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Focus on sex differences

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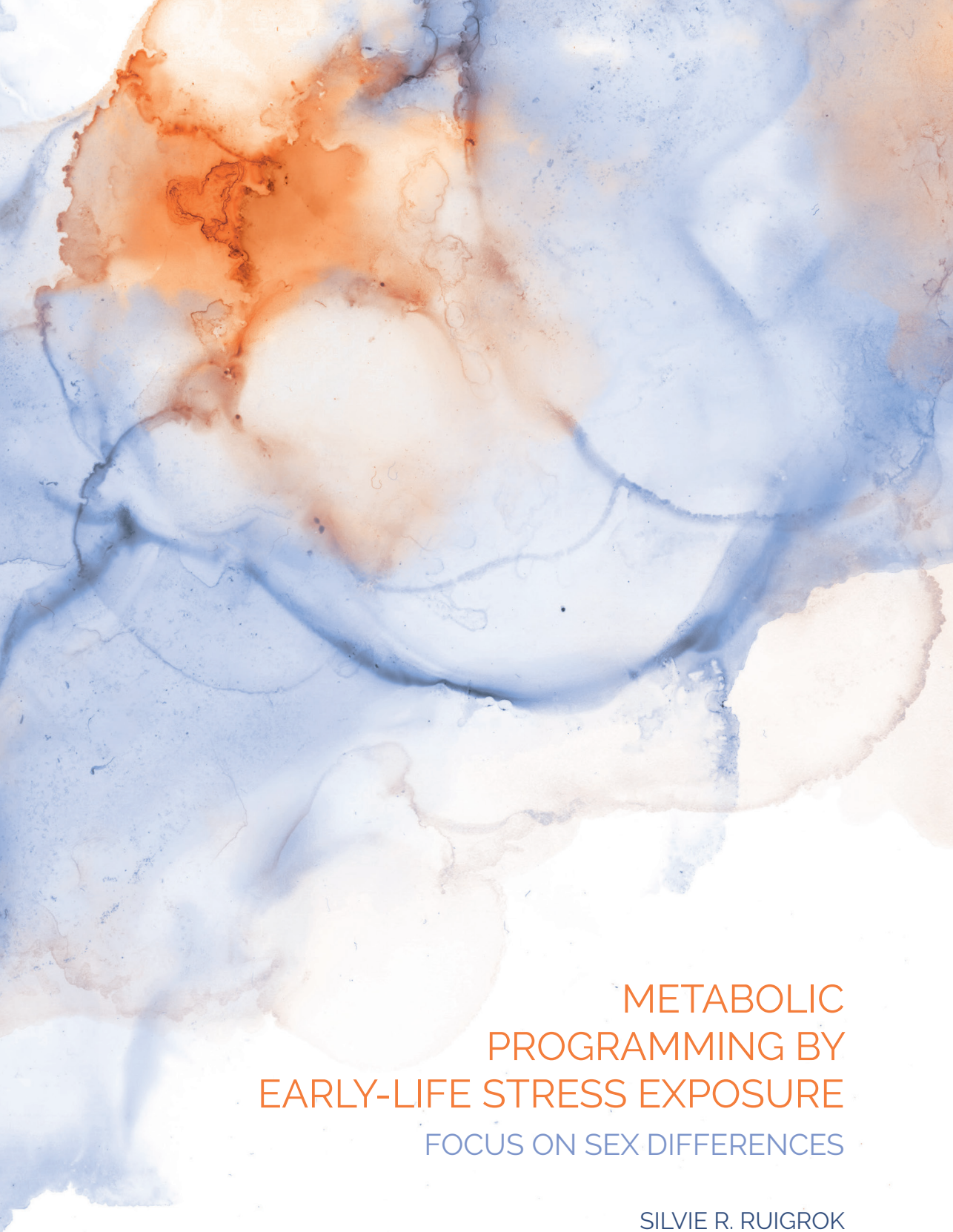

METABOLIC PROGRAMMING BY EARLY-LIFE STRESS EXPOSURE

FOCUS ON SEX DIFFERENCES

SILVIE R. RUIGROK

Metabolic programming by early-life stress exposure

Focus on sex differences

Silvie Rosalie Ruigrok

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Preface

Early life stress programs individuals for life

Exposure to early-life stress (ES) is very common in modern society. In the US, approximately 61% of adults have experienced some form of early-life adversity, including, but not limited to parental separation, domestic violence and abuse (physical, emotional or sexual) (Merrick et al., 2018). Notably, exposure to ES increases the risk for developing a wide range of disorders later in life, including metabolic diseases such as obesity, as well as mental and cognitive disorders (Chugani et al., 2001; Danese and Tan, 2014; Green et al., 2010; MacMillan et al., 2001; Roseboom et al., 2006; Saleh et al., 2016). Prevention of ES is difficult, and an increased understanding of the biological underpinnings of such long-lasting effects is needed in order to develop effective intervention strategies.

The idea that the early environment is important for later life health was initially proposed by David Barker (Barker, 2007, 1995). His Developmental Origins of Health and Disease hypothesis (DOHaD) was initially based on geographical studies linking child mortality (attributed to a low birthweight), to ischaemic heart disease (Barker and Osmond, 1986). Barker therefore hypothesized that *in utero* undernutrition can 'program' the development of individuals, which would lead to an increased risk of coronary heart disease later in life. Today, it is known that many types of stressors, including those of a psychological or nutritional nature, occurring in pre- or postnatal life, can all increase the later risk on disease. This extends beyond the cardiovascular domain, and includes obesity, diabetes, cognitive dysfunction and mental disorders (Alciati et al., 2013; Bellis et al., 2015; Chugani et al., 2001; Danese and Tan, 2014; Green et al., 2010; MacMillan et al., 2001; Roseboom et al., 2006; Saleh et al., 2016).

The concept of the early-life environment shaping the offspring for life is called 'programming'. In humans, the first 1000 days after conception are considered to be a critical window for such programming, whereas in rodents the gestational period up to the first two postnatal weeks represent an extremely sensitive developmental period, during which brain structures including the hypothalamus and hippocampus continue to develop (Bouret and Simerly, 2006; Cusick and Georgieff, 2016; Pleasure et al., 2000). However, little is known about exactly which elements in the early-life environment are involved in programming by ES. Moreover, while a substantial amount of literature has been devoted to the effects of ES on mental and cognitive functions, less is known about the effects of ES on later metabolism, or about the subcellular substrates underlying the programming by ES. Since metabolic and brain disorders are often comorbid (Nguyen et al., 2014; Nooyen, 2010), similar underlying pathways could be involved. Finally, although some studies indicate a sexually dimorphic effect of ES (Boynton-Jarrett et al., 2010; Hay et al., 2008; Kozyrskyj et al., 2011; Park et al., 2018), this is largely unexplored so far.

Scope of this thesis

Elements in the early environment: focus on nutrition

The early life environment encompasses many essential elements that interact with one another. This includes both exogenous factors (e.g. tactile stimulation, temperature and nutritional provision) and endogenous modulators (e.g. stress hormones, inflammation) (Hoeijmakers et al., 2015; Lucassen et al., 2013). Indeed, in the past decades, it has become evident that tactile stimulation (e.g. high versus low licking and grooming) affects offspring development (Francis and Meaney, 1999; Liu et al., 1997). However, since these elements interact and affect one another, it is likely that ES programs offspring by affecting multiple of these essential elements (Hoeijmakers et al., 2015; Lucassen et al., 2013). Nutrition provides the energy and building blocks needed for proper development, and breast milk offers an optimal nutrition in the early postnatal period. For example, breast feeding reduces the risk on obesity, diabetes type 2, infection and cognitive impairments (Cusick and Georgieff, 2016; Horta et al., 2015; Lee et al., 2016; Mameli et al., 2016; Mayer-Davis et al., 2006). Yet, whether early-life nutrition mediates programming by ES, or whether early-life nutritional interventions can modulate the effects of ES on offspring, is largely under-investigated. In particular the lipid quality during the early life period seems critical for proper brain and metabolic development (Schipper et al., 2020). In this thesis, we therefore focus on two aspects; the fatty acid composition of maternal milk and the physical properties of the lipid droplets in an early-life dietary intervention study.

Metabolic programming

Early in life, a metabolic setpoint seems to be established which determines later-life metabolic health (Bouret and Simerly, 2006; Dearden and Ozanne, 2015; Levin, 2006). Stress during the critical developmental period seems to alter this metabolic setpoint, but little is known about how ES programs metabolic functions. It is suggested that the later-life nutritional environment determines the ES-induced metabolic outcome, and that hence ES-exposed animals may be particularly vulnerable for unhealthy diets (Murphy et al., 2018, 2017; Yam et al., 2017). However, why ES-exposed animals respond differently to such diets and whether ES also affects their choice for unhealthy food, is unknown. Moreover, if and how ES affects the brain circuits that regulate food intake and energy expenditure remains to be understood. The hypothalamus regulates energy homeostasis by regulating food intake and energy expenditure (Timper and Brüning, 2017), while the reward circuitry (including the ventral tegmental area; VTA) is implicated in the hedonic aspects of food intake (De Macedo et al., 2016). In rodents, the hypothalamus and VTA continue to develop into the first two weeks of postnatal life and adolescence, respectively (Bouret and Simerly, 2006; Kalsbeek et al., 1988; McCutcheon et al., 2012; Teicher et al., 1995; Voorn et al., 1988), suggesting these brain circuits could be particularly vulnerable for programming by ES. In this thesis, we focus on both these circuits.

Sex differences

ES has been proposed to affect both sexes differently. For example, ES may increase the metabolic vulnerability predominantly in girls/females, while the risk to develop cognitive problems may be more profound in boys/males (Boynton-Jarrett et al., 2010; Hay et al., 2008; Kozyrskyj et al., 2011; Park et al., 2018). Due to logistic feasibility, there is a strong male-bias in (rodent) research. However, it is crucial to gain insights into sex-specific effects of ES. This will aid to a develop a deeper understanding of differences between the sexes under both basal circumstances and in response to ES, and help to develop appropriate (sex-specific) intervention strategies.

Comorbidity

Metabolic diseases (e.g. obesity, diabetes) often occur together with cognitive disorders (Dye et al., 2017; Nguyen et al., 2014; Nooyen, 2010). This clear comorbidity suggests that similar underlying pathways and/or subcellular substrates are involved in the programming by ES. Mitochondria are implicated in both metabolic and cognitive disorders (Guo et al., 2017; Khacho et al., 2017; Sivitz and Yorek, 2010), mediate the stress response and are affected by stress (Picard et al., 2014). It has therefore been hypothesized that mitochondria could be involved in the programming by ES (Hoffmann and Spengler, 2018; Zitkovsky et al., 2021). If a common subcellular substrate can be identified, this can serve as an interesting target for future intervention studies.

Animal models for ES exposure

Animal studies are crucial to investigate the mechanisms that underlie programming by ES in more mechanistic detail, since they allow to control for confounding factors like recall bias, genetic differences and environmental factors (e.g. diet)(Nestler and Hyman, 2010). The three most commonly used ES models are maternal deprivation (removing the mother a single time for 24h), maternal separation (removing the mother repeatedly for 3-8h) and the limited bedding and nesting material (LBN) model. The latter relies on creating an impoverished environment from postnatal day (P)2-P9, and results in fragmented maternal care (Naninck et al., 2015; Rice et al., 2008), without removing the mother from the cage or frequent interaction of the experimenter. This model therefore better recapitulates ES exposure in humans, where the parent is typically present but not able to provide the appropriate care. Exposure to LBN results in metabolic alterations and cognitive impairments later in life (Naninck et al., 2017; Rice et al., 2008; Yam et al., 2017), making it a suitable model for studying effects of ES in rodents.

