cognitive impairments and mental disorders throughout later life.

The fact that nutritional deficiencies drive altered brain development and later-life brain functions, led to the idea that supplying the developing baby or infant with extra nutrients could benefit brain development and ultimately may also have a positive impact on brain functions and behaviour in adulthood. While not all nutritional interventions studies are efficient, it has been hypothesized that in the broader context of impaired functioning, stress or disease, there is an increased need for optimal nutritional, leading
to increased chances for effective nutritional interventions in these circumstances. During brain development, the brain is extra sensitive to nutritional deficiencies but also to other environmental perturbations like stress. Exposure to ELA indeed leads to an altered brain development and in addition, also affects the nutritional status of both mother and offspring, leading to a sensitive state of the brain of the infant in need for optimal nutrition1,2,24,25.

2.2 Opportunities for PUFAs

While all nutrients are essential for brain growth, some specifically support neurodevelopment, including the macronutrients long-chain polyunsaturated fatty acids (PUFAs) and the micronutrients iron, choline, iodine, vitamins A, D, B6 and B1222,24. Indeed, we and others have previously shown protective effects of both early dietary PUFAs and micronutrients (Chapter 4;15,26,27 against ELA-induced cognitive deficits. For this thesis, we have specifically focused on early dietary PUFAs and their implications for ELA exposed offspring, due to their critical role in brain development and inflammatory signaling, which are both affected by ELA16,28.

PUFAs are essential fatty acids that the body cannot synthesize and for which we thus rely on from dietary sources. PUFAs are main components of the phospholipid bilayer of membranes of all neural cells. PUFA-containing phospholipids are mostly localized around membrane proteins, meaning that variation in the PUFA composition of synaptic membranes can have large impact on membrane-bound proteins such as receptors and ion channels, thereby affecting neuronal and glial functions as well as neuron-glia communication29. PUFAs are divided in ω3 and ω6 PUFAs, both are key for inflammatory signaling, with ω3 PUFAs and their derivatives having mostly anti-inflammatory properties while ω6 PUFAs and their derivatives are mostly pro-inflammatory compounds30–32. Over the years, there has been a shift in the general consumption in western style diets of PUFAs towards a higher intake of especially ω6 PUFAs and less of the ω3 PUFAs, resulting in a high ω6/ω3 ratio33, which is thought to contribute to the increased risk for mental health disorders and chronic disease20,34–37. It has been shown that the duration of PUFA-based nutritional interventions that are required to alter brain phospholipid composition, depends on the developmental stage of the animal, with impact on brain phospholipids being most efficient during developmental stages when ω6 and ω3 PUFAs accumulate in the brain38. In adulthood, once PUFAs are incorporated in membranes, the composition of phospholipids remains fairly consistent. Accordingly, some studies have not detected any changes in brain DHA following a 7 month ω3 deficient diet39 while others have shown changes but only very slowly40,41. This is consistent with the low rate of fatty acid turnover in adulthood, which decreases further under conditions of low ω3 PUFA availability42. In this thesis, we have assessed an early dietary intervention with either a high (15) or low
Chapter 8

(1.1) \( \omega_6/\omega_3 \) ratio, thereby modulating developmental \( \omega_6 \) and \( \omega_3 \) PUFA availability in the periphery and brain (15; chapter 4, 5 and 6).

In the next section we will discuss the existing scientific proof for several underlying biological mechanisms involved in the impact of ELA and early dietary PUFA’s on later-life brain function related to cognitive function and mental health. It is important to note, as discussed in chapter 6 and 7 that ELA also increases risk for the development of metabolic health disorders such as obesity, diabetes and inflammatory bowel disease43–46. Some of the underlying mechanisms discussed in this thesis might therefore be of importance for understanding the early-life origin of both mental and metabolic health.

3. Biological mechanisms underlying ELA and PUFA effects on the adult brain, and the interrelations between these mechanisms

3.1. Microglia mediate the effects of ELA and early dietary PUFAs on hippocampal function

Microglia are the resident macrophages of the brain and, depending on the brain region, make up approximately 10 - 15% of all brain cells47,48. As discussed in chapter 2, they enter the brain during the onset of neurogenesis and play crucial roles in several developmental processes such as neuro-/glio-/angiogenesis, axonal outgrowth, synaptogenesis and synaptic pruning. Considering their key role in refining neuronal networks during development, their relatively low turnover, their involvement in glucocorticoid signaling (chapter 1, 49), and large variety of expressed receptors, we and others have proposed microglia to be central cells in contributing to the long-lasting effects of ELA and dietary fatty acids on brain functioning50–53.

3.1.1 Early-life adversity and microglia

Indeed there is evidence for ELA induced alterations in microglia morphology and gene expression15,16,53–55. In particular, we have shown that immediately following ELA exposure at P9, hippocampal microglia display altered morphology, i.e. reduced Iba1 coverage in the dentate gyrus and reduced complexity of Iba1 positive cells in the hilus of the hippocampus16. This was remarkably not accompanied by gene expression changes of microglia extracted from the whole hippocampus at P9 (chapter 3), suggesting that there might be subregion-specific effects on microglia morphology and functions or that the effects at the gene expression level might be latent. Importantly, ELA has persistent effects on both morphology (i.e. increased immune reactive morphology subtypes) and gene expression (i.e. altered expression of inflammatory genes) of hippocampal microglia in adulthood, which was accompanied by an altered transcriptional response to a systemic lipopolysaccharide (LPS)
challenge (chapter 3). In addition we have previously reported long-lasting effects of ELA on microglia morphology in response to chronic amyloid pathology in an APP/PS1 mouse model\textsuperscript{16}. Our data is in line with other studies showing ELA-induced microglial changes throughout life\textsuperscript{53–55} and suggest long-term ‘programming’ of microglia properties and their environment by ELA in such a way that they respond differently to (inflammatory) challenges in adulthood. Such programming of microglia and its differential and often exaggerated response to later life challenges could potentially contribute to the ELA-induced increased risk to develop psychopathologies and neurodegenerative disorders later in life, which have a strong (neuro)immune component\textsuperscript{56–59}.

