Early nutritional strategies to modulate the early-life stress- and Alzheimer's disease-induced changes in the brain

Focus on microglia

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General Discussion

CHAPTER SEVEN

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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 period (1–4). As such, the brain is very sensitive to disruptions during this period and can e.g. be 'programmed' by exposure to environmental factors like stress and nutrition (5–8). Indeed, adverse early-life conditions, including nutritional deficiencies and chronic stress have been shown to exert lasting effects on (cognitive) health (9,10). We explored here the potency of beneficial programming in the form of nutritional programming, which could have short- and long-term positive effects on the brain and behavior, as has been shown before for tactile stimulation (11,12) and positive childhood experiences (13,14).

The aims of this thesis included:
i) reviewing the existing knowledge on how early-life nutrition affects the early-life stress (ES)-induced decline in behavior later in life, and the biological mechanisms that could be involved (**chapter 2**)
ii) elucidating whether some of these biological mechanisms (specifically microglial functioning) can be targeted by supplementation with early coffee polyphenols (**chapter 3**) or early polyunsaturated fatty acids (PUFAs) in wildtype mice as well as in an Alzheimer's disease (AD) mouse model (**chapter 5** and **6**)
iii) exploring the role of the PUFA/oxylipin milieu and its relation to microglia in different brain regions and sex (**chapter 4**).

In this general discussion, I will first touch upon how early-life factors, like nutrition, can exert life-long effects. Then, I will introduce the field of nutritional psychiatry in the context of ES. Subsequently, possible mechanisms linking early-life factors and later-life outcomes will be discussed, including lipid metabolism and neuroinflammation, highlighting the interplay between these factors in both ES and AD.
While the focus is mainly on microglia, I will also briefly address other processes that could be affected. As nutrients pass through the circulation, there are e.g. also indirect peripheral effects on the brain possible, which will be discussed. Finally, I conclude with critical gaps in the literature that deserve to be bridged in future studies.

Early nutrition as a determinant factor for later-life outcomes

Early nutrition plays a critical role in determining long-term health and well-being. On the one hand, human data show that pre- or postnatal undernutrition can affect later-life vulnerability to neuropsychiatric disorders and cognitive deficits (15–18). In addition, nutrient status both pre- and postnatally, has been shown to be of key importance here (19). For example, micronutrient (such as iron, zinc and iodine) deficiency in infants has been associated with significant later cognitive deficits (19).

The fact that nutritional and caloric deficiencies can alter brain development and dif-

ferences in later-life physiology has led to the idea that a supplementation of the developing offspring with extra nutrients, in this case coffee polyphenols (**chapter 3**) or PUFAs (**chapter 5** and **6**), could benefit brain development and thereby have a positive impact on brain functions and behavior in adulthood, and as we studied here, both in a stressed (e.g. ES in **chapter 3** and **5**) or diseased context (a mouse model of AD in **chapter 6**).

Nutritional psychiatry in ES-exposed individuals

Nutritional psychiatry is a research field that provides evidence for diet quality as a modifiable risk factor for psychiatric disorders and tries to develop translatable intervention studies. In humans, a healthy diet has been linked to a lower risk for psychiatric disorders, whereas an unhealthy diet has been associated with a higher risk for psychiatric disorders or worse symptoms in patients (20–22). By examining the role of diet in the prevention and management of mental health disorders, nutritional psychiatry vouches for relatively cheap and most often easily implementable treatments instead of supporting pharmacological treatment. What will help drive this field forward, is identifying who needs a nutritional intervention, consisting of how much and which nutrients, and the optimal timing. This, however, is complex due to a large individual variability and multifactorial outcomes, and it demands properly powered studies (preferably randomized-controlled trials (RCTs) in humans) and a deep understanding of the biological mechanisms involved, which is not trivial given the complexity and variation in the human diet.

While the studies contributing to the field of nutritional psychiatry mostly aim for therapeutic efficacy of nutrients in patients with mental health disorders, we focused in **chapter 2** specifically on ES-exposed individuals, which are known to have an increased vulnerability to neuropsychiatric disorders as well as cognitive deficits (23–25). We compiled evidence that diet is a modifiable risk factor for behavior in ES-exposed individuals, a very specific vulnerable target group. Next to contributing to nutritional psychiatry, this thesis contributes to nutritional neuroscience, exploring the effects of nutrients on the brain.

Mechanisms behind early-life factors and later-life outcomes

The relationship between early-life experiences (such as ES, early nutrition and their interaction) and later-life health outcomes has been quite well established in both preclinical as well as clinical studies, but the mechanisms underlying the process are largely unknown. In the next section, I will discuss how the findings in this thesis complement the existing evidence for two biological mechanisms involved in the effect of ES and early diet: lipid metabolism (specifically PUFAs) and neuroinflammation. Finally, I will highlight the interplay between these mechanisms and their potential in later-life health outcomes such as ES-induced cognitive deficits and (aspects of) AD.