Thesis aims and outline

The main objective of this thesis is to increase our understanding of the mechanisms by which ES programs offspring for life. A large part of this thesis will be devoted to metabolic programming by ES, and whether these effects are sex-specific. However, due to the high

comorbidity of metabolic and cognitive disorders, we also studied the effects of ES on metabolism and brain functions simultaneously. By doing so, we aim to identify common underlying pathways, which could aid the development of novel therapeutic strategies.

Human and animal studies have repeatedly shown an association between ES exposure and an increased risk on later metabolic, mental and cognitive disorders, with some reporting sex differences. Besides the fact that metabolic diseases and mental disorders are often comorbid, they also have a sex-specific prevalence. It is unclear if ES could be at the origin of such sex differences in disease prevalence. In **chapter 1**, we review the clinical and pre-clinical evidence for effects of early-life adversity (ELA; including pre- and postnatal stress) on offspring metabolic and mental outcome, focussing on sex differences. We discuss whether ELA affects both mental as well as metabolic health, if ELA-induced effects differ between males and females, and if this is similar for pre- and postnatal stress. To understand if preclinical research is a useful tool to develop novel intervention strategies, we describe if clinical and pre-clinical studies converge in their findings on the impacts of ELA. We furthermore propose mechanisms (placenta and breastmilk) by which ELA could program offspring for life.

In **chapter 2,** we increase our understanding of the elements in the early-life environment that might mediate the programming by ES. Due to their significant effect on both metabolic and cognitive function, we hypothesize a role for (maternal milk) fatty acids in ES programming. Using a mouse model for ES, we study the effects of such stress exposure on milk, as well as offspring brain and erythrocyte fatty acid status. We do so in both male and female offspring, and correlate milk and offspring FA status to each other.

Moreover, while ES exposure increases the risk for obesity, breast feeding evidently decreases this risk. It is thought that, next to the nutritional composition of milk, the physical properties of human milk lipid droplets contribute to this health benefit. Emerging evidence indicates that peripheral and central inflammation are involved in obesity development and its associated negative health outcomes. In **chapter 3**, we therefore investigate whether ES exposure alters the peripheral and central inflammatory profile, and whether a concept infant milk formula (IMF) mimicking the physical properties of human breastmilk could protect against such effects.

In **chapter 4**, we aim to further understand what factors contribute to the increased risk on obesity after ES exposure. We have previously shown that ES increases fat accumulation when exposed to an unhealthy diet in mice. However, in the human condition, an important aspect of obesity is the choice of unhealthy foods, hyperphagia and snacking behavior. Yet, whether food choice also contributes to ES-induced metabolic vulnerability, and if this is sex specific, has not been investigated. In addition, we also study if ES affects the physiological

response to such a diet and the brain circuits that regulate food intake, focusing on the hypothalamus and reward circuitry.

The hypothalamic circuitry regulates food intake and energy expenditure by sensing circulating hormones and nutrients. As fatty acid metabolism within the hypothalamus functions as a sensor for energy availability, and integrates both nutritional and hormonal signals, we investigate in **chapter 5** if ES alters these hypothalamic nutrient sensing pathways in male and female mice.

In **chapter 6**, we aim to identify a common subcellular substrate by which ES could program both metabolic and cognitive functions, focussing on mitochondria. Mitochondria are cellular powerhouses and are implicated in both metabolic disease and cognitive dysfunction. We study the effects of ES on mitochondria biology directly after stress exposure and in late adulthood, and relate this to metabolic and cognitive readouts.

Mitochondria in microglia have been implicated in diet-induced obesity development. In **chapter 7**, we investigate whether ES affects mitochondrial density and structure in hypothalamic microglia. We study these parameters at two ages (early in life and in late adolescence), under standard dietary circumstances and upon exposure to a high-fat high-sugar diet. In addition, using an *in vitro* approach, we study the effects of ES on microglial mitochondrial respiration in response to different nutritional environments. In this preliminary chapter we also focus on potential sex differences.

In **chapter 8**, the main findings of this thesis and their implications are summarized and discussed.

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CHAPTER 1

1

Early origin of sex-dependent vulnerability to mental and metabolic disorders: a comprehensive review and comparison of the effects of early-life adversity in human and rodents

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In preparation

Abstract

There is a large body of evidence from human and animal studies indicating that early-life adversity (ELA) increases the risk to develop mental and metabolic disorders. Notably, mental and metabolic disorders often co-occur and some of these disorders exhibit sex-dependent prevalence. However, how the early-life origin, the comorbidity and sex-differences in these disorders are related to each other is currently not well understood. We set out to comprehensively review the evidence from human and rodent studies that; i) studied effects of pre- or postnatal stress on mental and/or metabolic health outcomes and ii) investigated effects in both sexes. In this comprehensive review we find that: i) ELA impacts on both mental as well as metabolic health, ii) ELA-induced effects often differ between the sexes, iii) depend on timing of stress exposure and iv) evidence from human and rodent studies largely converge in their findings on the impacts of ELA. In particular, ELA-exposed boys/males seem more often negatively affected on the cognitive and externalizing domain, while for the internalizing behaviours and disorders (e.g. anxiety and depression) both sexes are negatively affected depending on the type and timing of ELA. In the context of metabolic outcomes, human and rodent studies show that girls/females are more vulnerable to develop overweight/adiposity, and exposure to ELA in rodents seems to more often result in reduced insulin sensitivity in males. We conclude that evidence from studies in both humans and rodents suggest that the long-term health of men/males and women/females is differentially impacted by ELA. Therefore, we stress that differences between the sexes must always be investigated in both human and animal ELA studies and that results cannot be generalized to the other sex. Finally, in the context of population studies, intervention trials, treatments and diagnosis we recommend to systematically take into account the sex, early-life history and comorbidities of the individuals, laying ultimately the basis for personalized treatment.

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1.Introduction

Exposure to early life adversity (ELA) during critical developmental periods prenatally[1–3] and early postnatally[4,5] increases the risk to numerous health problems later in life, including psychopathologies[6,7], cognitive dysfunctions[8,9], as well as metabolic and cardiovascular diseases[10,11] (see systematic review Hughes and colleagues (2017)[12]). Such early life adversity can take many different forms, from psychological to more metabolic in nature, or most often a combination of those (e.g. war, under/over nutrition, maternal depression, or being exposed to neglect and sexual or physical abuse. Remarkably, different forms of early life adversity can lead to strikingly similar health outcomes impacting both mental and metabolic health[6–11], suggesting a shared early-life origin for mental and metabolic health risks.

Next to this possible shared early-life origin, two additional aspects are important to note about mental and metabolic disorders: they are i) often comorbid and ii) exhibit sex differences in their prevalence. Concerning their comorbidity, for example, there is emerging evidence for a bidirectional relationship between depression and obesity: obesity increases risk for depression with 55% and depression increases risk for obesity with 58%[13–16]. Another example is that obesity and diabetes have been associated with cognitive decline and Alzheimer's disease[17–19]. The co-occurrence of these mental and metabolic disorders suggests the possibility that similar pathways might be involved and/or interact. As to the sex differences in their prevalence, in general, women seem to be more at risk for developing internalizing disorders such as depression[20] and anxiety[21], while men are at higher risk for developing externalizing disorders such as attention deficits, hyperactivity and aggression[22]. Sex differences in dementia are observed mostly >85-90 years of age, when older aged women are at higher risk than men[23–26]. Regarding metabolic diseases, there are somewhat more subtle sex-differences as well. Worldwide, obesity is more prevalent in women[27,28] while diabetes is slightly more prevalent in men, especially at middle age[29–31], with prevalence variations within and between countries[27,30] .

Despite the very large body of literature from human and rodent studies[32] addressing effects of ELA on various health outcomes, it remains unclear how ELA impacts on the vulnerability to develop mental and metabolic disorders, and if their sex-specific prevalence and comorbidity indeed has an early-life origin. However, these are very relevant questions. In fact, there is increasing evidence that ELA impacts the biologic makeup of an individual such that the mechanisms and pathways at play in an ELA-induced mental or metabolic disorder might be different compared to the same disorder triggered by other factors or life events[33]. This can have major implications for diagnosis and treatment strategy and efficacy. Similarly, when evaluating results of human cohort studies and trials on factors influencing disease, or when evaluating treatment efficacies, stratification based on ELA becomes thus relevant. We suggest that the observed co-occurrence between mental and metabolic diseases and the sex-differences in disease prevalence might (at least in part) have an early

origin, and stem from sex-specific alterations in the development of brain and metabolic tissues in response to ELA, dependent on timing of ELA exposure[34–37].

The reason that such evidence is lacking so far, is (at least in part) due to the fact that, despite the increasing interest and acknowledgment of the *comorbidity* of these diseases, historically metabolic and mental illnesses have been studied by different experts, not in the context of each other and even less so when thinking of how ELA modulates these disease risks. In addition, concerning the sex differences in disease risk, in human studies sex is often a factor that is taken into account but rarely specifically tested for, and rodent studies only very recently are more systematically performed in both sexes. Understanding how ELA act upon women and men and across diseases is crucial for more precise and personalized diagnosis, treatment and study outcome evaluations. When disentangling the effects of ELA, the *timing* of ELA exposure deserves particular attention as well (i.e. prenatal versus postnatal stress) for the risks of disease development. Notably most current research papers discuss either pre- or postnatal stress. Lastly, in order to best evaluate if the current rodent ELA models are suitable tools to study the underlying mechanisms, to identify novel targets and to test proof of concept intervention strategies, is key to establish if and to what extent the ELA-induced effects are consistent across *human and rodent literature*. This comprehensive review sets out to get further clarity about these key aspects and assess based on the large body existing literature if and how pre- and postnatal stress impact on mental and metabolic disease risk, and the sex specificity of these effects in human and rodents.

2. Methods

2.1 PubMed search

A comprehensive literature search was conducted in PubMed up to March 2020 (Fig. 1). For the search terms used for the human and rodent literature see supplementary material 1. The search was aimed at identifying relevant papers examining the sex-specific effects of early-life adversity on mental and metabolic health outcomes from human and rodent prospective cohort studies. Titles and abstracts of retrieved papers were examined by four authors (LB and JK for the human and SR and KR for the rodent literature). We acknowledge that there is also literature on the effects of early life adversity in other species (e.g. primates[38], sheep[39] and pigs[40]). However, for the purpose of this review, due to the wealth of evidence originating from rodent and human studies, we decided to focus on those.

For the *human* aspect, the PubMed search led to the identification of 52 research papers covering mental and/or metabolic health outcomes. The inclusion criteria were (1) prospective cohort studies examining associations between (2) early life stress exposure (either occurring prenatally or postnatally up to 2 years postpartum) including stress, anxiety, depression and other forms of stress (e.g. bereavement, violence, a disaster) (See for details

Supplementary table S1.A). In human studies the exact timing of stress exposure is often hard to define, as a mother who is suffering from a depression during pregnancy may still have depressive symptoms postnatally and woman suffering from postnatal depression might have had high levels of stress during the pregnancy as well. When both prenatal stress (PRS) and postnatal stress (POS) were investigated in the same study and analysed separately, the specific study was included for both PRS and POS analyses. Studies had to assess either (3) mental health outcomes (including cognitive functions, externalizing or internalizing behaviours) and/or (4) metabolic parameters (including insulin/glucose (sensitivity), body weight and body composition). We selected papers that included (5) both girls and boys, and (6) either stratified the analyses for sex, or tested for an interaction between ELA exposure and offspring sex for any of the outcomes. We included all ages at which the outcomes were measured in the offspring. In the included studies, this ranged from 1 up to 32 years of age with most studies investigating either children or adolescents. 52 human research papers met the inclusion criteria and were included in the current review.