While in this thesis we have specifically addressed the role of microglia in programming of the brain by ELA and early diet, other glial cells such as astrocytes and oligodendrocytes are evidently crucial during brain development too, making them potential substrates for brain programming by early environmental factors (chapter 1 and 2). Several types of ELA including stress, inflammation and malnutrition have been shown to have direct and long lasting effects on astrocytes\textsuperscript{60} and developmental oligodendrogenesis was impaired in mice exposed to ELA which was regulated by neuronal activity\textsuperscript{61}. As discussed earlier, ω3 PUFAs have immense effects on microglia functionality but there is evidence for their impact on astrocytes and oligodendrocytes too. Recently ω3 PUFAs were demonstrated to promote astrocyte differentiation partly by modulating expression of BDNF and glial-derived neurotrophic factor (GDNF)\textsuperscript{62} and ω3 PUFA deficiency alters oligodendrocyte maturation and myelin integrity during brain development\textsuperscript{61}. Thus, all neural cell types, neurons and glial cells, are vital during brain development and therefore could play their part in brain programming by ELA and dietary PUFAs.

3.1.2 PUFAs, their derivatives and microglia

Next to being affected by environmental stress, there is ample data showing that dietary PUFAs modulate microglia morphology and function both in vitro as in vivo\textsuperscript{63}. For example, docosahexaenoic acid (DHA) can block microglia-induced activation of the NFκb pathway in microglial cell cultures and maternal ω3 PUFA deficiency alters microglial phenotype, reduces their motility and increases microglial-mediated phagocytosis of synaptic elements in vivo\textsuperscript{62,64}. Effects of PUFAs on microglial phagocytic capacity seem to depend on their local environment and on the substrate, since supplementation of DHA and eicosapentaenoic acid (EPA) increased microglial phagocytosis of amyloid-β in an Alzheimer’s disease mouse model\textsuperscript{65,66}. Moreover, we have previously demonstrated that ELA-induced cognitive impairments were accompanied by a reduction in the survival of adult born neurons in the hippocampus together with an increased expression of microglial CD68, a marker for phagocytosis, suggesting possible involvement of microglia in the integration or survival in neural networks. The early low ω6/ω3 PUFA diet not only reversed the effect on neurogenesis, it also reduced the ELA increase in microglial CD68 expression\textsuperscript{15},
supporting the idea that increased ω3 PUFA availability early-in life can program microglia and/or their local environment, leading to long lasting effects on microglial phagocytic capacity. Additionally, in chapter 4 we detected long term effects of the early PUFA diet on microRNAs (miRNAs) and target genes associated with the hippocampal molecular pathway “phagosome formation”. While in chapter 4 we analyzed whole hippocampal RNA and not specific to microglia, nonetheless this too points to long-term effects of early dietary PUFAs on later-life molecular processes related to phagocytosis.

PUFAs esterified in neural cell membranes also modulate intracellular processes after enzymatic cleavage into a variety of bioactive derivatives, also referred to as oxylipins because they are generated through the oxidation of PUFAs\(^6\). Some researchers refer to them as specialized pro-resolving mediators (SPMs) due to their importance in the resolution of inflammation\(^6\). Oxylipins derived from ω3 PUFAs DHA and EPA and their precursor a-linolenic acid (ALA) represent mostly anti-inflammatory and pro-resolving metabolites while oxylipins derived from arachidonic acid (AA) and its precursor linoleic acid (LA) are mostly pro-inflammatory compounds\(^30,69,70\). Microglia can both respond to and produce oxylipins themselves\(^32,71\). Three main enzymes are involved in the conversion of PUFAs into oxylipins: cyclooxygenase (COX), lipoxygenases (LOX) and cytochrome P450 (CYP), which are all three used for both the synthesis of ω6 and ω3 oxylipins. As mentioned earlier, Madore and colleagues (2020) beautifully showed that long term dietary ω3-PUFA deprivation induced increased microglial phagocytosis of synaptic elements. Notably, this was mediated by 12-hydroxyeicosatetraenoic acid (HETE) converted from AA by 12/15 LOX\(^6\). In another study, two ω3 derived oxylipins, i.e. resolving E1 and resolving D1, were shown to inhibit LPS-induced microglial activation \textit{in vitro}, via regulation of the NFkb signaling pathway and specific microRNA (miRNA) expression\(^72–74\). In the context of Alzheimer’s disease, ω3 derived oxylipins Maresin 1 and Resolvin D1 reduced β-amyloid (Aβ)\(^{42}\)-induced inflammation in human microglial cultures and stimulate microglial phagocytosis of Aβ\(^{42}\)\(^66,71\).

While not specific for microglia, there is additional evidence that PUFAs modulate brain oxylipins: a 2-month dietary ω3 PUFA supplementation (as compared to mice that were deprived of ω3 dietary PUFAs), increased ω3 derived oxylipins and decreased ω6 derived oxylipins, which were measured directly following the dietary intervention. This was true both under basal conditions as in response to an inflammatory LPS challenge\(^75\), suggesting that dietary PUFAs can promote the resolution of inflammation through modulation of oxylipins. We demonstrated in chapter 5 that both ELA and early dietary ω6/ω3 ratio led to changes in the adult hypothalamic PUFAs and related oxylipins. Notably, the impact of ELA was highly dependent on the early diet. For example, ELA decreased the ω3 eicosapentaenoic acid (EPA), which was reversed by the low ω6/ω3 diet. This is in line with our previous study showing that the low ω6/ω3 diet increased hippocampal levels of EPA both directly after the dietary intervention and into adulthood\(^15\). In addition we
showed that an acute peripheral LPS challenge in adulthood increased hypothalamic prostaglandin E2 (PGE$_2$, chapter 5), an ω6 AA derivative previously reported under inflammatory conditions$^7$. Next to the common increase in PGE$_2$, the LPS induced alterations depended on both ELA and diet. For example, specifically in mice fed a low ω6/ω3 ratio PUFA diet, in response to LPS we detected a reduction ω6 AA and its derived oxylipins (5-HETE, 8-HETE, 5-KETE, 12-KETE, 15-KETE, TxB2, PGF$_2$). This data supports the importance of dietary PUFAs early in life, as well as their protective capacities and long-lasting impact on inflammatory signaling, which likely plays part in their beneficial effect on brain and behaviour.

![Diagram of oxylipin synthesis pathways.](image)

**Figure 1. Oxylipin synthesis pathways.**


Next to oxylipins, another class of bioactive lipids derived from PUFAs that can modulate microglial functions are endocannabinoids (eCBs)$^{63,77}$. The most abundantly expressed eCBs in the brain are the AA metabolites ethanolamides anandamide (AEA) and 2-arachidonoylglycerol (2-AG), eCBs bind to cannabinoid receptors CB1 and CB2$^{78}$, with CB2 being most abundantly expressed in microglia$^{79}$. Endocannabinoids seem to regulate microglia activity under inflammatory conditions$^{80,81}$. For example, microglia from CB2 knockout mice have reduced phagocytic capacity and an CB2 antagonist reduced microglia motility in vitro$^{82}$. 
Additionally, endocannabinoids play important roles in microglia-neuron interactions. For example, by secretion of extracellular membrane vesicles containing AEA on their surface, microglial AEA can activate neuronal CB1 thereby inhibiting presynaptic neurotransmitter release\textsuperscript{83}. Additionally, epoxide derivatives of ω3 eCBs (Epoxyeicosatetraenoic acid-ethanolamide (EEQ-EA) and epoxydocosapentaenoic acid-ethanolamide (EDP-EA) are potent anti-inflammatory regulators of BV2 microglial cells too\textsuperscript{84}. While we did not measure eCBs directly, we did detect an ELA mediated increase in DAG in the adult hypothalamus, which could possibly impact 2-AG signaling since DAG is the main precursor for 2-AG. Others have shown effects of ELA on developmental endocannabinoid signaling in the hippocampus\textsuperscript{85}, which could possibly impact neuronal and synaptic plasticity.