Early life factors mediate later-life outcomes via lipid metabolism

Lipids in the brain

Lipids in the central nervous system (CNS) are of particular interest due to their high abundance in the brain. The brain contains a lot of lipids, (grey matter contains 36-40%, white matter contains 49-66% and myelin consists of 78-82% lipids (26)) making it the second highest lipid content behind adipose tissue (27). Many of these lipids are built up by PUFAs, and the brain contains high levels of PUFAs (about 25-30%), that are mainly docosahexaenoic acid (DHA) and arachidonic acid (AA)(28). Lipids needed for normal functioning of the body are ingested via the food or synthesized by adipocytes or hepatocytes from carbohydrate precursors (a relatively small percentage of the total fat balance in humans)(29). Lipids are synthesized and metabolized by enzymes according to their necessity, but PUFAs (being essential fatty acids) cannot be produced in the body and can only be ingested through diet. These PUFAs play a role in forming and maintaining cellular integrity and structure of the cell membrane (30–32). In **chapter 4** and **5**, we focus on their role outside of the membrane, as free PUFAs or their derivatives oxylipins in their role acting as signalling molecules (30–32).

PUFA metabolism

Within the body, linoleic acid (LA) and α-linolenic acid (ALA) undergo elongation, desaturation, and β-oxidation, resulting in the formation of long-chain (LC)-PUFAs. This metabolic process requires specific enzymes, Δ6 and Δ5 desaturases, and elongases, which are shared between both N-6 and N-3 PUFAs, leading to competitive metabolization (33). LC-PUFA biosynthesis primarily occurs in the liver (34)(**Fig. 1A**). However, the brain also has the necessary enzymes to synthesize LC-PUFAs, though this process is less efficient (35,36). As mentioned before, LC-PUFAs, in particular AA, DHA and eicosapentaenoic acid (EPA) have a significant presence in the brain (37). LC-PUFAs are predominantly esterified in phospholipids, but can be further metabolized to free bioactive oxylipins.

The canonical pathway of oxylipin biosynthesis starts with the release of PUFAs from membrane phospholipids by phospholipase A2 (PLA2) enzymes (37,38). Once released, non-esterified PUFAs serve as a substrate for three main enzymatic reactions catalysed by cyclooxygenases, lipoxygenases or cytochrome P450 monooxygenases to become active oxylipins (39)(**Fig. 1B**). Oxylipins derived from N-3 PUFAs (e.g. ALA, DHA and EPA) are mostly described to have anti-inflammatory and pro-resolving actions, while oxylipins derived from N-6 PUFAs (e.g. LA and AA) are mostly pro-inflammatory lipid mediators (37,40,41).

PUFA metabolism between tissues

Blood levels of PUFAs have been commonly used as a biomarker to assess an individual's dietary intake and overall fatty acid status. While blood PUFA levels are useful for confirming dietary uptake, they fall short in providing a comprehensive picture of PUFA status within specific tissues like the brain. Indeed, studies have indicated that PUFA blood levels do not consistently reflect brain PUFA levels due to the brain's tightly regulated mechanisms for maintaining its own lipid metabolism and balance (35,43–46). For example, in rats, while dietary DHA supplementation (44) or ALA supplementation (46) both increased blood DHA levels, brain DHA levels remained more stable and changed much more slowly in time (44) or did not change at all

(46). These studies show discrepancies between amount of N-3 PUFA intake, blood levels, and brain PUFA content, particularly for critical fatty acids like DHA. This emphasizes a tight regulation of brain PUFA content. Although we are not sure about whether this discrepancy in peripheral and brain PUFAs also exists in ES-exposed individuals specifically, we do know that these differences are also evident in AD (47–49), precluding the possibility to use blood PUFA status as a possible biomarker for stratification for N-3 PUFA interventions in this population.