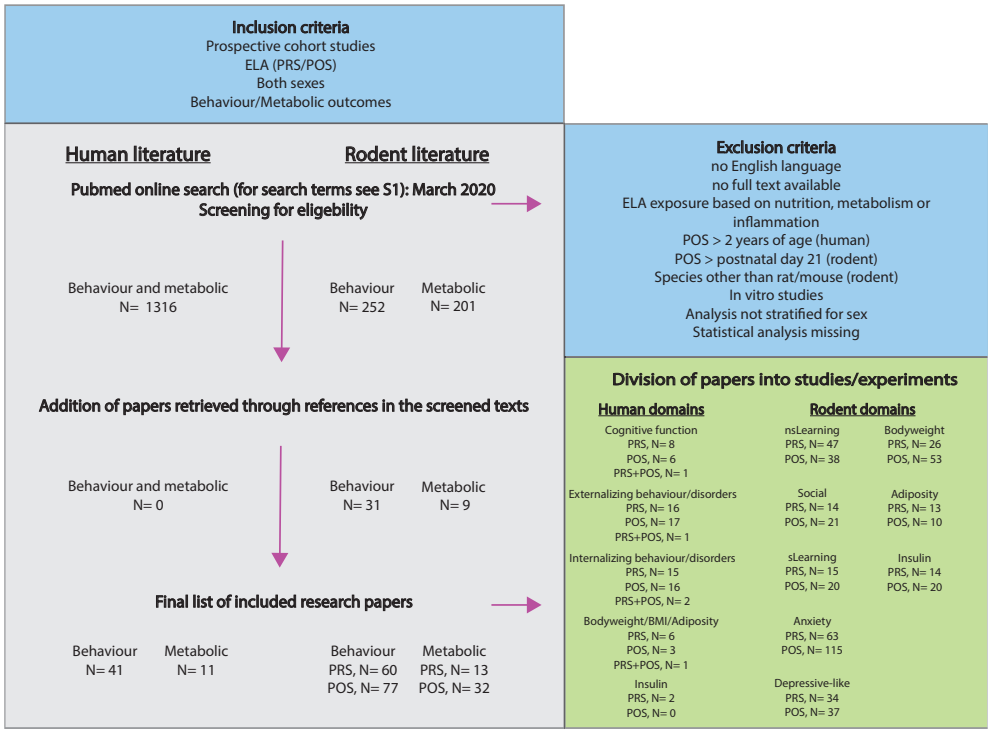

Figure 1. Inclusion criteria and selected papers

For the *rodent* aspect the PubMed search led to the identification of 182 research papers covering behavioural and/or metabolic health outcomes. We included (1) prospective rodent research papers that employed an early life stressor of physical or psychological

nature and excluded those that are nutrition-, metabolism- or inflammation-related. These encompassed either stressors occurring prenatally (e.g. prenatal restraint or variable stress) or postnatally up to 21 days postpartum (e.g. maternal separation or the limited nesting and bedding material paradigm) (Supplementary table S1.B). The included research papers had to assess either (2) *behavioural outcomes* (including cognitive, social, anxiety or depressive-like behaviour) or (3) *metabolic outcomes* (including bodyweight, adiposity, or insulin/glucose levels/sensitivity) and (4) include both sexes. Finally, (5) considering the importance of the later-life environment (second-hit) for the ELA-induced phenotypes, for the papers focusing on behavioural effects, studies with secondary stressors (e.g. later-life acute or chronic stress) and for those focusing on metabolic parameters, papers with high-caloric diet exposure later in life were also included. The behavioural studies with a secondary stressor are not included in the main analyses per domain, but separately discussed in paragraph 4.4.3. Results of mouse and rat experiments were collapsed unless there was a different effect between species. Another relevant aspect in conceptualizing the findings across many studies is the age at which the outcome was assessed; therefore, we divided rodent studies based on the age of outcome (up to adolescent: <60 days old; adulthood: >= 60 days old). 449 rodent experiments (45 on metabolic parameters; 404 on behavioural parameters) met these inclusion criteria and were included in the current review.

2.2 Behavioural and metabolic domains

As mentioned above, we divided our outcome parameters into several relevant domains to be able to better dissect out the effects of ELA on specific aspects of mental health/metabolism for the human literature and behaviour/metabolism for the rodent literature. In order to do so, we divided the various studies/experiments within each research paper and assigned them to the specific domain it was addressing and scored for each study (human) or experiment (rodent) what the effect of ELA was on that specific domain.

Concerning mental health for the *human* research papers, the domains included were: cognitive functions, externalizing and internalizing behaviours and disorders. Cognitive function includes IQ, school performance, attention disengagement, and executive functions, and is measured with e.g. cognitive tests or attention tasks. Externalizing behaviour and disorders include aggression, hyperactivity, and attention problems, and is assessed via questionnaires and/or the presence of the externalizing disorders Attention Deficit Hyperactivity disorder (ADHD) and Conduct Disorder (CD) (symptoms) as readout. Finally, internalizing behaviour and disorders encompass emotional problems, anxiety and depressive symptoms, and is assessed via questionnaires or diagnostic criteria for these disorders (see Supplementary table S2.A for all included readouts per domain). Concerning behavioural outcomes for *rodent* research papers we divided the various outcome parameters in the following domains: cognitive (non-stressful and stressful learning), social behaviour and emotional behaviour (anxiety and depressive-like behaviour). Within the cognitive domain, “non-stressful learning (nsLearning)” is assessed with neutral or non-

stressful learning tasks (e.g. Object Location Task (OLT), Object Recognition task (ORT) and Morris Water Maze (MWM)) and "Stressful learning (sLearning)" is assessed under stressful circumstance e.g. fear acquisition, but also forgetting of stressful events (e.g. "Fear learning/extinction" and "Active or Passive Avoidance"). Social behaviour is tested e.g. via social interaction/recognition paradigms that in addition can specifically assess social play and aggressive behaviour. Within emotional behaviour anxiety-like behaviour is assessed by investigating exploratory activity in the aversive environment like an open, brightly lit and/or elevated space (i.e. Open Field Test (OF), Elevated Plus Maze (EPM), Dark-light box (Da-li-box)). Measures such as coping behaviour (i.e. Forced swimming Test (FST), Tail Suspension Test (TST)) or anhedonia (i.e. Sucrose Preference Test (SPT)) are considered to reflect depressive-like behaviour (Supplementary table S2.B).

Concerning metabolic health, we assessed measures of body composition and insulin sensitivity available among the included studies. In the case of human studies, these consisted of bodyweight, body mass index (BMI) and skinfold thickness, circulating glucose and insulin levels as well as HOMA-IR (a measure of insulin resistance). For rodent experiments, bodyweight, adiposity (DEXA scan or weights of specific adipose tissue depots), insulin/glucose levels, HOMA-IR, insulin tolerance tests (ITT) and glucose tolerance tests (GTT) were the measures within the included studies.

2.3 Scoring of sex differences in study and experiment outcomes

In order to better understand sex differences in the effects of ELA on mental and metabolic health, we scored the effects in both sexes for all studies/experiments. *For human metabolic studies as well as for rodent behaviour and metabolic experiments*, we scored the effects in boys/males and girls/females separately, resulting in one male and one female score per study/experiment. For example, if in a specific study bodyweight was increased in girls but not boys, girls were scored 'increased' and boys 'no effect'. If a preclinical study found that ELA impaired learning and memory in males but not females, males were scored 'decreased' and females 'no effect'. As final outcome we calculated for both sexes how often ELA decreased, increased, or had no effect on the outcome in each domain.

In human studies of mental health, often multiple aspects belonging to the same domain are investigated, with possible differential effects for boys and girls in these sub-domains. In this case a composite score of the sub-domains leads to the definition of one of the following cases 'boys more affected', 'girls more affected' or 'both sexes affected', resulting in one score per study. For example, in a study investigated the effects of ELA on full scale IQ, as well as the separate IQ components such as verbal IQ, boys were affected on all IQ components, while girls were affected only on verbal IQ. In this case, the study was scored as 'boys more affected'. In the end, for all domains, we calculated how often boys were more affected, girls were more affected, and in which studies both sexes were equally affected.

Glossary

- **ELA**: early-life adversity, encompassing an early-life adverse event. For clinical studies between conception and 2 years postpartum. For preclinical studies between conception and 21 days postpartum
- **PRS**: prenatal stress, from conception until birth
- **POS**: postnatal stress, from birth until postnatal day 21
- **Research paper**: a publication
- **Study:** comparison between CTL and ELA exposed children for a specific readout, from a *human* research paper
- **Experiment:** comparison between CTL and ELA exposed offspring for a specific readout, from a *rodent* research paper
- **Behavioural paradigm**: experimental method to measure behaviour in a standardized manner

3. Effect of ELA on mental health outcomes depends on period of exposure, the specific behavioural domain as well as sex

Based on our inclusion and exclusion criteria (see method section and Fig. 1) we selected 41 research papers, of which 15 studies addressed cognitive functions, 34 externalizing and 33 internalizing behaviour and disorders.

3.1 Boys appear to be more vulnerable for the effects of ELA, especially postnatal stress, on cognitive function

When investigating the effects of ELA on the cognitive domain, we found that for PRS exposure, boys were more affected in 2 out of 8 studies, and girls were more affected in 1 out of 8 studies. Upon POS exposure, boys were more affected in 3 out of 6 studies, versus girls in 1 out of 6 studies. For the remaining studies either equal effects in boys and girls or no effects of ELA were observed (see Table 1.A for a detailed overview of all results).

When comparing outcome parameters of several studies with each other it is important to keep in mind that the result of the study often depends on characteristics of the ELA and the method of assessment of cognitive functions. Indeed, concerning PRS, for example, prenatal anxiety was associated with worse performance in a reaction time task and difficulty with sustained attention more so in boys compared to girls[41,42]. But, prenatal perceived stress and anxiety have also been found to be negatively associated with attention disengagement[43] and shifting[44], as well as lower visuospatial working memory performance[44,45] in both sexes, and there have been studies where maternal depressive symptoms during pregnancy did not impact IQ in either sex[46,47]. Similarly, when looking at the effects of POS, boys exposed to postpartum depression exhibited lower full scale IQ at 3.5 years of age[48]. These effects of POS on IQ in boys seem to persist beyond early childhood, as postnatal maternal depression was

negatively associated with full scale IQ[46] and with poorer results on academic performance in adolescent boys[49]. But postpartum depression has also been associated with lower school adjustment in both sexes and lower verbal intelligence specifically in girls[50].

When only looking at the '*larger*' studies (defined as N>500, 2 studies), one study with over a 1000 participants found that while PRS or POS alone did not affect IQ, high levels of depressive symptoms pre- and postnatally decreased IQ scores in children at 5-6 years of age with boys exhibiting lower full IQ cognitive scores compared to girls, while affecting verbal IQ in both sexes[47]. This is in line with the second larger study included, which found an association between prenatal anxiety and poor performance in a reaction time task in both sexes, but in an additional analysis of highly anxious mothers boys were more affected in the reaction time task compared to girls[41].

Overall, both when looking at all included studies or only the large ones (N>500), we find that upon ELA exposure boys seem more vulnerable to develop cognitive problems, which was especially found in the POS studies. Girls, rather than being entirely resilient, often exhibit a lower verbal IQ. Because different ELA exposures and aspects of cognitive functions were assessed and due to the relatively low number of longitudinal prospective human studies investigating the association between ELA and cognitive functions, these conclusions should be taken with caution and highlight the need for additional studies in this direction.