Thus, there is abundant evidence for ELA and (early) dietary PUFAs, partly via their bioactive derivatives, can impact microglia functionality into adulthood. While not cell-type specific, in chapter 4 we additionally demonstrated using a microarray approach on whole hippocampal tissue, that both ELA and early dietary ω6/ω3 ratio affect the miRNA and gene expression response to an inflammatory LPS challenge later in life. Several pathways associated with the immune response were differentially regulated depending on ELA and early diet, to which glial cells such as astrocytes and microglia most likely contribute.

In summary, the large variety of receptors expressed by microglia make them responsive to many molecules, ranging from cytokines, chemokines, stress hormones and neurotransmitters to bioactive lipids, that regulate their homeostasis and activity (chapter 1; chapter 2; chapter 3). Due to the critical activities of microglia during brain development, changes in their physiology can have major impact on brain functions including the regulation of neuronal plasticity and neuroinflammation. Indeed, we and others have provided evidence for ELA and early dietary PUFAs to impact microglial transcriptional profile and functionality into adulthood, which is associated with neuronal plasticity and inflammatory signaling. Several of the properties of PUFAs and their effects on microglia, e.g. modulation of inflammatory signaling via the production of oxylipins and phagocytic capacity, might contribute to their beneficial effects on hippocampal functions in particular for risk groups exposed to ELA in which these processes seem to be altered to begin with. In the next section, we discuss the role of hippocampal plasticity in the impact of ELA and dietary PUFA on cognitive functions.

3.2 ELA and dietary PUFAs impact cognitive functions via alterations in hippocampal plasticity

The term “neuronal or brain plasticity” refers to the ability of neural networks in the brain to change, adapt and reorganize its structure, connections and functions, e.g. through growth and in response to hormones or experiences\textsuperscript{86}. These processes are key
for cognition, learning and memory\textsuperscript{87}. During brain development the brain is extremely "plastic", but also in adulthood there is still a certain degree of brain plasticity, which is essential for learning, memory and recovery from stress or damage. A brain region often focused on is the hippocampus, a very plastic brain region also in adulthood that is a key for learning and memory. Therefore the term “hippocampal plasticity” is frequently used as well\textsuperscript{88,89}.

Examples of hippocampal plasticity-related processes are; adult neurogenesis, synaptogenesis, network reorganization and neuronal activity changes. Together with impaired cognition (chapter 4;\textsuperscript{15}), we and others have shown ELA to affect measures of neuronal and synaptic plasticity, as shown by a reduced hippocampal volume, reduced adult neurogenesis and altered neuronal excitability\textsuperscript{14,15,90}. While in this thesis we have not specifically focused on ELA-induced changes in hippocampal plasticity, we did demonstrate in chapter 4 that ELA induced alterations in the hippocampal expression of miRNAs and target genes. These were associated with learning and memory such as ephrin and PKA signaling, possibly underlying the ELA-induced effects on aspects of hippocampal plasticity. In addition, by taking an unsupervised approach of looking at hippocampal gene expression via weighted gene co-expression network analysis (WGCNA), we detected a gene co-expression module that correlated with ELA. This network was associated with processes related to structural and functional components of the synapse, also pointing towards ELA mediated effects on hippocampal and synaptic plasticity (chapter 4).

Also dietary PUFAs have been associated with changes in neuronal plasticity\textsuperscript{91,92}. For example, long-term ω3 PUFA supplementation can increase gene and protein expression related to dendritic arborization and spine formation, and lead to increased adult neurogenesis and synaptic plasticity\textsuperscript{91,93,94}. Previously we have demonstrated that the ELA induced reduced survival of adult born hippocampal neurons could be prevented by an the early low ω6/ω3 ratio diet, suggesting long term programming of hippocampal plasticity by early dietary PUFA’s. Furthermore, as discussed in chapter 4, we detected a diet specific gene co-expression network module that was associated with nervous system development and neuronal plasticity (chapter 4), and demonstrated that the low ω6/ω3 PUFA diet lead to long term changes in miRNAs and genes associated with hippocampal plasticity such as cAMP and CREB signaling, which was most prominent in mice previously exposed to ELA. This data highlights possible molecular substrates of the beneficial effect of the diet. Moreover, in response to an inflammatory LPS challenge in adulthood, specifically in mice fed a high ω6/ω3 diet miRNAs and target genes were mostly associated with an activation of inflammatory signaling while in mice fed the low ω6/ω3 diet rather an inhibition was detected of pathways associated with hippocampal plasticity. Both the respective activation and inhibition of inflammatory and neuronal
plasticity pathways were further modulated by previous ELA exposure. The low ω6/ω3 diet specific inhibition of these hippocampal plasticity pathways, further supports the notion that these processes were activated by the diet under basal conditions (chapter 4). Importantly, the effect of the diet on hippocampal plasticity pathways was strongest in mice previously exposed to ELA, supporting the hypothesis that dietary interventions may have the largest impact in risk groups.

It remains intriguing to understand what the specific mechanisms are that lead to the specific long-term alterations observed after ELA or dietary PUFAs at the various levels. A very likely and highly investigated candidate mechanism translating environmental factors into changes in gene/protein expression and thereby alters brain physiology, function, behaviour and risk for disease, is epigenetics. While there are multiple aspects of epigenetic mechanisms, these include microRNAs, which has been our focus in this thesis and will be discussed in the next section in the context of ELA and dietary PUFAs.

3.3. Epigenetic mechanisms translating environmental factors into changes in gene expression and brain function
As discussed throughout this thesis, ELA and early diet are predisposing events contributing to the risk to develop mental and metabolic disorders later in life. Epigenetic mechanisms are key in many of the adaptive and maladaptive biological processes changed during early life. They regulate gene expression without changing the genome, via for example DNA methylation, post-translational histone modifications, post transcriptional RNA editing and non-coding RNAs. Notably, all these epigenetic mechanisms have been associated with ELA and early diet.