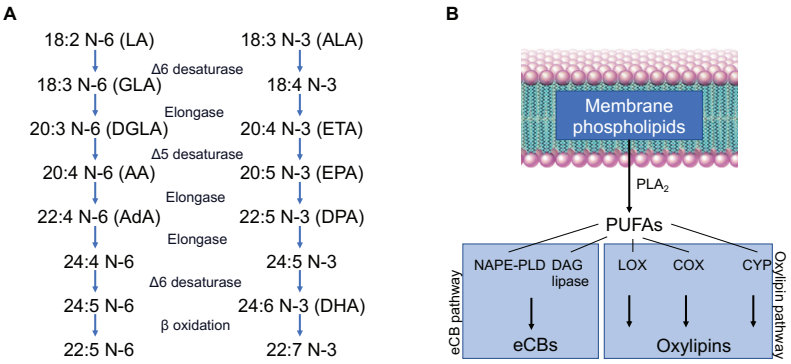

Figure 1 – PUFA biosynthesis pathway and metabolization pathways A) biosynthesis and β-oxidation in the liver. Adapted from (42), B) release and metabolization of PUFA from membrane phospholipids (37–39). *Abbreviations: AA Arachidonic acid; ALA α-linolenic acid; AdA Adrenic acid; COX Cyclooxygenase; CYP Cytochrome P450; DAG Diacylglycerol; DGLA Dihomo-γ--linolenic acid; DHA Docosahexaenoic acid; DPA Docosapentaenoic acid; eCBs Endocannabinoids; EPA; Eicosapentaenoic acid; ETA Eicosatetraenoic acid; GLA γ-linolenic acid; LA Linoleic acid; LOX Lipoxygenases; NAPE-PLD N-acyl phosphatidylethanolamine phospholipase D; PLA2 Phospholipase A2; PUFAs Poly-unsaturated fatty acid*

ES and PUFA metabolism

There is emerging evidence from clinical studies that ES might affect plasma lipid metabolism (50,51). For example, ES exposure was associated with reduced PUFA levels in blood of low-income children (50). Evidence from pre-clinical studies, at least concerning fatty acids in blood erythrocytes, have indicated no differences in fatty acid status right after ES, 3-8 weeks (52,53) and > 2 months after ES ((53), **chapter 5**). The hippocampus in the same study, displayed vast membrane-bound PUFA changes directly after stress, increasing the N-6/N-3 ratio (which could have effects on neuroinflammatory systems in these animals). These effects did disappear over time (53). This emphasizes that fatty acid status in periphery and the brain are not necessarily correlated and that their interrelation deserves further study.

Next to our work on PUFA metabolism, not much work is available on free PUFAs and their derivatives in the ES brain. Our earlier work indicated that ES could induce lasting alterations in free PUFA- and oxylipin species within the hypothalamus at P156 (i.e. long after the stress paradigm had ended)(54). As the hippocampus is found to be implicated in the cognitive decline in ES (55,56), we studied this brain area in **chapter 5**. At P60, there was no specific effect of ES on the levels of free PUFA- and oxylipin species when assessed in a standard dietary background. Others have shown an increase in PUFA to oxylipin turnover and their me-

tabolism with increasing age (57–59). In support, the number of PUFA- and oxylipins species detected in **chapter 4** and **5** at P60 is smaller than at P156, which may suggest that ES-associated changes may become apparent later (when larger numbers and species PUFAs and oxylipins can be detected) and unveil themselves with increasing age (P60 versus P156). It is also plausible, given our data in **chapter 4**, that the baseline differences between the hippocampus and hypothalamus underlie a differential responsiveness to ES. This warrants further investigation.

The lack of a lasting effect of ES on the PUFA/oxylipin profile at P60 in **chapter 5** does not necessarily indicate the absence of an effect on any PUFA level. First, it is probable that the mechanisms that are acutely implicated by ES wash out over time, like we also observed in our earlier work where we saw lasting effects on cognition at 4 months of age, even though the membrane-bound fatty acid alterations (acutely) induced by ES in the hippocampus have disappeared by then (53). Of note is further that this process is probably slower than the normalization of any ES-induced PUFA/oxylipin profile changes, as membrane-bound PUFAs are more stable than the volatile free PUFA and oxylipins that have a short half-life (60,61). Furthermore, ES affects at least one aspect of PUFA metabolism—whether in terms of availability, metabolism, or oxidation—as shown in **chapter 5**, where a specific PUFA- and oxylipin response to a dietary intervention that increases N-3 PUFAs, was observed exclusively in ES animals, but not in controls.

An example of a mechanism that is acutely affected by ES and that could have effects on PUFA metabolism, is oxidative stress. Oxidative stress is characterized by an excess of reactive oxygen species (ROS), which can cause damage to proteins, lipids and nucleic acids in the cell (62). Several studies have reported an increased oxidative stress profile after ES ((63–65), **chapter 2**). Interestingly, ES induces an increase in lipid peroxidation (64), a process for which PUFAs are an important substrate (66). In addition, ES-induced alterations in the enzymes involved in the N-3 and N-6 pathways could have specific effects on PUFAs (67,68). For example, a liver transcriptomic analysis revealed that the pathways that were specifically enriched after ES, were all processes related to fatty acid metabolism (67). For example, mRNA for the enzymes involved in the process of ALA conversion to EPA, such as fatty acid desaturase (FADS)1 and FADS2, were found to be reduced after ES (67). In addition, ES was shown to upregulate proteins involved in fatty acid β-oxidation, such as Decr1, Hadha and Hadhb (68). All of these alterations might already exist right after the stress paradigm, with ES-exposed animals potentially adapting their PUFA metabolism later in life, a change that could be viewed as a form of ‘priming’ or programming by ES, where temporary changes in fatty acid metabolism can imprint on later-life PUFA-related processes (such as the de-esterification of EPA, as proposed in **chapter 5**) , which could again have later consequences for e.g. network function or cognition.