Table 1. Overview of human and rodent studies/experiments and the respective behavioural outcomes for the specific domains

A. Human studies assessing mental health per domain, timing of ELA and sex

Domain	Timing ELA	Boys more affected	Girls more affected	Both sexes equally affected	No effect in either sex	Total studies	References
Cognition	PRS	2	1	3	2	8	41–47,283
	POS	3	1	1	1	6	43,46–50
	PRS + POS	1	0	0	0	1	47
Externalizing behaviour & disorders	PRS	8	3	3	2	16	46,51,60–63,67,70,52–59
	POS	9	2	4	2	17	46,50,66–72,51,53,56,58,59,63–65
	PRS + POS	1	0	0	0	1	73
Internalizing behaviour & disorders	PRS	3	6	4	2	15	46,51,52,56,58,60–63,67,70,76–78,284
	POS	6	2	3	5	16	46,51,70,75,77–80,56,58,63,65–69
	PRS + POS	1	0	1	0	2	73,285

B. Rodent experiments assessing behaviour per domain, timing of ELA, outcome age, species (if relevant) and sex

Domain	Timing ELA	Outcome age	Species	Increased	Decreased	No effect in either sex	Total experiments	References
nsLearning	PRS	Adolescence	rats+mice	M: 2; F: 1	M: 12; F: 10	7	22	84,85,153,286,287,86–93
		Adulthood		M: 2; F: 1	M: 5; F: 9	11	25	88,93,102,107,123,126, 140,288–291,94–101
	POS	Adolescence	rats	M: 0; F:0	M: 3; F: 2	6	10	103–106,108,117,144
			mice	M: 0; F:0	M: 1; F: 1	0	1	120
		Adulthood	rats	M: 0; F:0	M: 4; F: 4	11	16	109–116,118,119
			mice	M: 0; F: 0	M: 8; F: 2	3	11	120–124
sLearning	PRS	Adolescence	rats+mice	M: 0; F: 0	M: 2; F: 3	2	5	86,87,92,292
		Adulthood		M: 2; F: 3	M: 4; F: 1	3	10	99,123,125–130
	POS	Adolescence	rats+mice	M: 0; F: 0	M: 2; F: 2	0	2	293
		Adulthood		M: 3; F: 1	M: 3; F: 4	9	18	114,122,123,131 135,167,294,295
Social behaviour	PRS	Adolescence	rats (no mice studies)	M: 3; F: 1	M: 4; F: 2	2	7	136–139
		Adulthood	rats+mice	M: 0; F: 0	M: 3; F: 3	3	7	107,123,140–142
	POS	Adolescence	rats+mice	M: 6; F: 3	M: 1; F: 0	2	11	144–149
		Adulthood		M: 0; F: 0	M: 3; F: 2	6	10	119,123,124,133,143,150–152
Anxiety-like behaviour	PRS	Adolescence	rats+mice	M: 7; F: 12	M: 2; F: 0	11	27	87,92,136,137,139,153–156,172
		Adulthood		M: 14; F: 11	M: 1; F: 2	16	36	84,107,290,296-298,123, 125, 136,160,161,178,288, 289,93,98,141,157,158, 162,102,129,140,159,160, 163,181
	POS	Adolescence	rats	M: 14; F: 9	M: 1; F: 0	17	37	103,104,117,144,147, 164,182, 299–305 106,116,184,187, 226,108,165,185
			mice	M: 0; F: 1	M: 1; F: 1	7	10	120,148,170–172
		Adulthood	rats	M: 16; F: 9	M: 1; F: 2	23	42	109,110,151,164,166–169,188,189,231,295, 111,306–313,112,118, 119,133–135,150
			mice	M: 7; F: 10	M: 2; F: 2	10	26	121,122,176,123,131,152, 170, 171,173–175
Depressive-like behaviour	PRS	Adolescence	rats (no mice studies)	M: 4; F: 2	M: 4; F: 1	3	11	94,136,137,139,154,177
		Adulthood	rats+mice	M: 4; F: 6	M: 2; F: 1	12	23	84,94,181,123,128,136,140, 160,163,178,179
	POS	Adolescence	rats+mice	M: 15; F: 10	M: 0; F: 3	5	21	103,148,299,170,171,182–187
		Adulthood		M: 2; F: 3	M: 2; F: 2	8	16	110,111,188,189,113,121–123,150,166,170,171

3.2 Boys are more at risk for externalizing behaviour and disorders upon ELA exposure

Concerning the effects of ELA on externalizing behaviours and disorders, we find that boys are at higher risk after both exposure to PRS (boys more affected: 9; girls more affected: 3, out of 17) and POS (boys more affected: 10; girls more affected: 1, out of 17).

When reviewing the effects of PRS, prenatal maternal depression and anxiety respectively correlated to externalizing problems measured with the CBCL in boys at 2.5 years of age[51], and hyperactivity and attention problems (SDQ) at 5 years of age in boys but not girls[52]. These effects of PRS extend beyond early childhood, as prenatal maternal depression was also associated with externalizing problems at 16-17 years of age, particularly in boys[53]. Moreover, death of a close relative or perceived stress during pregnancy, has been associated with an increased risk for developing ADHD especially in boys[54,55]. When looking at conduct disorder (CD) risks, also interesting sex differences emerge. Indeed, maternal distress decreased CD symptoms in girls while it increased it in boys[56], and similarly prenatal maternal depression has been associated with higher risk to commit a crime in boys[57]. Some studies also show associations between PRS and externalizing problems in both sexes. For example, prenatal stressful life events and maternal anxiety respectively predicted infant negativity at 6 weeks and 12 months of age[58] as well as childhood conduct problems[59] equally in boys and girls. The few studies concluding girls to be more affected than boys[60–62] investigated externalizing behaviour at a rather young age (1-4.5 years of age) while of the 6 studies investigating externalizing behaviour in offspring at 5 years and older, none of them concluded girls to be more affected[46,52,53,55,59,63], highlighting the importance of the age at which the various outcomes are studied. More studies that follow children into an older age are needed to be able to understand the temporal dynamics of the impact of PRS on behaviour.

Similar as PRS, POS is associated with externalizing problems specifically in boys. For example, maternal depressive or PTSD symptoms predicted boys externalizing behaviour at 33 months of age measured with ITSEA[64], as well as measured with CBCL at 1.5 and 3 years of age[65–67]. The effects of POS in boys seem to persist into adolescence, as postnatal depressive symptoms were associated with externalizing problems at 16-17 years of age, specifically in boys[53]. Yet, postnatal depression was associated with externalizing problems in both boys and girls as measured with CBCL at 4[68] and 12 years of age[69], or with SDQ up until 13 years of age[59], suggesting girls are not completely resilient. Only one study concluded girls were more affected by POS compared to boys and showed that postnatal anxiety symptoms predicted CD symptoms in girls but not boys at 4 years of age[70]. Interestingly, whereas exposure to postnatal depression seems detrimental mostly for boys' behavioural development, girls exposed to a depressed mother postpartum seem to display a more 'mature' phenotype compared to unexposed girls. For example, girls born to women with postnatal depression are better adapted in class and less distractible[71], are less likely to use physical aggression[72] and have less externalizing problems[50]. In line, pre- and postnatal anxiety increased risk

to develop CD symptoms in boys, while it reduced these risks in girls[73]. Interestingly, ELA has been shown to also accelerate sexual maturation in girls[74]. These findings indicate that girls might adapt to maternal emotional states possibly via accelerated maturation of externalizing behavioural regulations.

When looking at the large studies (N>500, PRS: 8; POS: 7), a similar picture emerges for PRS, while for POS the increased vulnerability in boys is less apparent. For example, in 5 out of 8 large studies, maternal prenatal distress, bereavement, anxiety and depression were associated with increased risk for CD[56] and ADHD[54], hyperactivity and attention problems[52] and criminal offenses[57], in boys but not girls. However, two large studies found associations between PRS and externalizing behaviour problems in both sexes[59,70]. For POS, only 2 out of 7 studies reported specifically boys and not girls to be affected; maternal depression and anxiety as well as paternal depression were associated with increased externalizing problems and conduct symptoms in boys[63,67]. Three studies found effects of postnatal stress and depression on conduct problems and externalizing behaviour in both sexes [59,68,69] and in another large study postnatal maternal anxiety correlated with conduct problems specifically in girls and not boys[70].

In conclusion, from the analysis of all included studies it appears that both PRS and POS are associated with externalizing behaviour problems mostly in boys. However, when specifically looking at the 15 included large studies (N>500), the increased vulnerability in boys disappears which calls for the need of more large studies in this area. In addition, several studies report girls to exhibit a somewhat more mature behaviour and although this adaptation might seem to be beneficial at first, on the long term it may lead to other emotional and health problems[75]. These results highlight the key importance of longitudinal studies that extend into adulthood and further to reach a better *understanding of the complex impact of ELA*.

3.3 ELA affects internalizing behaviours and disorders in both boys and girls depending on timing of stress exposure

Considering the effects of ELA on internalizing behaviours and disorders, girls seem more vulnerable for PRS (boys more affected: 3; girls more affected: 7; out of 16), while boys for POS (boys more affected: 6; girls more affected: 2; out of 17).

For example, prenatal maternal stressful life events have been related to internalizing problems at 1.5 years of age in girls only[58], and prenatal maternal anxiety predicted the CBCL score for internalizing behaviour and anxiety and depressive symptoms at 2.5 years of age more so in girls as compared to boys[62]. In addition, maternal prenatal anxiety and depression have been associated with depressive symptoms and emotional disorders in adolescent girls but not boys[46,76,77]. Although girls seem more vulnerable for the effects of PRS on internalizing problems, boys are not resilient. For example, maternal anxiety and distress during pregnancy predicted emotional symptoms in both sexes at 2 and 3-4 years of age, respectively[52,56]. In addition, depression and high stress during pregnancy predicted

internalizing problems at 1-2 year of age[61,67] as well as depression and anxiety symptoms at 20 years of age[78] more so in boys. It is unclear what specifically contributes to these differences in study outcome, but some studies suggest that the trimester in which PRS occurs may matter in this regard[60,70]. However, at this point these studies are inconclusive and more research on this matter is needed.

In contrast to PRS, POS seems to increase vulnerability to develop internalizing behaviour problems more so in boys than girls. For example, postnatal PTSD has been shown to be associated with toddler internalizing behaviour only in boys[65]. Similarly, postnatal depression has been found to predict internalizing problems at 3 and 4 years of age measured with CBCL[66,68] as well as at 7 years of age measured with SDQ[79] in boys but not girls. Notably, these effects seem to persist until adolescence as postnatal depression predicted offspring depression at 18 years in boys but not girls[77]. Although few studies showed that POS was associated with internal behaviour problems in girls, there were some that did point to such an association. For example, postnatal anxiety and depression predicted emotional problems and depressive symptoms in childhood and adolescence in both boys and girls[77,80] and postnatal stress trajectory predicted DASS scores for depression and anxiety symptoms in girls but not boys at 20 years of age[78].

When looking at the large studies (PRS: 7; POS: 8), for PRS the female vulnerability disappears and boys and girls seem equally affected, while for POS the increased vulnerability in boys compared to girls remains. For example, one study with over 7000 participants showed that exposure to prenatal depression was associated with depression in girls[77], while another study (n=2868) showed that high stress during pregnancy predicted depression and anxiety specifically in boys[78]. Both studies assessed depressive symptoms up into late adolescence (18-20 years of age), and it is unclear what factors contribute to this discrepancy. Two large studies investigating effects of PRS on internalizing behaviour (emotional symptoms and anxiety) at a younger age (3-5 years), observed similar effects in boys and girls[52,56]. For POS, also within the large studies, there is a heightened vulnerability for boys. For example, postnatal maternal depression and anxiety were associated with increased risk on emotional symptoms, internalizing behaviour problems, anxiety and depressive symptoms in boys[67,68,77,79], and only one study concluded that POS was associated with increased risk on depression and anxiety symptoms specifically in girls[78].