Non-coding RNAs include both long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) and play important roles in regulation of gene expression. miRNAs are comprised of 20-30 nucleotides and downregulate gene expression post-transcriptionally by binding to the 3'- untranslated region (UTR) of their mRNA targets. Expression of approximately 30% of all protein-encoding genes are fine-tuned by miRNAs. In this thesis we have assessed miRNAs and their ability to regulate gene expression. Indeed, in chapter 4 we demonstrated a role for miRNAs in regulating ELA and early dietary PUFA-induced changes in hippocampal target gene expression associated with molecular pathways related to inflammation and hippocampal plasticity, as discussed in the previous paragraph 3.2. Both under basal conditions and in response to LPS, the of ELA on miRNA and target genes strongly dependent on the early diet. This data shows that miRNAs might be guiding factors in the ELA and diet induced gene expression changes detected in adulthood, also in response to an adult inflammatory challenge. Moreover, the observed diet mediated changes in miRNAs might be key for its protective effects on hippocampal plasticity and learning and memory, specifically in risk groups exposed to ELA (chapter 4).
3.3.1 Epigenetics and microglia

Since epigenetics are involved in the regulation and reprogramming of cell phenotypes, they are critical for regulating cellular plasticity, also for microglia. During brain and microglia development, epigenetic factors regulate which genes are expressed and which are repressed. In addition, epigenetic processes play a crucial role in the concept of “immune memory”, which poses that immune cells are affected by early experiences, thereby influencing future responses, also related to the “second-hit hypothesis” and “match-mismatch” hypothesis of early stress exposure. Microglia have a relatively long half-life, which makes them great candidates for epigenetic modifications to regulate their responsiveness to later-life challenges. Indeed, preconditioning/priming of microglia by LPS causes several changes in their epigenetic landscape (i.e. repressive histone modifications), leading to blunted pro-inflammatory response to a later-life LPS challenge. In the context of ELA and microglia, both microglial histone modifications and DNA methylations patterns have been reported to be affected by ELA. As discussed in chapter 3 and paragraph 3.1 of this general discussion, we did not detect any transcriptional changes directly following ELA exposure at P9, whereas these became apparent in adulthood, both under basal conditions as in response to LPS. Possibly, at P9 epigenetic modifications in microglia are at play that do not translate to altered gene expression at basal state, but that eventually, together with following stimuli from the microenvironment across life, lead to an altered trajectory of microglia gene expression across development into adulthood and ultimately to the detected changes in adulthood.

Of all the potential epigenetic regulators of microglia, miRNAs are probably the most studied ones. miRNAs have been proven to be key for the differentiation of progenitor cells into microglia during development and in activation processes into adulthood. In fact the variety of phenotypes and activation states of microglia can be characterized by distinct miRNA signatures. Differentially expressed miRNAs were also detected upon LPS stimulation of primary microglia, demonstrating acute miRNA responses thereby possibly affecting gene expression. If and how miRNA contribute to the changes that we observed in microglia gene expression remains to be determined.

3.3.2 Epigenetics and dietary PUFAs

Nutri-epigenomics, is the field that studies interactions between nutrition and the epigenome. Firstly, epigenetic factors can control gene expression involved in nutrient metabolism, which may thereby contribute to differences between individuals in their nutrient requirements and susceptibility to disease. Secondly, dietary intake of nutrients may alter epigenetic mechanisms. This bidirectional interaction between the diet and the epigenome implies that nutritional interventions may reprogram epigenetic marks that are associated with increased risk for disease. For example, DNA methylation is regulated by micronutrients, also called methyl-donors due to their involvement...
in 1-carbon metabolism. We have previously reported that an early diet enriched in micronutrients can rescue part of the ELA induced cognitive deficits. In this study neither ELA nor the diet were associated with changes in hippocampal global or GR specific (Nr3c1) DNA methylation\(^{26}\), thus further investigations will have to address which are the specific epigenetic mechanisms, next to the observed miRNA (chapter 4) that are also taking place in response to ELA and mediating the beneficial effect of the diet.

There is also evidence from both animals and human studies that dietary fatty acids can affect epigenetic processes as well as epigenetic factors regulating fatty acid metabolism, however, this was so far mostly studied in peripheral tissues\(^{116}\). For example, animal studies have shown that changes in maternal intake of PUFAs during pregnancy and lactation can persistently change DNA methylation and histone modifications of genes in peripheral tissues related to metabolism\(^{103,116,117}\). Concerning the brain, Maekawa and colleagues demonstrated that dietary PUFA deficiency during development (both arachidonic acid (AA) and docosahexaenoic acid (DHA)) induced phenotypical changes in the offspring in adulthood, that were associated with schizophrenia symptoms and which was associated with hyper-methylation of nuclear receptor promoters involved in oligodendrocyte integrity and the gamma-aminobutyric acid (GABA)-ergic system\(^{118}\). In humans, next to several studies reporting epigenetic modifications upon altered dietary PUFA intake in peripheral tissues, Haghini and colleagues (2015) showed that plasma PUFA composition was linked to DNA methylation patterns for genes involved in PUFA synthesis in major depressive disorder patients\(^{119}\). It is currently unclear how exactly dietary fatty acids modify the epigenome. Regarding histone modifications, there is evidence that SCFA can inhibit histone deacetylase activity resulting in histone deacetylation\(^{120}\).

Nutri-mironomics specifically studies the interactions between nutrition and miRNAs as epigenetic modulators. Also here, most evidence comes from studies examining fatty acid intake and epigenetic marks in peripheral tissues or cells. For example, using a macrophage cell line, it was shown that ω3 EPA and DHA could modulate the expression of several miRNAs both under basal conditions as in response to an inflammatory stimulus\(^{101}\). Recently, several studies have described alterations in plasma and/or brain miRNAs in Alzheimer’s disease patients associated with inflammatory signaling\(^{121}\) and miRNAs were proposed as potential mechanisms via which dietary DHA can have beneficial effects on AD symptoms\(^{122}\). We have added a piece to the puzzle in chapter 4, in which we demonstrated long-term effects of dietary ω6/ω3 PUFA diet on hippocampal miRNA expression both basally as well as in response to an inflammatory LPS challenge, which was accompanied by changes in target gene expression. As mentioned earlier, these diet modulated miRNAs and target genes were mostly associated with hippocampal plasticity pathways, which were activated under basal conditions and inhibited in response to LPS (chapter 4). Importantly these effects were most prominent in mice previously exposed
to ELA, suggesting that modulating early dietary PUFAs could be an effective strategy to support molecular pathways associated with hippocampal plasticity and learning and memory, key processes that are affected by ELA and in ELA-induced disease states such as depression.