N-3 PUFA supplementation and PUFA metabolism

So far, there is limited evidence (53,54) showing that early N-3 PUFA supplementation mediates later-life outcomes via the programming of PUFA metabolism. As discussed in several

chapters (**chapter 5** and **6**) of this thesis, the focus in previous studies has generally been on measuring the acute effects of circulating supplemented PUFAs, rather than exploring the later and long-term programming effects of N-3 PUFA supplementation. It is unknown whether wash-out effects play a strong role in our studies, but it is highly probable this is relevant for the effects we found of N-3 PUFA supplementation at P60, as half-life of some free PUFA- and oxylipin species is as short as 8 to 13 minutes (60,61,69).

While there are thus clear programming effects on PUFA metabolism, it remains unclear how exactly an early enrichment with N-3 PUFAs can modulate later-life PUFA metabolism. We propose that the effects of this enrichment could be ascribed to e.g. a sustained incorporation of extra N-3 LC-PUFAs in cell membranes during postnatal LC-PUFA accretion (which is a critical build-up to the adult LC-PUFA levels) (70,71). Acute effects of N-3 PUFA treatment on cells have been described and an increased incorporation of these PUFAs in membranes was indeed observed. For example, after treatment with DHA, BV-2 cells (a microglial cell line) exhibited an increased DHA incorporation in their membranes (72). This membrane uptake of PUFAs could be sustained (i.e. from P42 to P60), but especially at later stages, changes in responsivity could become apparent only upon e.g. stimulation by ES or lipopolysaccharides (LPS), as we observed in previous studies (54).

An alternative option is that transcriptional- or epigenetic programming events could be induced by N-3 PUFAs. This is indeed the case in the brain (73) and liver (74) directly after supplementation. In a previous study with a similar design, a programming effect of N-3 PUFA enrichment was found in an integrated mRNA-miRNA approach (75), supporting the occurrence of lasting programming effects in the adult hippocampus. It is plausible that such effects can also be observed in individual molecules relevant for specific PUFA pathways (like in (74)), that may have been missed in studies on fatty acid pathways per se, as such molecular modifications would not have appeared in the pathway enrichment analyses (73,75).

Though we have shown brain region effects on a standard chow diet in **chapter 4**, no sex effects were found for PUFA/oxylipin species in this chapter. We propose that there may be a different metabolic homeostatic setpoint in the hypothalamus and the hippocampus, leading to different responsivity to N-3 PUFAs. Although males and females present with similar homeostatic setpoints for the detected PUFA and oxylipins (**chapter 4**), we have observed that their responsivity to N-3 PUFAs is different (*Data not shown*), indicating possible differences in metabolism, that have also been described for metabolic programming between males and females before (76–79).

Early life factors mediate later-life outcomes via microglial modulation

ES and microglial modulation

We and others have proposed that microglia are important cells contributing to the long-lasting effects of ES. As the main resident immune cell of the CNS, microglia are altered in ES-exposed animals, and affect their morphology, transcriptomic expression, cytokine patterns, phagocytosis capacity, and more ((53,80–85), **chapter 3** and **chapter 5**).

We show in **chapter 3** that ES increased the CD68 coverage in the dentate gyrus and the cornu ammonis of the hippocampus at 4 months of age, which was not accompanied by morphological changes of these cells. Seemingly in contrast, we observed in **chapter 5** that ES decreased CD68 expression in these same brain regions, and was accompanied by an increase in their morphological complexity at 2 months of age. As already touched upon in this chapter, the combined findings indicate a dynamic switch in the neuroinflammatory response that appears to be different between the time points of; the end of first week of ES (80–85) in young-adulthood ((80) **chapter 5**), and in late adulthood ((53,84,85) **chapter 3**). Directly after ES, an increased inflammatory profile is observed, while on the other hand, impaired synaptic pruning occurs. Together, this could indicate a premature shift from synaptic pruning to immune-surveillance, as under standard conditions, such changes in microglial gene expression, and their roles in cell cycle regulation and synaptic pruning, only occur during a later stage of development (86,87).