In conclusion, when taking into consideration all included studies, the effects of PRS on internalizing behaviour problems seems somewhat stronger in girls. However, when looking at specifically the large studies this trend disappears. For POS, both when considering all studies as well as focusing on the large studies, boys seem more vulnerable for the effects of POS. It is important to note that there are some discrepancies in the literature of which the source is not always clear and thus more research is needed to better understand if and how different factors (e.g. timing of PRS, nature of PRS and POS, age of outcome and the specific assessment method used) contribute to the differential effects of PRS and POS on internalizing behaviour problems in boys and girls. This calls not only for more research

in this field, but also to a higher level of standardization in categorizing ELAs and outcome measures.

Where human research is very powerful to find out if and what relationship exists between ELA and outcomes in specific domains in the human population, animal models are essential to imply causality and to further disentangle the biological mechanisms underlying the impact of ELA on offspring. Indeed, in human studies it is hard to establish causality between an early life event and a later outcome as time-periods of adversity can be difficult to exactly identify, often ELA's are not isolated events, there are a multitude of additional confounding factors in the surrounding environment that are often hard to control for (e.g. genetic background, diet, socio-economic status), and there is a likelihood of trans-generational transmission of psychopathologies. For these reasons, the complementarity of human and rodent studies is essential to gain a complete understanding. Indeed, animal models allow for better controlled (longitudinal) study designs, the investigation of specific time-periods and intensities of ELA, and the opportunity to study underlying mechanisms at multiple levels. Therefore, in the next paragraph we will report rodent ELA studies and their effects on later-life behaviour in both sexes.

4. Evidence of sex differences in the effects of ELA on rodent behaviour

To study the effects of ELA exposure on rodents, multiple animal models have been developed which can be divided into PRS and POS exposures (Supplementary table S1.B). In total 60 PRS research papers which were divided into a total of 173 experiments addressing cognitive, emotional or social behaviour were selected, with restraint stress (39%) and variable stress (25%) being the most commonly used PRS models. In total 77 POS research papers were included which were divided into 231 experiments for which maternal separation (MS; 56%), maternal deprivation (MS; 30%) and the limited bedding and nesting material paradigm (LBN; 31%) were the most often used POS models. For both PRS and POS anxiety-like behaviour was the most frequently investigated domain (PRS; 39% and POS; 51%). In this section we will discuss in detail the findings stratified by behavioural domain, period of ELA exposure and age of outcome. The experiments were divided over the domains as follows: PRS: cognitive (non-stressful (47) and stressful (15) learning, social behaviour (14) and emotional behaviour (anxiety (63), depressive-like behaviour (34)) and POS: cognitive (non-stressful (38) and stressful (20) learning, social behaviour (21) and emotional behaviour (anxiety (115), depressive-like behaviour(37)) (Fig. 1).

4.1 Impact of ELA on cognitive functions depends on nature of learning task, type and timing of ELA exposure and sex

To get insights into the effects of ELA on cognitive functions, it is important to divide the experiments addressing this question into non-stressful and stressful learning. In fact, there is evidence that different processes might be at place when learning under non-stressful or stressful circumstances and that if exposed to ELA, one could be best striving in stressful situations, while having impaired learning when the conditions are not stressful. This hypothesis is also known as the match-mismatch or predictive-adaptive-response hypothesis[81–83]. We will first review the effects of PRS and POS on non-stressful learning (nsLearning), followed by the effects on stressful learning (sLearning) (for the included behavioural paradigms see supplementary table S2.B).

4.1.1 non-stressful learning

PRS does not lead to sex-specific nsLearning impairments when animals are tested during adolescence (males: 12; females: 10, out of 22 experiments) while when tested in *adulthood*, PRS-exposed females slightly more often display memory impairments as compared to males (males: 5; females: 9, out of 25 experiments). POS leads to differential effects in rats and mice and therefore we report these results separately. For POS rat studies, no sex-dependent nsLearning impairments were present either when tested *during adolescence* (males: 3; females: 2, out of 10 experiments) or *in adulthood* (males: 4; females: 4, out of 16 experiments). For POS mice studies, effects on learning of adolescent mice has not been sufficiently studied (1 experiment showed learning impairments in both sexes), however when tested in *adulthood*, POS clearly impairs nsLearning specifically in males without affecting females (males: 8; females: 2, out of 11 experiments). See table 1.B for a full overview of the results per domain, outcome age, and if relevant species. In Box 1 we elaborate on the unaffected experiments (i.e. where neither males nor females showed altered behaviour in response to ELA).

PRS exposure, when tested *during adolescence*, most often reported memory impairments in both sexes[84–89]. Because these experiments were extracted from multiple research papers, have used either rats or mice, several PRS models and various behavioural paradigms, it makes a strong case for PRS-induced learning impairments in young rodents, independent of sex. However, some papers report male specific memory impairments[85,90,91] or increased memory[92,93] as well as female specific impaired[92] and increased memory[91] have been reported. The specific reason for this discrepancy is not entirely clear, but could be due to for example the specific PRS model used (i.e. early PRS[91] versus late PRS[93]) or the exact outcome age[92]. When tested *in adulthood*, PRS-exposure mostly leads to learning impairments with overall most experiments finding specifically females to be impacted[88,94–97] but also some showing impairments in specifically males[98,99] or both sexes[94,100,101]. Next to impaired memory, increased memory has also been reported specifically in males[93,98] and females[102]. Based on the available information in our hand about these experiments,

there doesn't seem to be any specific experimental parameter that we could point to as responsible for these differences and calls for more careful description of experimental details to increase reproducibility.

When studying POS effects, as mentioned, we observed differential effects in rats and mice and we therefore discuss them separately. For rats, the majority of POS experiments did not report changes in nsLearning in either sex, both when tested *during adolescence*[103–108] and in adulthood[109–116]. Within the few experiments showing changes in learning capabilities, there was no sex-or age-dependent effect[104,106,110,113,116–119]. When POS-exposed *mice* were tested in adulthood, a male vulnerability to develop learning impairments is clearly present[120–122]. These research papers all used common ELA models (LBN and maternal separation) and behavioural tests (ORT, OLT, MWM) while the few studies that failed to show this sex effect, either used a less common ELA model (e.g. faecal smell stress,[123]) or nsLearning behavioural paradigm (e.g. "Attention-Set-Shifting-Task (ASST)" testing attention or the "T-maze" which rather tests working memory[124]).

In summary, when PRS and POS affect nsLearning, they mostly lead to impaired learning and memory. When tested *during adolescence* no clear sex-differences emerges, while *in adulthood*, PRS exposed females seem to be slightly more vulnerable and POS exposure leads to a clear male susceptibility in mice specifically and not in rats.

4.1.2 Stressful learning

When reviewing the studies addressing the effect of ELA on sLearning, the large majority of both PRS and POS experiments were performed in rats and behaviour was tested in adulthood. Based on the few studies concerning *the adolescent period*, PRS does not lead to sex-specific differences (less memory in males: 2; females: 3, out of 5 experiments), and the effects of POS have been only tested in two experiments showing a decrease in sLearning in both sexes. Due to the limited literature, the findings related to the adolescent age will not be discussed further. When tested *in adulthood*, PRS leads to minor sex-differences with adult males more often displaying less memory (more memory: 2; less memory: 4, out of 10 experiments) and adult females more memory (more memory: 3; less memory: 1, out of 10 experiments) of the stressful event. Also POS leads to differential effects in males and females, with males developing equally more or less memory of the stressful event (more: 3; less: 3, out of 18 experiments) and females displaying decreased memory of the stressful event (more: 1; less: 4, out of 18 experiments).

Upon PRS exposure, adult males more often display decreased memory of the stressful event[125–127] and adult females are either not affected or display increased memory[127,128] as compared to their respective controls. In addition, some reported both sexes to display increased[129] or decreased memory of the event[130]. For these experiments age of testing (P63 versus P100) and behavioural paradigm (contextual versus cued fear conditioning) could explain this difference. All above mentioned experiments used common PRS models

induced during late gestational period while for example PRS via faecal smell stress during mid gestational period did not affect sLearning in either sex[123].

Also for POS experiments, when tested *in adulthood*, sLearning often seems to be affected in a sex-dependent manner. Two papers showed decreased memory of the stressful event in females and not males[114,131] and others reported male-specific increases in memory during fear conditioning or active avoidance paradigms[132–134]. There is also evidence for impaired learning in both sexes[122] or findings in opposite direction depending on sex, with increased memory specifically in females[135] or decreased memory in males only[134]. Indeed Lehmann et al. (1999) reported that effectiveness of POS on sLearning highly depends on the timing of the POS model and behaviour paradigm used[134].

In summary, ELA modulates performance during sLearning paradigms. When tested *during adolescence* this does not seem to be dependent on sex, however, both the effects of PRS and POS on sLearning have not been studied sufficiently at a young age to date. *In adulthood*, both PRS and POS seems to differentially affect males and females. PRS-exposed males more often display less memory and females more memory of the stressful event while POS-exposed males develop equally often more as less memory and females specifically less memory of the stressful event. However, sex-differences are subtle and different findings exist among research papers. There is evidence that (at least part of) this discrepancy might lie in the specific PRS and POS models used. To fully establish this studies directly comparing the effects of multiple ELA models are awaiting.

4.2 PRS and POS lead to altered social behaviour in adolescent males

Within this section we will discuss papers that addressed the effects of ELA on social behaviour. This is a relatively new research field and only comprised approximately 8% out of the total experiments included in this review.

When effects of ELA on social behaviour were tested *during adolescence*, changes were most often reported in male rodents exposed to PRS (males: 7; females: 3; out of 7 experiments) or POS (males: 7; females: 3; out of 11 experiments). When animals were tested *in adulthood* often no behavioural change was observed and when altered behaviour was found, this was equally often changed in male and female rodents (3 out of 7 (PRS) or 10 (POS) experiments for both sexes).

When PRS-exposed rats were tested *during adolescence* (no mice studies met the criteria for this review), males seem to be more often affected. Two experiments showed that specifically males displayed a decrease in social behaviour[136,137], while no experiment showed this for females. However, females are not completely resilient since behavioural changes in both sexes have also been reported[137–139]. The used rat strain and type and timing of PRS seem to greatly matter for the outcome of social behaviour experiments in young rats. For example, Schroeder and colleagues reported that daily restraint stress during the last week of pregnancy did not alter social behaviour in neither Wistar Kyoto (WKY) nor Wistar

rats, while 7 days of restraint stress applied in a random fashion throughout pregnancy led to decreased social behaviour in specifically male WKY rats and increased social behaviour in male and female Wistar rats[137]. Others have reported that restraint stress during the last trimester decreased social behaviour in male and female Sprague-Dawley rats[138,139], while in Long-Evans the same PRS paradigm decreased social behaviour only in males[136]. When PRS-exposed rats were tested *in adulthood*, no sex-dependent behavioural changes were observed. Some report both sexes to be affected[107,140], one specifically males[141] and one specifically females[142]. However Grundwald and colleagues only found the female-specific effect with a social memory task and not with a social preference task[142]. Additional research is needed to be able to better understand the impact of PRS on adult social behaviour.