Thus, there are indications that PUFAs can modulate epigenetic regulation including miRNAs, however additional research is necessary with a focus on the brain and molecular pathways via which dietary PUFAs exert their actions on epigenetic mechanisms.

3.3.3 Epigenetics and the gut microbiota
As discussed in chapter 7, the gut microbiota can be modulated by a variety of environmental stimuli thereby influencing health of the host. All types of epigenetic factors have been implicated in translating these environmental stimuli into changes in biological processes, by interacting with the gut microbiota and leading to gene expression changes. In addition, it has been suggested that microbiota itself is an epigenetic player, since they are in fact an “outside source” that can effect gene expression of the host without modifying the DNA.

While it is not clear how exactly bacteria in the gut affect epigenetic mechanisms, there is evidence for microbiota generated SCFA, vitamins and neurotransmitters altering the host epigenome including alterations in DNA methylation, acetylation and histone modifications. Subsequent gene expression changes can lead to altered intestinal permeability and gut homeostasis. Concerning miRNAs, a bidirectional interaction has been reported between the gut microbiota and host miRNA expression: specific gut microbes can alter expression of miRNAs, and miRNAs can promote or inhibit growth of gut microbes.

There is also evidence that altered gut microbiota can affect brain miRNA. Conventional raised mice exposed to antibiotics and mice that lack all microorganisms from birth (i.e. germ free (GF) mice) were shown to exhibit aberrant expression of miRNA in the amygdala and PFC, two brain regions important for stress and fear behaviours. Pathway analysis on the target genes of the differentially expressed miRNAs suggested roles in neurodevelopment and plasticity. Re-colonization of GF mice by environmental microbes partly restored miRNA levels to some extent while some miRNAs remained altered following post-weaning exposure to microbes dependent on the brain region, suggesting the presence of a critical neurodevelopmental window during which the gut microbiota can modulate brain development. These results indicate a gut microbiota-dependent regulation of brain miRNAs, which additionally relies on the neurodevelopmental stage and brain region involved. Exactly which gut-microbiota-brain communication pathways are responsible for these observations remains to be established.
Epigenetic mechanisms seem thus essential for translating environmental factors into changes in gene and protein expression, thereby leading to alterations in biological functions. Exactly how environmental cues such as ELA and diet alter epigenetic signaling is often not clear. Regarding miRNA expression, several mechanisms have been proposed, including: i) DNA methylation, ii) altered miRNA processing, ii) interaction with endogenous (hormones, cytokines, peptides) and exogenous (xenobiotics) compounds. Next to DNA, RNA/genes and proteins, lipids are also main compounds of eukaryotic cells with essential functions in (inter)cellular structure, homeostasis and cellular communication, which will be discussed in the next section.

3.4 Brain lipids in ELA and PUFA mediated risk for brain disorders
There is emerging evidence for a role of brain lipid dysregulation in later-life mental disorders including cognitive impairments, for which ELA and PUFA deficiencies are major risk factors. Lipids may function as energy storage molecules but can also be bioactive and determine fundamental properties and functions of neurons and glial cells via modulation of the integrity of cell membranes and cell-cell signaling among others. Five main lipid categories exist: fatty acids, phospholipids, sterol lipids and sphingolipids, that perform specific biological functions depending on the brain region, cell-type and even organelle. Brain lipid composition can be influenced by environmental factors, making them a promising target for research into how ELA and nutritional interventions affect brain functions and increase risk for later-life disease.

As discussed extensively in chapter 5, we have demonstrated ELA and early ω6/ω3 diet-mediated long-lasting changes in the hypothalamic lipidomic profile, both basally as well as in response to an inflammatory LPS challenge. For example, ELA increased hypothalamic diacylglycerol DAG and a low dietary ω6/ω3 ratio in early life decreased several lipids in adulthood including TAGs, FFAs, DAGs and CER. LPS decreased ceramides and lysophosphotidylcholine while it increased hexosylceramides. Notably, the ELA-mediated changes in the lipid profile were highly dependent on the early diet. In particular it appears that the low ω6/ω3 ratio diet leads to a more anti-inflammatory and neuroplasticity promoting lipid profile. These data suggest that brain lipid changes might (at least in part) contribute to the mechanisms via which the low ω6/ω3 diet protects ELA exposed mice against long lasting effects on neuroinflammatory and hippocampal plasticity processes as well as learning and memory.

To our knowledge we were the first to study the effects of early-life environmental factors on the adult brain lipidomic profile. So far, we do not know how exactly ELA and early dietary PUFAs can exert these long-lasting effects. In this paragraph we will elaborate on several properties and functions of brain lipids that could be involved in the effects of ELA and early dietary PUFAs on the brain.
3.4.1 Brain lipid metabolism

The importance of lipids to biological systems is highlighted by the fact that in humans 5% of genes are involved in lipid synthesis of thousands of different lipid species, for which substantial energy resources are used. The brain contains all major lipid classes of which most (except from essential PUFAs that need to be taken up via the diet) can be synthesized both in the brain (de novo synthesis) as in the periphery after which they may be trafficked over the blood-brain-barrier (BBB), either via passive diffusion or transport-protein mediated pathways (e.g. lipoproteins for TAGs and fatty acid binding proteins (FABPs) for fatty acids (FA)). Furthermore, intercellular exchange of lipids play a large role in brain lipid homeostasis, for example through micro-vesicles, lipoproteins and non-esterified FAs. Without going into detail, the structure of individual lipid classes affects their intracellular localization and synthesis. For example, the main site for lipid synthesis is the endoplasmic reticulum (ER) while some (e.g. the certain phospholipids) are synthesized in the mitochondrial membrane. Both dysfunctions of the ER and mitochondria have been associated with brain disorders for which ELA and malnutrition are risk factors. Moreover, mitochondria, the energy producing organelles within cells, also produce reactive oxygen species (ROS), which can be modulated by ELA and dietary PUFAs. Whether the in chapter 5 described ELA and diet-induced changes hypothalamic lipids are accompanied by changes in physical and chemical properties of mitochondria remains to be investigated.

3.4.3 Lipids and neuronal plasticity

Lipids, for example phospholipids and sphingolipids, shape the physical properties of all cell membranes, which makes them key modulators of neuronal and synaptic plasticity. Several studies have shown that neuronal membranes are not static but remain fairly dynamic in their lipids composition and thus also in their physical and chemical properties, which is most prominent during development. The physical properties of the membrane may impact how neurotransmitters interact with membrane-bound signaling proteins. Given the importance for lipids in neural cell function and plasticity, it is not surprising that altered lipid composition may contribute to changes in neuronal plasticity and to the etiology of mental disorders, including cognitive impairments.