While the observed changes in microglial expression and activity in early-life and later adulthood after ES, reflect this theory, during intermediate ages (P60), a reduction in microglial activity was found (**chapter 5**). I propose that this decrease in activity may be due to a phase of adaptation and compensation, where microglia downregulate their inflammatory responses to maintain homeostasis after the initial stress response in early-life. The heightened activity in late adulthood is further also consistent with the 'second-hit' theory: microglia become 'primed' right after ES, potentially leading to a higher and possibly exaggerated activation when exposed to an additional stimulus or inflammatory agent in late adulthood, thereby increasing vulnerability to psychopathologies and neurodegenerative diseases (88,89), where neuroinflammation often is a key pathological process (89,90).

N-3 PUFA (supplementation) and microglial modulation

In addition to microglia being influenced by environmental stress, there is substantial evidence that dietary N-3 PUFAs, or a lack thereof, can alter microglia morphology and function in both *in vitro* and *in vivo* studies (53,72,91–95). For example, in young mice, maternal deficiency of N-3 PUFAs led to an enhanced microglia-mediated phagocytosis, probably via changes in AA-derived oxylipin signaling (91). In addition, fish oil supplementation (rich in N-3 PUFAs) modulated microglial cell number and morphology in response to an intracerebroventricular injection with amyloid-β (Aβ) 1-40 (95). We have previously demonstrated in late adult mice that ES-induced cognitive impairments were accompanied by an increased expression of microglial CD68, a marker for phagocytosis, both of which could be reversed by early N-3 PUFA enrichment (53). These abovementioned studies all have in common that there is a response of microglia to PUFA levels, that seems to depend on whether the context is disadvantageous, such as a dietary deficiency, in disease, or the combination of ES and older age. At P60 (**chapter 5**), using a similar paradigm, we did not observe such anti-inflammatory changes and the hippocampus may thus exhibit intrinsically lower levels of inflammation or microglial activation at this age, which would reduce the impact of early dietary N-3 PUFA supplementation. In a system that is more disturbed, such as an AD model (or aged ES animals, as was studied ear-

lier (53)) or in the studies mentioned above, the benefits of such interventions may thus become more apparent as they counteract the heightened microglial activity and inflammation, as supported by our data in **chapter 6**, where we found reduced CD68 expression in hippocampal microglia in a model for AD, where an increased microglial activity is common.

As discussed in **chapter 4**, the microenvironment - specifically the intrinsic oxylipin/PUFA profile - can significantly influence microglial morphology and potentially their function at baseline. In this chapter, we demonstrate that specific hippocampal microglial morphology parameters correlate to specific oxylipin- and PUFA-species, highlighting the importance of lipid profiles in shaping microglial behavior and possibly responsiveness to N-3 PUFA supplementation. PUFAs can be incorporated into the phospholipid membrane of microglia. This incorporation alters the physical properties of the microglial membrane, and further affects the autocrine release of membrane-bound PUFAs, to which microglia are responsive via specific lipid-sensitive receptors such as TREM2, CD36, Toll-like receptors (96). As discussed before, BV2-cells stimulated with DHA exhibited an increased DHA incorporation in their membrane, which in turn lead to a reduction in their pro-inflammatory response to LPS (72). This could be due to a displacement of the LPS receptors, which is commonly observed for receptor proteins in membranes rich in N-3 PUFAs (97,98).

Therefore, alterations in microglial membranes can lead to an altered oxylipin synthesis by microglia, which possibly leads to the reduced availability of signaling receptors, such as LPS receptors. In addition, it is known that both hydroxyeicosatetranoic acids and prostaglandins (which are AA-derived oxylipins) are produced from microglial membranes in an autocrine manner (99,100) in culture. As these oxylipins are known to interact with the p38MAPK, NF-κB and PPAR-γ inflammatory pathways (101,102), a lower amount of their precursor in membranes, e.g. as a result of increased N-3 PUFA uptake in the membranes, might have less pro-inflammatory effects.

Next to oxylipins, another class of bioactive lipids that derives from PUFAs modulating microglial functions, are endocannabinoids (eCBs), particularly anandamine (AEA) and 2-arachidonoylglycerol (2-AG)(See **Fig. 1B**). These eCBs bind to cannabinoid receptors (CB)1, and 2, with CB2 being predominantly expressed in microglia (37). Under inflammatory conditions, CB2 activation promotes an anti-inflammatory, pro-resolving state in microglia (103).

While PUFAs can thus directly affect microglial function, their incorporation into or interaction with other CNS cells, such as neurons, astrocytes and oligodendrocytes, can also lead to indirect effects on microglia. These other cells have been not been studied in detail in this thesis, but are also highly responsive to PUFAs (37).

Despite the theories presented here, how exactly an early enrichment with N-3 PUFAs exerts its effects on the neuroinflammatory response, is currently unknown. In the experiments presented in **chapters 5** and **6**, we have not investigated whether membrane-bound N-3 PUFAs changed as a result of our dietary intervention, neither do we know whether this has happened in microglia specifically. This would, however, shed light on possible mechanisms behind a seemingly reduced microglial proinflammatory phenotype by early N-3 PUFA

supplementation, as observed in **chapter 6**.