In POS-exposed rodents, similar observations were made: *adolescent* males displayed altered social behaviour more often as compared to females. For rat experiments, the exact age of testing seems to be a key determinant of the observed effects. In fact, the 3 experiments that showed specifically males to display decreased social behaviour tested the animals around P40[143–145], while the studies reporting only females[145,146] or both males and females[147] to be affected used 25-day-old rats. Kundakovic and colleagues reported that for mice, the outcome depended on the used mouse strain, MS led to increased social behaviour in male C57BL/6 mice while no POS effect was found in Balb/c mice using a social approach task[148]. On the other hand, male MS-exposed Balb/c were more aggressive as compared to control animals, while this was not observed in C57BL/6 mice. Venerosi and colleagues also reported MS-exposed male CD-1 to be more aggressive during a social interaction task as compared to control mice[149]. While most papers did not report any behavioural changes when POS-exposed animals were tested in adulthood[123,124,150,151], some reported behavioural changes in specifically males[133], females[152] or both sexes[119,143]. Kentrop and colleagues reported that the outcome depended on the behavioural paradigm used: no behavioural change was found in either sex using a social interest paradigm, while both sexes performed less well on a social discrimination paradigm, possibly also measuring social memory[143].

Overall, based on the limited papers available studying ELA effects on rodents we found that when tested *during adolescence* both PRS and POS-exposed males are more often affected than females. *In adulthood* often no change in behaviour was observed, and when PRS or POS did affect social behaviour it was mostly similar in both sexes.

4.3 ELA induced effects on behaviour problems related to anxiety and depressive-like behaviour is highly dependent on sex, ELA-model, behaviour paradigm and age tested

4.3.1 Anxiety-like behaviour

When tested *during adolescence*, PRS females seem to be somewhat more vulnerable to develop increased-anxiety-related behaviour as compared to males (males: 7; females: 12, out of 27 experiments), while when tested *during adulthood* no clear sex-difference

becomes apparent (males: 14; females: 11, out of 36 experiments). Opposed to PRS, POS has differential effects in rats and mice and therefore we will discuss them separately. When POS-exposed mice were tested *during adolescence*, anxiety-related behaviour was often not affected and when it was this did not depend on sex (males: 1; females: 2, out of 10 experiments). *In adulthood*, POS-exposed *mice* did develop behavioural changes, however the sex-specificity of these effects is not clear cut in mice with females seemingly more vulnerable (males: 9; females: 12, out of 26 experiments), while in *rats*, POS-exposed males seem more vulnerable, both when tested *during adolescence* (males: 14; females: 9, out of 37 experiments) and *in adulthood* (males: 16; females: 9 out of 42 experiments).

When PRS effects were tested *during adolescence*, there were several experiments showing that specifically females, and not males, developed increased anxiety-related behaviour[87,137,153]. Others have also reported increased anxiety-related behaviour in both sexes[154,155] or in males specifically[136,139]. Based on these available papers it seems that even though PRS young female rodents more often develop increased anxiety-related behaviour as compared to males, this likely depends on the rodents' strain and the type and intensity of PRS[137,156]. In adulthood, PRS induced sex-differences were also modest; mostly both PRS-exposed males and females displayed increased anxiety-related behaviour[98,125,157–160]. However, from the experiments showing a sex-effect: increased anxiety-related behaviour was more often reported in specifically males[102,107,136,159,161,162] than in females[141,158,163], possibly indicating increased vulnerability in males.

From POS experiments in *rats*, both *during adolescence* and *adulthood*, approximately 50% did not show altered behaviour in either sex. However, when behaviour was affected, POS increased anxiety-like behaviour in males more often than in females, both when tested during adolescence and adulthood. The experiments that found the male vulnerability *during adolescence* mostly used MS as POS model and tested behaviour via OFT and EPM[108,144,164,165]. Similarly, when tested *in adulthood*, the majority of the experiments showed increased anxiety-related behaviour specifically in POS-exposed males[109,133,166–169] while only one paper reported this for females and they demonstrated this only based on behaviour during an EPM and not OFT[164].

In *mice*, there was no sex-effect of POS when evaluating anxiety-related behaviours. In fact, when tested *during adolescence*, in 70% of the experiments POS had no effect on behaviour in either sex[120,148,170–172]. *In adulthood*, behavioural changes are observed for both sexes. The specific effect of POS on adult anxiety-related behaviour seems to depend, at least partly, on the behavioural paradigm used to assess anxiety-related behaviour[123,152,170,173–176]. Indeed, for example, POS-exposed males displayed increased anxious behaviour assessed with OFT, while specifically females did when assessed with EPM as compared to their respective controls[152]. Another paper reported that, both for the EPM and OFT, POS males were more anxious as compared to their controls while females were less anxious during the OFT[175]. Interestingly, they only found this decrease in anxiety-related behaviour in females that were in the diestrous phase of their estrous cycle, indicating hormonal fluctuations

may affect POS-induced anxiety-related behaviour in females. Interestingly, Goodwill et al. (2019) reported females to be more vulnerable to POS for the anxiety domain, but that this could only be picked up by continuous home cage video monitoring, while a standard OFT showed both sexes to be more anxious as compared to their respective controls[170]. Lastly, Weiss et al. (2011) reported differential effects depending on the type of POS, they found a male vulnerability in offspring of MS dams that were additionally stressed by restraint of swim stress during the dam-pup separations while in regular MS offspring, females and not males were found to display increased anxiety-related behaviours for the same task, a free exploratory paradigm[174].

Taking everything together, PRS-exposed *adolescent* female rodents seem to be more vulnerable than males while *in adulthood* increased anxiety-like behaviour was observed in both sexes equally. Specifically, POS-exposed male rats more often develop anxiety-related behaviour changes as compared to females, both when tested *during adolescence* and *adulthood*, while for mice the outcome seems to be highly dependent on the specific POS model and behavioural paradigm.

4.3.2 Depressive-like behaviour

For ELA effects on depressive-like behaviour, when tested *during adolescence*, we found altered behaviour more often in males as compared to females exposed to either PRS (males: more: 4, less: 4; females: more: 2, less: 1; out of 11 experiments) or POS (males: more: 15, less: 0; females: more: 10, less: 3; out of 21 experiments). While when tested *in adulthood* behaviour was often not affected and when it was no clear sex-differences became apparent when rodents were exposed to either PRS (males: more: 4, less: 2; females: more: 6, less: 1; out 23 experiments) or POS (males: more: 2, less: 2; females: more: 3, less: 2; out of 16 experiments).

For example when reviewing the effects of PRS on depressively-like behaviour *during adolescence*, male rats (no mice studies met the requirements for this review) were more likely to be affected as compared to females[94,137,139]. In the three experiments showing that females were affected, males were also affected[137,154,177]. Similar as to anxiety-like behaviour, the outcome depended on the rat strain and timing of PRS model used[137]. *In adulthood*, firstly, half of the experiments did not show behavioural changes in either males or females[84,94,123,128,136,140,178]. Of note, six of these experiments were part of the same paper in which several PRS time-windows were tested of which the majority was not effective in modulating depressive-like behaviour in mice[178]. In fact, variable stress applied during first week of gestation led to increased depressive-like behaviour in specifically males, while the same stressor applied during mid or late gestational period did not affect depressive-like behaviour in either sex[178]. However, van der Hove et al. (2014) reported increased depressive-like behaviour specifically in male rats exposed to restraint stress during late gestation. This discrepancy could be due to the use different species (mice versus rats) and PRS model (variable stress versus restraint stress)[163,178]. Apart from these two papers,

most affected experiments actually reported increased anxiety-related behaviour in female rodents exposed to mid or late gestational stress[84,136,179–181], suggesting a female vulnerability depending on timing of PRS.

For POS, when tested *during adolescence*, several experiments showed specifically males to develop depressive-like behaviour[148,182–184]. However, female offspring is not resistant as behavioural changes in both male and female offspring have been reported as well[103,148,171,182,184–186]. All of these papers used MS or MD as POS model while the exact timing and used behavioural paradigm (see e.g.[184]) differed among experiments. Lastly, only one experiment, using a relatively late maternal deprivation (MD) model at P9, reported females and not males to display increased depressive-like behaviour[187]. From the details in these papers it becomes clear that even though young males seem to be more vulnerable for the effects of POS on depressive-like behaviour, the type and timing of POS and the behavioural paradigm used to assess behaviour greatly matter. *In adulthood*, half of the included experiments reported neither males nor females to be affected by POS[111,121–123,171,188,189] and, within the affected studies, there was no clear sex-difference. Some reported increased depressive-like behaviour specifically in MS exposed male rats[166], MS-exposed female rats[113] or LBN exposed female mice[170]. Decreased depressive-like behaviour has also been reported for MS-exposed male rats[113], MD-exposed female rats[150] or both male and female rats exposed to the LBN paradigm[110]. Thus, where in some cases species or POS model explain the different outcome, in other cases this is not entirely clear.

In summary, both for PRS and POS, when animals are tested *during adolescence*, males seem to be more vulnerable to display depressive-like behaviours. When tested *in adulthood*, both PRS and POS affect behaviour only in about 50% of the experiments and within the affected experiments no clear sex-difference becomes apparent. For all ELA experiments regarding depressive-like behaviour, timing and type of ELA and behavioural paradigm used seem to greatly matter for the outcome of an experiment.

4.4 Second-hit in ELA experiments related to behaviour

The two-hit hypothesis poses that ELA increases the sensitivity to later-life challenges resulting in increased allostatic load[190]. A secondary challenge might unmask early-programmed behavioural changes that might not be apparent under basal circumstances. Concerning the behavioural domains, we looked whether secondary stressors (e.g. acute or chronic stress) affected behavioural outcomes. Unfortunately, the effects of secondary stressors on the behaviour of ELA-exposed rodents has not been studied extensively with the inclusion of both sexes (PRS: 4.0%, POS: 13.9% of all included experiments). The low number of experiments per domain makes it difficult to draw firm conclusions on this matter. For example, for depressive-like behaviour, no sex-dependent behavioural changes were found when animals where only postnatally stressed early in life and tested in adulthood. However, when POS-exposed animals were exposed to a secondary stressor, specifically adult females exhibit increased depressive-like phenotype and males not, as compared

to controls exposed to the same secondary stressor[111,166,191]. This example highlights the importance of a second-hit in revealing new aspects of the ELA-induced programming. Thus, we encourage the inclusion of second hits when possible, to study the response of various systems not only under resting/basal state but also under demand, while being activated to respond to the current challenge.

5. Assessment of the effects of ELA on body composition and metabolic parameters in human studies

The metabolic effects of ELA are understudied compared to the effects mental health outcomes and behaviour, which is reflected by the number of included research papers in this review (human mental health outcomes: 41; human metabolism: 11; rodent behaviour: 131; rodent metabolism: 45). We will review and discuss in this chapter the current human literature with focus on the pre- versus postnatal stress exposures, sex differences, and the effects on bodyweight, adiposity and thereafter measures of insulin sensitivity.

5.1 Girls seem at higher risk for effects of ELA on body composition

Overall, girls seem somewhat at higher risk than boys to develop overweight or adiposity upon ELA (girls: 7; boys: 4, out of 10 studies) (table 2.A). When taking into account timing of ELA exposure, upon PRS exposure specifically, out of the 6 included studies 4 reported girls to exhibit increased risk to develop overweight or adiposity. In contrast, for boys, only 2 studies showed such increased risk and 2 studies found opposite effects, with boys showing a leaner phenotype. For POS, girls showed increased risk to develop overweight or adiposity in 2 out of 3 studies. In boys, such increased risk was also observed in 2 out of 3 studies, but only in a subgroup of boys (see below). Moreover, 1 study investigating PRS+POS exposure found increased risk for obesity only in girls.