As discussed throughout this thesis and general discussion (paragraph 3.2), the composition of phospholipids and PUFAs can greatly alter neuronal plasticity processes. Other lipids too have been implicated in neuronal plasticity such as sphingolipids, including sphingomyelin and ceramides. Sphingolipids have been proven important for synaptic plasticity (long-term potentiation (LTP)) and learning and memory, which seems to be at least partly mediated by N-methyl-D-aspartate (NMDA) receptors. This is likely due to the fact that the majority of NMDA receptors are localized in sphingolipid-enriched lipid rafts in the membrane of mostly excitatory synapses and from there,
respond to the neurotransmitter glutamate. Ceramides are composed of sphingosine and a fatty acid and play key roles in a variety of fundamental cellular processes including cell proliferation, growth, differentiation, survival and apoptosis. An increase in ceramide concentration may induce neuronal apoptosis via diverse mechanisms, including direct generation of reactive oxygen species, mitochondrial dysregulation and caspase 3 activation, see excellent reviews. In addition, the impact of ceramides on the fluidity of lipid rafts in synaptic membranes might contribute to reshaping synaptic structures and learning memory. Indeed reduced C16 ceramide levels in the hippocampus have been associated with learning and memory. More specifically, extinction learning coincided with a decrease in the activity of sphingomyelinase (ASM), which catalyzes turnover of sphingomyelin to ceramide. The stronger the decline in ASM activity, the better was the extinction learning. Interestingly, as discussed in chapter 5, the early dietary low ω6/ω3 diet reduced hypothalamic C16 ceramide levels. Even though these results were obtained in the hypothalamus, it is tempting to speculate that these changes might also occur in the hippocampus and contribute to the beneficial effects of the diet on neuronal plasticity and cognitive behaviour.

Another lipid class associated with neuronal plasticity is diacylglycerol (DAG). DAGs can either directly activate effector molecules (e.g. protein kinase C (PKC)), or they can be converted, by DAG lipases, into the endocannabinoid 2-AG. 2-AG is known to play crucial roles in synaptic signaling, axonal growth and adult neurogenesis, processes known to be affected by ELA. Precious studies have shown ELA to affect developmental endocannabinoid signaling. In chapter 5 we demonstrated that exposure to ELA led to an increase in hypothalamic DAG in adulthood. Interestingly, also adult chronic stress increases DAG levels in several brain regions. Currently we do not know if the effects of stress on DAG also affects DAG lipases and/or 2-AG levels in the brain, and whether this may thereby possibly affect neuronal plasticity.

3.4.4 Lipids and microglia

Microglia are the (immune) guards of the brain, extensively discussed in chapter 1, 2 and 3 and paragraph 3.2 of this general discussion. These innate immune cells appear to be key in all situations, ranging from physiology to pathology. In addition of expressing receptors for cytokines, stress molecules and neurotransmitters, they also express numerous receptors to sense alterations in lipid composition. Once disturbances are detected, the microglial lipidome and transcriptome can be altered leading to functional changes associated with neuronal plasticity, cell migration, phagocytosis and inflammatory signaling. Different aspects of lipid metabolism, such as lipid sensing, synthesis and oxidation control essential aspects of microglial biology. Due to the critical roles microglia play both in health and disease, it is not surprising that disturbances in
these processes have been ascribed to the development and phenotypical aspects of several brain disorders, including for example depression\textsuperscript{58,165,166} and Alzheimer's disease\textsuperscript{167}.

Apart from PUFAs, oxylipins and endocannabinoids (paragraph 3.2 of this general discussion), other lipids too have been demonstrated to interact with microglia and to play a role in neuroinflammation and microglial functions. For instance, saturated fatty acids (SFA) and ceramides (CER) have been implicated in (neuro-)inflammatory diseases. Notably microglia dysfunction has been reported in such diseases, e.g. microglia are affected by obesity but also contribute to obesity induced cognitive impairments\textsuperscript{168,169}. Both \textit{in vitro} and \textit{in vivo} studies have demonstrated that SFA, especially long-chain, can modulate microglia and support an important role for microglia to sense rising levels of SFA in the hypothalamus and subsequent inflammatory signaling\textsuperscript{63}. For example, early-life maternal obesity impacts microglia in the offspring, i.e. increased hippocampal microglial activation markers at birth and microglial cell density in adulthood, which was accompanied by an altered response to an inflammatory challenge during development or in adulthood\textsuperscript{170,171}. In addition, neonatal overfeeding sensitizes microglia in the hypothalamus, contributing to a basal pro-inflammatory profile and altered response to an inflammatory challenge throughout life. Moreover, the most abundant SFA palmitate can induce \textit{de novo} CER synthesis in primary microglia cultures via modulation of its synthesizing enzyme serine palmitoyl transferase. Subsequently increased ceramide levels may stimulate the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome assembly in microglia, which could lead to the production of the pro-inflammatory cytokine IL1b by microglia\textsuperscript{172}. Thus, apart from the role of ceramides in neuronal plasticity processes, they seem to be essential for microglial mediated neuroinflammation as well.

Due to the various functions lipids have in the cell, they likely represent an excellent substrate to mediate effects of ELA and dietary PUFAs on brain functions. Indeed we have shown ELA and diet dependent effects on brain lipids in adulthood, with the low $\omega_6/\omega_3$ diet specifically promoting lipids previously reported as to be anti-inflammatory and pro-neuronal plasticity. Future studies are warranted to investigate as to how exactly ELA and the early diet lead to these changes but also what the functional changes are of these lipid alterations.

So far I have mainly addressed central mechanisms that take part in programming by ELA and dietary PUFAs. The last biological system that I will elaborate on in this general discussion is the gut microbiota, and specifically the microbiota-gut-brain axis, and address how it can take part in ELA and dietary PUFA programming mediated effects on brain and metabolic programming.
3.4 The microbiota-gut-brain axis as a peripheral system translating early environmental factors into changes in brain function

The early life gut microbiota composition is influenced by various factors, including the host genome, stress hormones, mode of delivery, antibiotic usage and diet\textsuperscript{175}. Moreover, alterations in the gut microbiota have been implicated in mental (depression) and metabolic diseases (inflammatory bowel disease) for which exposure to ELA or early-life malnutrition are risk factors. Animal research under specific conditions, has now provided evidence for a link between gut dysbiosis and several brain processes such as neurogenesis, neurotransmitter signaling, myelination and even alterations in brain microglia\textsuperscript{174}. How exactly the gut microbiota are involved in ELA and dietary PUFAs induced risk for disease, remains unknown.