Opportunities for PUFAs targeting neuroinflammation in ES and AD

In the previous sections, I have discussed how PUFAs could have great potential in targeting neuroinflammation. We focus on the conditions of ES and AD, both of which are associated with chronic neuroinflammation, and where microglia play a key role ((85); **chapter 3**). We will next focus on what opportunities exist for PUFA interventions in these specific contexts, as nutritional approaches to prevent or alleviate chronic neuroinflammation are attractive as they have many advantages, among others being cost-effective and relatively easy to implement.

Early-life stress (ES)

Our previous studies have demonstrated i) a chronic ES-induced neuroinflammation in adulthood ((85); **chapter 3**), and ii) that early dietary supplementation with N-3 PUFAs leads to a reduction in pro-inflammatory microglia (53) and to behavioural improvements in adulthood (53,75). In young adulthood, at P60, we do not (yet) observe such a reduction of pro-inflammatory microglia (**chapter 5**). My hypothesis is that N-3 PUFA supplementation is particularly effective in reducing the neuroinflammation triggered by actual inflammatory triggers and in reducing chronic neuroinflammation, rather than that N-3 PUFAs constitutively inhibit microglial activation, as a possible preventative measure. I propose a more on-demand reduction of neuroinflammation, which could fit with the on-demand metabolization of PUFAs from membranes (which occurs through stimulation of microglial lipid-sensitive receptors (94)). In our experiments, this metabolization of PUFAs might be altered via resetting the sensitivity to metabolization enzymes via programming or by a change in membrane-bound PUFAs.

Alzheimer's disease (AD)

The data presented in this thesis further support the concept that PUFAs are able to reduce microglial activation in a model for AD, that is characterized, among others, by chronic neuroinflammation (**chapter 6**). This agrees with the hypothesis mentioned above. As discussed before, microglia are extremely sensitive to their lipid environment, via expression of several lipid receptors on their membranes. The effects of PUFAs on microglial phagocytic capacity appear to be highly context-dependent, and influenced by both the local environment and the nature of the substrate that is being targeted.

Whereas in mice that were N-3 PUFA deficient, microglia phagocytosed more synaptosomes (91), supplementation with DHA and EPA (93) or derivatives (104) could increase the phagocytosis of Aβ by a microglial cell line. This context specificity is further supported by findings in **chapter 6** of this thesis, where we demonstrated that *ex vivo*, microglial phagocytosis is upregulated in APPswe/PSEN1dE9 (APP/PS1) animals that received the N-3 PUFA diet early in life. In this case, the local environment of amyloid-β-primed tissue could have primed the microglia, and the presence of amyloid as a substrate may have enhanced their phagocytic activity *ex vivo*. This increased phagocytosis thus likely contributes to the observed reduction in amyloid load we found in these animals, indicative of a potential mechanism by which

PUFAs exert a potential neuroprotective effect in AD.

Untargeted effects of nutritional interventions in the brain

The impact of diet probably extends beyond the processes studied here, and is likely to influence a broader spectrum of brain cells and even peripheral systems. In studying the effects of nutrients specifically in microglia, as was done here, it is important to acknowledge that other processes occurring in different cells might possibly indirectly have contributed to the observed effects as well. For example, in **chapter 3**, we report beneficial outcomes on cognition in ES-exposed animals that were fed a polyphenol diet, but did not observe clear concomitant microglial changes. In the context of ES, it is plausible that other cellular processes or different microglial processes (e.g. alterations in gene expression) beyond what we have measured) are involved in the beneficial effects of polyphenols on behavior, such as oxidative stress and others ((105–107) see **chapter 2**).

Moreover, the impact of nutritional interventions can extend to peripheral organs. A bidirectional relationship between the brain and the periphery thereby complicates the study of (in)direct effects and the establishment of causes and consequences (108). An important example is the gut-brain axis, which is the bidirectional relationship between the gastrointestinal (GI) tract and the CNS (108), which is specifically relevant in the context of nutrients as they might have direct effects on the gut. Yet, the use of nutritional interventions in this context makes it difficult to draw clear causal conclusions. For example, one study observed that polyphenols reversed ES-induced depressive-like and anxiety-like behavior in rats, and observed profound changes on the gut microbiota and corticosterone levels (109). It is impossible to conclude whether polyphenols had a direct effect in the brain, or acted via indirect effects, where the gut microbiota altered corticosterone, or vice versa, where an shared mechanism (that e.g. affects both gut microbiota and corticosterone) is at play, or whether changes in gut microbiota and corticosterone are two independent observations that do not contribute to behavior in this setting.