For example, maternal report of intimate partner violence pre- or postnatally led to increased risk for obesity specifically in girls[192], and daughters of mothers with a psychiatric illness (and no use of SSRI's) during pregnancy were more vulnerable to develop overweight while no such effects were observed in boys[193]. Moreover, postpartum maternal distress was associated with increased waist-to-hip ratio in girls, whereas in boys this was only seen in those with high levels of stress reactivity[194], and prenatal depression was associated with increased BMI in girls, while for boys this was only true for those whose mothers required hospitalization due to depression[195]. While most studies suggest stronger effects of ELA in girls, there are also studies reporting ELA-induced metabolic vulnerability in both boys and girls for example after prenatal maternal stress due to an ice storm[196] and parental separation during pregnancy[197]. Interestingly, there are also studies reporting opposite effects in boys: prenatal maternal CORT levels were associated with marginally lower fat mass index (FMI) in boys, but higher FMI in girls at 5 years of age[198]. Similarly, depression

during pregnancy was associated with lower weight and smaller height among boys but not girls, while depression postpartum was associated with higher weight-for-height ratios among girls[195]. Thus, ELA most often increases bodyweight in girls. Boys seem less affected and there is some evidence for boys to exhibit a leaner phenotype. Importantly, even though there are not many studies available on the effects of ELA on body composition, almost all included studies are considered large (8 out of 10 had more than 500 participants) and thus the observed increased vulnerability for overweight or adiposity in girls seems solid.

5.2 Effects of ELA on insulin sensitivity parameters

There are no prospective cohort studies investigating associations between ELA and diabetes, however, two human studies on measures of glucose metabolism and insulin sensitivity after ELA exposure met the inclusion criteria for this review, and these showed no sex differences. In a large study with 1478 participants, prenatal psychosocial stress exposure did not correlate to fasting glucose and insulin resistance (HOMA-IR; measure for insulin resistance based on circulating glucose and insulin levels) in 5 to 6-year-old children[199]. A small study (32 participants) reported a positive association between prenatal stress due to an ice storm with insulin secretion at 13 years of age in both boys and girls[200]. As insulin insensitivity usually develops with age together with unhealthy lifestyle habits[201], it is important to study these insulin parameters in ELA-exposed adults instead of children.

Overall there is relatively few studies addressing ELA exposure on metabolic outcomes, however based on the evidence presented above ELA seems to increases risk on developing overweight more so in girls while no clear association was found between ELA and insulin sensitivity parameters in either sex. Studies in rodent models with their more controlled setting can increase our understanding on sex differences in metabolic programming by ELA, which will be discussed next.

6. Assessment of sex differences in the effects of ELA on rodent metabolic outcome parameters

It has been proposed that later life (nutritional) environment determines the ELA-induced phenotype: a healthy lifestyle or positive environment leads to resilience (lower adiposity, increased insulin sensitivity), while a negative environment or unhealthy lifestyle leads to vulnerability (increased adiposity, reduced insulin sensitivity)[202]. While in human cohorts later life dietary conditions are difficult to control for, this is not the case for rodent studies. Therefore, we considered not only the impact of ELA on metabolic health under basal circumstances, but also under different dietary conditions. We will thus next review and discuss the effects of PRS and POS on the various metabolic outcomes (body weight (BW), adiposity and insulin resistance) at two different ages (adolescence and adulthood), and

highlight when the outcomes were at basal or in response to a later life dietary challenge (unhealthy diet; UD).

6.1 Sex-dependent effects of ELA on bodyweight of rodents depend on nature of stress exposure and the later life diet

Under standard unchallenged dietary conditions, PRS does not affect body weight (BW) in most studies, and when it did there were no strong sex differences in either adolescence (males: increased: 2, decreased: 0; females: increased: 3, decreased: 0 out of 11) or adulthood (males: increased: 0, decreased: 2; females: increased: 0, decreased: 2, out of 10). In adolescence, POS mostly reduced BW (males: decreased: 12, increased: 2; females: decreased: 10, increased: 2, out of 26), while in adulthood POS either increased or decreased BW (males: increased: 4, decreased: 4; females: increased: 4, decreased: 4, out of 21) similarly in males and females. When challenging the system with an UD later in life, we find the following: only one study investigated the modulatory effect of UD (high-sucrose only) in adolescence, and observed increased BW in both POS exposed males and females. In adulthood, PRS exposure combined with UD either increased (males: 1; females: 2, out of 5) or decreased (males: 2; females: 1, out of 5) BW compared to control animals on UD without showing strong sex differences. In contrast, for POS a clear sex difference was observed: no studies showed an effect on adult BW in males, while 3 out of 5 studies (2 increased; 1 decreased) reported effects of POS in females. See table 2.B for a full overview of the results.

Under standard dietary conditions, PRS mostly does not affect BW in *adolescence*[203–205], although some studies reported an increase in adolescent BW in both sexes or in females only[206–208]. There are no clear differences in timing of PRS (early versus late in gestation), age at which BW was measured (early in adolescence versus later in adolescence), or used species that could explain these differences between studies. In adulthood, PRS mostly does not affect BW[206,209–211]. For example, chronic mild unpredictable stress during early and mid-gestation, or variable or restraint stress during late gestation did not affect BW in either sex[206,212–214]. Few studies showed a reduction in adult BW after PRS[215–217], while no studies reported an increase.

In contrast to PRS, POS has stronger effects on BW in both adolescence and adulthood. In *adolescence*, POS often leads to a reduction in BW similarly in both sexes[218–221]. For example, exposure to the limited bedding and nesting (LBN) paradigm with or without exposure to a substitute mother for one hour per day reduced BW at P27 and 9 weeks of age in males and females[110,222]. Similarly, maternal deprivation (MD) and maternal separation (MS) reduced BW throughout adolescence in male and female offspring[182,223–226]. However, some MS studies also showed an increase in adolescent BW[164,227,228]. It is unclear what contributes to these discrepancies. In adulthood, POS alters BW similarly in both sexes. LBN or MD resulted in a reduction[110,131,225], or no effect[121,224,229] on adult BW. In contrast, MS with or without early weaning either increased[164,230,231] or had no effect (e.g.[232–236] on adult BW. Taken together,

these studies show differences in the effect of ELA on BW depending on the timing of stress, and (for POS) the specific model used, but similar effects in both sexes.

When challenging the system with an UD later in life, a different picture emerges. PRS either decreased[217] or increased[206] adult BW when exposed to UD. For example, chronic variable stress early in pregnancy reduced BW in both males and females upon 17 weeks of UD, compared to their respective controls on UD[217]. In contrast, variable stress during late gestation has been shown to increase BW in male and female offspring when exposed to UD post weaning[206], while restraint stress during late gestation did not affect BW after 4 weeks of UD[237]. Due to the relatively limited amount of studies it remains difficult to understand what underlies the discrepancies in results among these studies. When looking at the effects of POS combined with UD, we observed that females were more susceptible compared to males. For example, MS combined with either sham injection or early weaning increased BW when fed a post weaning UD for either 12 or 17 weeks in females, but not in males[235,238]. In contrast, MD or MS alone followed by post weaning UD for 11 or 16 weeks, did not affect BW in either sex[225,236] suggesting that the type and severity of the postnatal stressor may influence metabolic outcome.

In conclusion, PRS in most studies does not seem to affect BW on chow diet and there are mixed results when exposed to UD later in life. POS, however, leads to a similar effect on BW in both sexes when fed standard chow, and upon UD later in life POS-exposed females are more affected than males.

Table 2. Overview of human and rodent studies/experiments and the respective metabolic outcomes for the specific domains

A. Human studies assessing metabolic outcomes: per domain, timing of ELA and sex

Domain	Timing ELA	Increased	Decreased	No effect in either sex	Total studies	References
BW	PRS	M: 2; F: 4	M: 2; F: 0	1	6	193,195–198,314
	POS	M: 2; F: 2	M: 0; F: 0	1	3	194,195,315
	PRS + POS	M: 0; F: 1	M: 0; F: 0	0	1	192
Insulin sensitivity	PRS	M: 1; F: 1	M: 0; F: 0	1	2	199,200
	POS	M: 0; F: 0	M: 0; F: 0	0	0	x
	PRS + POS	M: 0; F: 0	M: 0; F: 0	0	0	x

B. Rodent experiments assessing metabolic outcomes: per domain, timing of ELA, outcome age, species (if relevant) and sex

Domain	Diet	Timing ELA	Outcome age	Increased	Decreased	No effect in either sex	Total experiments	References
BW	standard diet	PRS	Adolescence	M: 2; F: 3	M: 0; F: 0	8	11	94,139,172,206,212,214–216,237,238,288
			Adulthood	M: 0; F: 0	M: 2; F: 2	7	10	94,206,212–216,237,238,316
		POS	Adolescence	M: 2; F: 2	M: 12; F: 10	11	26	110,116,164,182,222–228,231–233,235,241,242,313,317–323
			Adulthood	M: 4; F: 4	M: 4; F: 4	11	21	110,121,131,164,224,225,227, 229-233,235,236,242,313,317, 320,322,324
	unhealthy diet	PRS	Adolescence	M: 0; F: 0	M: 0; F: 0	0	0	x
			Adulthood	M: 1; F: 2	M: 2; F: 1	2	5	206,212,213,237,238
		POS	Adolescence	M: 1; F: 1	M: 0; F: 0	0	1	241
			Adulthood	M: 0; F: 2	M: 0; F: 1	2	5	225,233,235,236,239
Adiposity	standard diet	PRS	Adolescence	M: 0; F: 0	M:1; F: 0	2	3	94,206,212
			Adulthood	M: 0; F: 0	M: 2; F: 0	4	6	94,206,212,213,215,237
		POS	Adolescence	M: 0; F: 0	M: 0; F: 0	1	1	325
			Adulthood	M: 2; F: 2	M:1; F: 1	2	5	164,229,233,235,236
	unhealthy diet	PRS	Adolescence	M: 0; F: 0	M: 0; F: 0	0	0	x
			Adulthood	M: 1; F: 3	M: 2; F: 1	0	4	206,212,213,237
		POS	Adolescence	M: 0; F: 0	M: 0; F: 0	0	0	x
			Adulthood	M: 0; F: 2	M: 0; F: 1	1	4	233,235,236,239
Insulin	standard diet	PRS	Adolescence	M: 0; F: 0	M: 0; F: 0	1	1	206
			Adulthood	M: 1; F: 4	M: 2; F: 2	2	8	206,212,213,215,216,237,238,316
		POS	Adolescence	M: 1; F: 1	M: 2; F: 1	0	3	223,225,241
			Adulthood	M: 1; F: 0	M: 5; F: 3	3	10	164,224,225,227,230,233, 235,236,242,317
	unhealthy diet	PRS	Adolescence	M: 0; F: 0	M: 0; F: 0	0	0	x
			Adulthood	M: 1, F: 1	M: 1, F: 0	2	5	206,212,213,237,238
		POS	Adolescence	M: 0; F: 0	M: 0; F: 1	1	2	225,241
			Adulthood	M: 0; F: 2	M: 2; F: 0	1	5	225,233,235,236,239

6.2 ELA exposure increases adiposity in female rodents when exposed to an unhealthy diet

Adiposity can be affected by ELA independent of bodyweight[229], and is therefore another important indicator of metabolic health to assess with regard to ELA. Under standard dietary conditions, both in adolescence and in adulthood in some cases PRS exposure decreased adiposity in males (adolescence: 1 out of 3 studies; adulthood: 2 out of 6 studies) while this was never the case for females. For POS, only one study investigated its effects on adiposity in adolescence and observed no difference in either sex, while in adulthood, POS affected adult adiposity in both sexes in 3 out of 5 studies. When animals are subjected to an unhealthy diet (UD) in adulthood, a clear picture emerges: females specifically gain more adiposity when they are stressed during either pre- or postnatal life (females: 5; males: 1,

out of 8). When taking into account timing of ELA, for PRS, 3 out of 4 studies show increased adiposity in adult females, while this was true for only 1 out of 4 studies for males. For POS, adipose tissue accumulation in response to UD was never increased in males compared to their respective controls (4 out of 4), while females showed increased adiposity in 2 out of 4 studies, and decreased adiposity in 1 out of 4 studies.