As discussed in \textit{chapter 6}, we and others have shown that exposure to early postnatal stress and dietary PUFAs can modulate gut membrane permeability and the gut microbiota composition throughout life\textsuperscript{175–177}, which has been associated with altered behavioural outcomes as well\textsuperscript{176,178,179}. We specifically demonstrated that ELA and early dietary ω6/ω3 PUFA ratio, mostly in interaction with each other, modulated β-diversity ((dis-)similarities in microbiota composition between samples) and the relative abundance of bacterial groups at several taxonomic levels on the short and long-term. Several of the detected diet mediated changes were in line with literature (e.g. modulation of butyrate producing species (see paragraph 3.4.2) showing beneficial effects of dietary ω3 PUFA supplementation on brain and metabolism. We therefore hypothesized that the early diet could contribute to a stable and diverse microbiota thereby affecting developmental processes during early sensitive windows that could have long-term impact on later-life health (\textit{chapter 6}).

Several communication pathways and mechanisms have been proposed to play key roles in the crosstalk between the gut microbiota and the brain, such as the vagus nerve and the circulation, via which immune cells and molecules, stress hormones and metabolites could reach the brain. These will be briefly discussed in the next sections, in the context of ELA and dietary PUFAs.

3.4.1 Communication routes from the gut to the brain and back: the vagus nerve and the circulatory system

Two main communication pathways seem to be responsible for transferring signaling from the gut microbiota to the brain and vice versa: the vagus nerve and the circulatory system, which are in close connection to each other. The vagal efferents send signals “down” from the brain to the gut (approximately 15\% of all fibers) and the vagal afferents send signals “up” from the intestinal wall to the brain (approximately 85\% of all fibers). Vagal afferents also interact with the circulation and thus the peripheral immune system such as pro- and
anti-inflammatory cytokines. For excellent reviews on these communication pathways, also in relation to diet and stress see\textsuperscript{173,180}.

As discussed in chapter 4 and 5, we and others have shown that exposure to ELA can have long-lasting effects on the expression of plasma cytokines (i.e. IL6, CXCL1 and CCL2), both under basal conditions and in response to an inflammatory challenge later in life\textsuperscript{16,181,182}. Whether these specific changes in plasma cytokines are mediated by and/or affect the gut microbiota is currently unknown. Nevertheless there is evidence for gut microbiota to regulate aspects of peripheral circulation and inflammation, via for example modulating intestinal barrier functions leading to altered entry of immune-stimulating and neuroactive substances into the circulation\textsuperscript{183,184} and by direct interaction with nearby immune cells\textsuperscript{185}. Additional studies are needed to examine relationship between ELA, the microbiota and peripheral inflammation and if and how this is translated to altered neuroinflammation possibly via i) peripheral immune cells crossing the BBB via the circulation or via affecting vagus nerve activity.

While there are, to the best of our knowledge, no studies testing specifically whether stress exposure during early life can affect vagus nerve signaling, there is evidence for a close relationship between the vagus nerve and HPA axis signaling. For example, the vagal afferent fibers can regulate HPA axis activity and adrenal gland corticosterone secretion, by which it can impact both peripheral and central inflammatory signaling\textsuperscript{186}. In addition, there is evidence for ELA to modulate the enteric nervous system (ENS), the semi-autonomous part of the autonomous nervous system (ANS) comprised of a web of sensory-, motor- and interneurons embedded in the wall of the gastrointestinal system, also in close contact with the vagus nerve. ELA was shown to affect cholinergic neural activity, which was independent of the gut microbiota\textsuperscript{178}.

Future studies are warranted to fully appreciate the contribution of the microbiota in mediating communication between the HPA-axis, the immune system and the brain.

3.4.2 Gut microbiota and the HPA axis

As discussed in chapter 1, stressful stimuli induce a cascade of events in the hypothalamic – pituitary – adrenal (HPA) axis, leading to the secretion of glucocorticoids from the adrenal glands. The HPA axis is a self-regulatory network, using its released products to provide negative feedback at all levels of the HPA axis\textsuperscript{187}.

The response of the HPA-axis to stressful stimuli is known to affect several physiological systems, including the immune system and the gut microbiota\textsuperscript{173,188}. The gut microbiota themselves have also emerged as a major regulator of the HPA-axis activity, especially during conditions of stress\textsuperscript{180}. Indeed, studies performed on germ-free (GF) rodents
demonstrated a bidirectional communication between corticosterone and the gut microbiota: stress and corticosterone are able to modify growth of gut microbiota, and vice versa, gut microbiota can alter the stress response\textsuperscript{180}. For example, GF mice exhibited an exacerbated HPA-axis response to an acute stressor resulting in elevated plasma levels of corticosterone, which could be reverted by colonization with commensal bacteria\textsuperscript{189}. Moreover, exposure to pre- and pro-biotics could also alter the HPA-axis response to stress\textsuperscript{190,191}. While we did not test for HPA-axis activity or corticosterone levels in chapter 6, we and others have shown ELA induced differences in HPA-axis signaling\textsuperscript{26,192,193}. Notably, we demonstrated that the ELA induced rise in corticosterone and adrenal gland hypertrophy could be prevented by an early diet enriched in micronutrients, supporting the importance of early-life nutrition\textsuperscript{26}. Micronutrients have been shown to impact the gut microbiota\textsuperscript{194}, however whether the observed ELA and diet induced changes in HPA-axis signaling were modulated by or led to changes in the gut microbiota needs further investigation.

3.4.2 Gut microbiota and their metabolites

Gut microbiota produce hundreds of metabolites that can affect our physiology ultimately leading to changes in the brain too. They can impact on the intestinal epithelium, the enteric nervous system (vagus nerve) or enter the circulation. Several of these microbe-derived neuroactive metabolites have been described as being capable of contributing to the gut-brain crosstalk, including short-chain fatty acids (SCFA; butyrate, propionate, acetate) but also neurotransmitters including acetylcholine, GABA, serotonin\textsuperscript{195–197}.

As discussed in chapter 6, we showed that while ELA seems to reduce species involved in the production of butyrate (e.g. Lachnospiraceae) (Chapter 6; 133–135), increasing dietary ω3 PUFA availability, either in the early diet, or throughout life, can promote several species associated with butyrate production (e.g. Clostridiales)\textsuperscript{201,202}. Although there is also some evidence for diet induced changes in bacterial derived butyrate\textsuperscript{173,203,204}, it is currently unclear if ELA and early dietary ω6/ω3 ratio indeed affect butyrate levels in the gut, whether it gets to the brain and which functions it exerts there.