Food for thought

Considerations for experimental designs containing dietary interventions

In animal dietary studies, there are several inherent aspects that have to be considered in relation to the experimental design as it can affect the validity and generalizability of the results. In this section, I outline some of the key considerations relevant for the experiments presented in this thesis.

Synthetic diets

Synthetic diets are often used in experimental settings to ensure precise control over nutrient intake and composition. However, as mentioned in **chapters 3, 5,** and **6** synthetic diets might lack at least some of the essential fibers that are present in standard chow. As this might influence inflammatory changes and/or e.g. the microbiome, this may affect our outcomes (110–

112). Therefore, we opted for either a grain-based diet or a semi-synthetic diet with added fibers. Notably, possibly due to color and or palatability, intake of synthetic diet was shown to be less in rats compared to chow diets (113), which is important to take note of when calculating the dosage in the diets.

Mode of administration

The mode of administration is crucial as it can significantly influence the outcome of the study, affecting absorption and metabolism as well as behavioral responses. Multiple administration routes are used in literature, including intraperitoneal injection, oral gavage and *per os* (through feeding). Though polyphenol injections, but possibly PUFA injections as well, lead to more effective bioavailability due to the absence of some important barriers and metabolizing organs (stomach, GI tract and liver)(114), we preferred using pelleted diets over oral gavage and injection, for two reasons: i) we wanted to study dietary interventions and pelleted diet resembles the intake of humans better, ii) in our models, in which ES plays a key role (see **chapter 3** and **4**), a pelleted diet is preferred over oral gavage or injections, as the restraint stress for the individual animals and/or the disturbance of the nest during these procedures might interfere with our relevant (e.g. behavioral) outcomes (115,116).

Following the two-hit hypothesis, restraint followed by gavage or injection could be a second stressor to which ES animals are specifically sensitive, creating an exaggerated (immune) response in these animals (117–119). Important to note therefore is that some nutrients such as PUFAs and polyphenols, because they are volatile and sensitive to oxidation (120,121), need specific adaptations to the diet, e.g. polyphenols need an acidic environment with a lower temperature for stability (120). This in turn might require significant changes to the pellet that affect palatability and color. These alterations might exclude the opportunity to use a grain-based diet.

Can one supply more than necessary?

It is possible that when there is no deficit in a certain nutrient, administering these nutrients might lead to a supra-physiological dose (i.e. amounts significantly higher than the recommended dietary intake), and given the bioactivity of nutrients, this might cause relevant changes to specific outcome measures. In **chapter 3**, we observed that there was an increase in microglial cells after supplementing polyphenols to control animals. This observation was not linked to behavioral alterations, but could have other effects in these mice of which we cannot estimate the consequences besides what we have measured. Notably, polyphenols interact with ROS to inhibit their effects, but ROS also serve as cell signaling molecules for normal physiological processes (122,123). An excess of these polyphenols might therefore interfere with normal physiological processes in control animals, leading to possible adverse effects.

Moreover, high doses of one nutrient can interfere with the absorption or metabolism of others, as we show in **chapter 5** where we upregulate N-3 PUFA pathways by administering more ALA, inhibited erythrocyte membrane-bound N-6 PUFAs in WT animals. Due to these interactions, it could be that a dietary excess and the negative consequences of that, may out-

weigh the potentially positive effects of a nutritional intervention per se. In support, one interesting observation in AD mice showed that benefits of N-3 PUFAs were indeed neutralized by a high dietary intake of N-6 PUFAs and SFAs (124).

Finally, in case of a nutrient deficit, it might be good to evaluate if there are metabolic problems present that could cause this, because in such instances, supplementation might not be effective and other strategies have to be employed. For example, FADS gene variants, which encode desaturase enzymes, can impair the body's ability to convert ALA into EPA and DHA, or LA into AA (125), and reduces effectivity of lowering the ratio of LA/ALA, but in this case DHA supplementation could be a successful alternative.

Critical gaps in literature

Sex differences

The need and relevance to study males as well as females is becoming increasingly important (126–128), but experiments in females still lack behind. This is also emphasized by the limited studies on the effect of nutritional intervention on ES-induced behaviors performed in females that we were able to include in **chapter 2**. In this thesis, we have only studied both sexes in **chapter 4** mostly due to logistic reasons. The other studies were performed in males only, which limits generalization, because there is a sex-specific vulnerability to ES (129,130) and AD (131). Moreover, sex differences in microglia have been described (which we extensively elaborate on in **chapter 4**).

We have observed that the PUFA metabolism, at least in erythrocytes, is different in males and females (**chapter 4**), and as a result, the biological response to a nutritional intervention might be sex-specific. Indeed, we observed that the effect of N-3 PUFA supplementation on the brain PUFA/oxylipin profile was not only dependent on brain region, but also on sex (*Data not shown*). These factors might thus be of considerable influence in the efficacy of a nutritional intervention, but whether the sex-specific response to N-3 PUFA supplementation observed (*Data not shown*) leads to functional differences in these mice, is currently unknown.