SCFA, such as butyrate, propionate and acetate are products of anaerobic bacterial fermentation of fibers and carbohydrates in the gut. They are lipophilic in nature allowing them to reach the brain by crossing the blood-brain barrier, where they could possibly affect the expression of neurotrophic factors and interact with neurons and glial cells\textsuperscript{205–207}. At the same time, SCFA are involved in keeping intestinal permeability in check, affecting the passage of molecules into the circulation\textsuperscript{208}. Lower levels of SCFA may contribute to increased intestinal permeability and increased peripheral and central inflammation, which are known risk factors for several mental disorders. In fact, there is evidence for a link between peripheral levels of these metabolites and mental disorders. For example, fecal
levels of acetate and propionate correlated inversely with core symptoms of depression\textsuperscript{209}. Moreover, several animal studies have shown antidepressant effects of SCFA\textsuperscript{183,210}. Nevertheless, much is still unknown about how and in which situation bacterial produced SCFA reach the brain and the exact mechanisms by which they influence brain functions. Nevertheless, it is tempting to speculate that the observed ELA and diet mediated changes in species involved in butyrate production might contribute to the respective detrimental and beneficial effects of ELA and the low $\omega_6/\omega_3$ ratio diet on hippocampal functions.

### 3.3.3 Gut microbiota and brain microglia

As mentioned above, there is evidence for the gut microbiota influencing peripheral inflammation thereby possibly also affecting central neuroinflammation. In fact, microglia, have been suggested to be key for the communication between the gut and the brain\textsuperscript{174,211,212}. For example, differences in microglia gene expression, morphology and function have been reported between specific pathogen-free (SPF) and germ free (GF) mice, which was dependent on sex and age\textsuperscript{212}. Microglia from adult GF mice exhibit an immature gene expression profile and altered response to an adult inflammatory challenge (LPS). The differences between microglia of SPF and GF mice were, at least in part, regulated by bacterial derived SCFA\textsuperscript{211}. But also proper vagal nerve signaling has been shown to be essential for modulation of microglia and neuroinflammation. For example, electrical stimulation of the vagus nerve, in combination with peripheral or central LPS challenge, has been reported to decrease microglial release of pro-inflammatory cytokines in the brain, which was no longer observed following vagotomy\textsuperscript{213}. In addition, in the context of aging, it has recently been shown that the gut microbiota, via increased intestinal permeability and accumulation of N6-caboxymethyllysine accumulation in microglia, impacts oxidative stress pathways and mitochondrial functions in microglia\textsuperscript{214}.

Thus, the gut microbiota i) is required for microglial maturation (both at the level of gene expression and morphology), ii) controls microglial functions under basal conditions and in response to an inflammatory challenge throughout life and iii) does this in a sex and age dependent manner. In addition, as mentioned earlier, microglial effects on neuroinflammation can be translated to the gut via efferent fibers of the vagus nerve. The in \textbf{chapter 3} and \textbf{6} discussed alterations in microglia and the gut microbiota mediated by ELA and early dietary $\omega_6/\omega_3$ ratio are therefore possibly interrelated.

In this section I reviewed several ways via which the gut microbiota could be involved in mediating the effects of ELA and dietary PUFAs on brain programming. Importantly, the gut microbiota are also a key player in chronic metabolic diseases. In the next section the comorbidity and sex-dependency of ELA induced metabolic and brain disorders will be discussed.
4. Factors mediating the effects of ELA on later-life phenotypes

4.1 Comorbidity and sex-dependency of ELA induced metabolic and brain disorders
ELA increases risk for the development of both metabolic and mental disorders later in life\textsuperscript{45,215–217}. Notably, in the general population these diseases are often comorbid with sex-differences in their prevalence and presentation. In chapter 7 we extensively reviewed the human and rodent literature assessing effects of ELA (pre- and postnatal stress) on either metabolic and behavioural readouts in both sexes. We found that ELA impacts mental and metabolic health in a sex-specific manner, which additionally depends on type and timing of ELA exposure. This data calls for detailed assessment of individuals early-life history, sex and comorbidities for diagnosis and designing (personalized) intervention trials and treatments. Importantly, rodent and human studies largely overlapped in their findings, supporting high translational value of rodent research and the validity of rodent models to study biological mechanisms underlying ELA induced (brain) programming and risk for disease.

4.2 Resilience versus susceptible population
Due to the ELA increased risk for disease, ELA exposure is often assumed to solely have detrimental effects on later-life outcomes, however the opposite can be true is specific cases\textsuperscript{218}. Not all individuals exposed to ELS display the same neurobiological, cognitive and metabolic phenotypes in adulthood. According to the predictive-adaptive or match-mismatch hypothesis, the early-life event influences the development of the individual to adapt to a specific environment (i.e. the predicted environment). Depending on a match or mismatch between the early and later-life environment there will be a beneficial or detrimental response respectively, for example in terms the response to later-life stressor or inflammatory stimulus\textsuperscript{108,219–221} (chapter 7). Accordingly, some alterations during development therefore result in adaptations that lead to resilience to specific circumstances in adulthood. Several factors may play a role in ELA induced vulnerability for disease, including genetic vulnerability, epigenetic alterations and sex of the exposed individual but also type and timing of stress exposure (chapter 7). Understanding more of the factors leading to either vulnerability or resilience to ELA will aid the search for biomarkers and ultimately personalized interventions and treatments.
Figure 2. Mechanisms underlying brain programming by ELA and early dietary PUFAs.

Abbreviations: PUFAs: polyunsaturated fatty acids
4. Concluding remarks

ELA alters brain development and increases risk for mental and metabolic disease, for which several underlying biological mechanisms have been addressed in this thesis: HPA axis signaling, inflammation and microglia, neuronal/hippocampal plasticity, epigenetics (focused on miRNAs) and transcriptomics, brain lipids and PUFA derived oxylipins and the gut microbiota (figure 2). In this general discussion I attempted to demonstrate that these mechanisms are strongly interrelated, they affect and depend on one another. We demonstrated that an early-life dietary intervention based on lowering the ω6/ω3 PUFA ratio has far reaching effects on these neurobiological mechanisms and can be very effective in modulating/supporting them in particular for risk groups exposed to ELA. In some cases, an optimal early diet might reverse ELA induced effects where in other instances the diet may provide additional “support” to the system to better adapt to the alterations induced by ELA exposure. In order to develop effective personalized (nutritional) interventions to prevent or treat ELA and/or nutrition mediated mental and metabolic disorders, deep understanding of these mechanisms and their interrelations is essential.
References