How nutrition and ES shape programming

This thesis investigated the programming effects of ES on microglia, and we explored the potentially beneficial programming effects of nutrition. The interaction between these two types of programming is complex, as the mechanisms underlying them, remain largely unknown. Future studies should therefore focus more on whether the positive effects of nutrition might occur through converging mechanisms, where both ES and nutrition impact similar pathways and "even each other out", or through adaptive mechanisms, in which nutrition could mitigate or modify the effects induced by ES.

Epigenetic mechanisms are likely to be a common mediator of both alterations by stress as well as those induced by early nutritional interventions. Epigenetic programming in response to ES has been described previously (132,133) while nutrition also has the capacity to exert epigenetic programming, for example through folate and vitamin B (134,135). Interest-

ingly, early dietary supplementation of 1-C metabolism-associated micronutrients, which are known to promote DNA methylation during ES, has been shown to partially rescue cognitive decline later in life (136). This could mean that this dietary programming had interfered partially with ES programming. The interaction between the dietary interventions and the stress paradigm could have resulted in overlapping or counteracting epigenetic modifications, potentially contributing to the observed effect. It will be of interest to further study this in the context of the two dietary interventions used in this thesis.

Clinical and translational implications

The findings in this thesis provide important insights into the potential of early dietary interventions in the context of ES and AD, and into the (neuroinflammatory) mechanisms involved. Translating these findings into practical recommendations for a human population faces some important challenges, such as differences between the human and rodent diet; the limitations of modelling ES in the context of nutrition and the limitation of a genetic model for AD.

One of the key issues is the complexity of the human diet. Among humans, the diet is very diverse, and it is less amenable to isolated supplementation compared to the well-controlled diet and environment of rodent chow. For example, a close adherence to Mediterranean diet exerted beneficial effects on human cognition, and the vulnerability to mild cognitive impairment and AD as evidenced by a systematic review and meta-analysis (137), but there is one major outstanding question: how does one model the complexity of the Mediterranean diet in rodent diets? The Mediterranean diet consists of various nutrients, including vitamins, fatty acids and polyphenols (138). Instead of creating the exact same diet in rodents, we often opt for selecting key nutrients and fixed proportions of these nutrients, but with that strategy we do not know what interactions of nutrients we do not model.

Different nutrients in a diet could further interact in complex ways with each other, as is for example described for polyphenols and PUFAs (139), making it more challenging to isolate the effects of individual supplements. As discussed in **chapter 2**, the efficacy of specific dietary supplements in the context of ES in humans requires more studies, ideally these studies will be large-scale RCTs, but this may pose problems of adherence when long-term effects are to be considered.

An additional challenge for the field of nutritional neuroscience is the large role of socioeconomic status (SES) in the exposure to adverse childhood experiences (140), which also correlates with access to good quality food and overall health sources (141). Indeed, ES-exposed individuals more often have a low SES, with poor access to resources and a healthy balanced diet. Without addressing these disparities in health equity, any potential benefits of nutritional strategies might in practical terms be inaccessible to those most in need.

Finally, our research has largely focused on the amyloid-β pathology of AD using the transgenic APP/PS1 rodent model. While this model is valuable for understanding this specific pathway in AD, and does produce plaque pathology, it does not fully capture the complexity of the human disease ((142), **chapter 6**). Moreover, the genetic basis of this model is more relevant to familial AD, which involves for a smaller proportion of AD cases, that admittedly show a

similar neuropathological phenotype. Thus, the generalizability of our findings to sporadic AD need to be considered, as this form is more common and influenced by a combination of genetic, environmental, and lifestyle factors (143).

Concluding remarks

In this thesis, we have provided and reviewed evidence that supports the efficacy of nutritional interventions in mitigating detrimental behavioral effects in the context of ES. A preliminary conclusion from mostly rodent studies is that nutritional interventions are quite effective in alleviating ES-induced changes in behavior, and that the early-life therapeutic time window is of particular importance in this respect. We further found that neuroinflammation as a biological mechanism (specifically microglial functioning) is not effectively targeted by an early supplementation with polyphenols, but can be affected by local PUFA- and oxylipin species and by (early) N-3 PUFA supplementation.

Finally, we highlight that early N-3 PUFA supplementation could be of particular interest in AD, as it reduced amyloid pathology in a transgenic mouse model of AD. As such, this thesis contributes to a better understanding of how early-life factors, such as nutrition and stress, contribute to later-life neuroinflammation and cognition and may help provide new avenues for future nutritional intervention strategies.

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