Under standard dietary conditions, in adolescence, PRS either had no effect in either sex[206,212] or decreased adiposity in males specifically[94]. Similar effects were observed in adulthood: PRS (early or late in gestation) exposed males showed decreased adiposity, while females were unaffected[94,217]. However, other studies found no effect of PRS (early-mid or late gestation) in either sex[206,212,213,215]. Although it is unclear what contributes to these differences between studies, taken these altogether, they suggest males may be somewhat more affected by PRS, however showing a leaner phenotype.

POS similarly affected adult adiposity in both sexes, but the exact outcome (more or less adiposity) seems to depend on the POS paradigm. Whereas exposure to LBN led to decreased adiposity[229], exposure to MS increased adiposity[164,235] in adult males and females. Of note, although POS does not seem to result in clear sex differences in adiposity when fed standard chow, there might be more subtle sex differences. For example, MS increased adipose tissue depot weight in males and females, and while this was true for only one out of three depots in males, it was true for all three depots in females[164], and LBN led to a higher mesenteric fat percentage, which was particularly the case in females[229]. As the various fat depots have different functional implications239, this suggest the need for more detailed studies in this area.

When animals are subjected to an unhealthy diet (UD) in adulthood we see that females gain more adiposity when exposed to PRS or POS[212,213,235,238]. For PRS, in the studies specifically finding increased adiposity in females but not males, UD started in adulthood with a duration of 3-10 weeks, and PRS was applied either early-mid or late during gestation[212,213]. Notably, the one study that observed increased adiposity in both sexes used an UD starting directly after weaning[206]. This suggests that timing and length of exposure of UD is an important aspect to take into account when comparing the various studies and that starting UD exposure at younger age might overrule the otherwise observed sex differences.

When looking at the effects of POS, similar to PRS, a female-specific increase in adiposity upon UD is observed[235,238], although one study found that while POS did not affect male adiposity, females showed less adiposity after UD[164]. All studies used the MS paradigm and UD started either directly or soon after weaning. The two studies observing more adiposity added either early weaning or sham injections to the MS protocol[235,238], suggesting that the severity of the stressor might influence metabolic outcome.

In conclusion, under standard chow, males may be somewhat more affected by PRS, while POS affects adult males and females similarly. While PRS led to reduced adiposity, POS either increased or decreased adiposity, which is potentially related to the used POS model. When fed an UD, specifically ELA-exposed females accumulated more fat.

6.3 ELA tends to compromise insulin sensitivity in males

High levels of circulating glucose (hyperglycaemia) and insulin (hyperinsulimea) are signs of insulin insensitivity. Insulin sensitivity can be measured with insulin and glucose tolerance tests (ITT; GTT). Under healthy circumstances, blood glucose is expected to quickly decrease in the ITT as well as the GTT. Reduced insulin sensitivity is thus indicated by either high glucose or insulin levels, or slow glucose clearance. Only one study investigated the effects of PRS on insulin sensitivity during adolescence, and observed no effects in either sex. In adulthood, PRS increased insulin sensitivity measures more often in females compared to males (males: 1; females: 4, out of 8), while in 2 out of 8 studies for both sexes reduced sensitivity was observed. For POS, adolescent males were affected in 3 (2 decreased; 1 increased) out of 3 studies, and females in 2 (1 decreased; 1 increased) out of 3 studies. In adulthood, POS affected adult insulin sensitivity measures more so in males (males: 6; females: 3, out of 10). When reviewing the studies including an unhealthy dietary condition later in life, there are no studies addressing the effects of PRS in combination with UD on insulin sensitivity in adolescence. In adulthood, PRS affected insulin sensitivity in 2 out of 5 studies for males and in 1 out of 5 for females. For POS, one study investigated its effects in adolescence and observed detrimental outcomes only in females. In adulthood, POS either decreases (2 out of 5 studies) or does not affect (3 out of 5 studies) insulin sensitivity in males, while in females POS increases (2 out of 5 studies) or did not affect (3 out of 5 studies) insulin sensitivity measures when fed UD. When combining both adult PRS and adult POS studies, females never show decreased insulin sensitivity when fed UD, while ELA-exposed males on UD showed this in 3 out of 10 studies.

Under standard dietary conditions, in adulthood, PRS females more often showed increased insulin sensitivity measurements compared to males[206,213]. For example, exposure to prenatal variable stress during late gestation led to lower insulin levels, lower HOMA-IR and faster glucose clearance in adult females, but not males[206,213]. However, in another study PRS during late gestation did not affect glucose clearance in GTT or ITT[237], and also reduced insulin sensitivity is described upon either early-mid or late PRS exposure in females[215], males[216], and both sexes[212]. These discrepancies between studies do not seem to be explained by the gestational period in which the stress was applied, nor measured outcome (e.g. circulating insulin/glucose levels versus GTT/ITT) or used species, thus further research is needed to gain a better understanding of the effects of PRS on insulin sensitivity.

When looking at POS, both increased and reduced insulin sensitivity was observed in adolescence[223,225,240]. The studies of Mela and colleagues suggest timing of measurement matters: with the same POS model they found increased insulin levels in males at P36 without affecting females 223, but decreased insulin levels at P45 and P65 in both sexes[225]. In adulthood, males were more often affected, mostly showing reduced insulin sensitivity indicated by e.g. higher circulating glucose[241], and higher circulating insulin levels[233], although some studies also report such effects in both sexes[227,230]. In conclusion, when rodents are fed a standard chow diet, PRS more often increases insulin sensitivity in females compared to

males, while POS affects males slightly more often, mainly resulting in decreased insulin sensitivity.

In conclusion, when rodents are fed a standard chow diet, PRS more often increases insulin sensitivity in females compared to males, while POS affects males slightly more often, mainly resulting in decreased insulin sensitivity.

When exposed to an UD later in life, PRS-exposed males are reported to have both increased glucose clearing in GTT and ITT[212], as well as reduced glucose tolerance[206] after PRS during either early-mid or late gestation, respectively. Females were not affected in these studies. However, another study found increased insulin sensitivity measures specifically in females but not males exposed to PRS followed by UD in adulthood[213]. Differences between the studies are timing of stress, as well as the timing and duration of UD.

POS exposure followed by post weaning UD reduced insulin sensitivity measures in males but not females in two studies[225,236], or did not affect insulin sensitivity measures in both sexes[235]. No studies reported signs of reduced insulin sensitivity in POS-exposed females. In contrast, a study reported increased insulin sensitivity measures in females after post weaning UD, while males were unaffected[238]. These studies indicate sex differences in vulnerability to high caloric diets following POS.

Taken together, ELA-exposed males seem to more often show reduced insulin sensitivity, either when fed standard chow or UD, while ELA-exposed females more often show increased insulin sensitivity, depending on the timing of the ELA and diet. However, more studies focussing on sex-dependent effects of ELA on insulin sensitivity are needed, preferably investigating insulin sensitivity in multiple ways (e.g. ITT/GTT as well as insulin and glucose levels), at multiple ages, and in response to both standard and UDs.

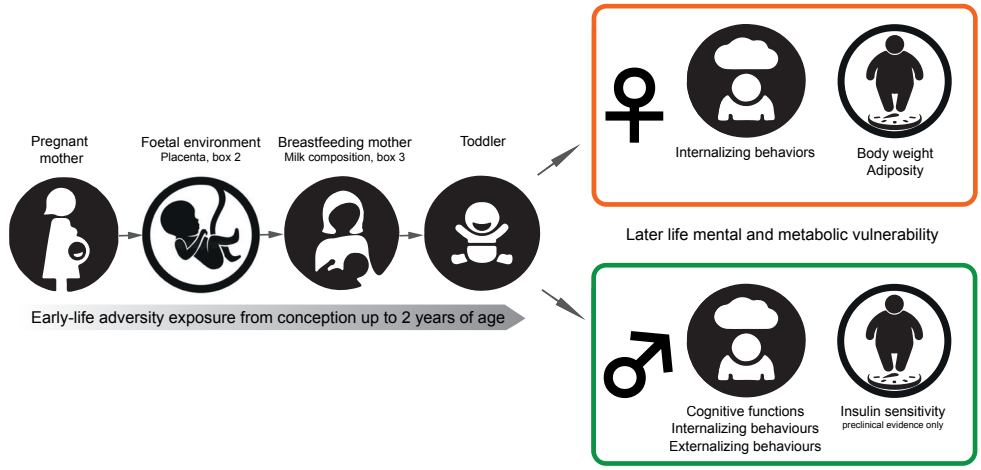

Figure 2. ELA exposure differently affects boys/males and girls/females depending on the specific domain

7. Discussion

With this extensive review we set out to unravel i) whether effects of ELA on mental and metabolic health outcomes are sex-specific, ii) to what extent the timing of ELA exposure matters for these outcomes, iii) considering the comorbidity of mental and metabolic diseases, whether sex-specific effects of ELA on these outcomes align, and iv) whether these ELA-induced effects are consistent across human and rodent studies. We will here integrate the so far reviewed evidence from the different fields and address these points. In addition, important considerations are being discussed on study designs of the included research papers (box 1), which may have implications for the outcomes of this review.

7.1 Sex dependence and convergence of human and rodent literature of ELA-induced effects

Concerning the data from mental health/behavioural *domains for human and rodent studies respectively*, we can carefully conclude that they mostly go hand in hand. Indeed, ELA leads to specific sex differences, and the overall conclusion after integrating human and rodent research and the PRS/POS-induced effects across the various mental health/behavioural domains is that males/boys seem to be most vulnerable for the effects of ELA. When looking at the considered mental health (cognitive, internalizing, externalizing) and behavioural domains (cognitive, social and emotional behaviour) the following picture emerges (Fig. 2):

Concerning *cognitive functions*, ELA exposed boys were more prone to perform poorer on cognitive tasks as compared to girls. Timing of ELA seems to matter, since the effect was stronger after POS exposure, with evidence for this effect to be lasting up to adolescence (human studies) and adulthood (rodent studies). Important to note is that girls do not seem to be entirely resilient to ELA as there is initial evidence that ELA might impact girls verbal intelligence, and similarly there is also evidence from rodent studies for females to be impacted at specific ages or settings (e.g. adult PRS-exposed females; and interestingly adult PRS-exposed females tend to develop more memory of stressful events). Concerning *externalizing behaviour* in children, such as attention problems, aggression and hyperactivity, especially boys were at risk to develop these type of behavioural changes, both after PRS and POS exposure. While this domain is harder to directly relate to rodent studies, social behaviour (e.g. measuring social interest and sometimes aggression) can be used as a proxy for aspects of externalizing behaviour. Indeed, in line with human literature, especially young male offspring of PRS or POS stressed dams display altered social behaviours. Finally, when considering *internalizing behaviours*, the picture is a little more complex. We found that both boys and girls exposed to ELA often develop internalizing behaviour problems (i.e. emotional problems, anxiety and depressive-symptoms, and that the specific result (the affected sub-domain and sex-specificity) highly depends on timing of stress exposure and age of outcome. More specifically, the effects of PRS on internalizing behaviour are somewhat stronger in girls, while boys seem more vulnerable for the effects of POS.

Similarly, evidence from rodent studies indicates that both sexes can develop behavioural changes related to anxiety and depressive-like behaviour and that this also depends on the type and timing of the ELA model, behavioural paradigm and the age at which the animals were tested. Thus, ELA-exposed boys/males seem to be more vulnerable for the cognitive and externalizing domain, while for the internalizing disorders (e.g. anxiety and depression) both sexes are affected depending on type and timing of ELA. This suggests that ELA might contribute to the sex differences in disease prevalence in the general population together with other genetic and environmental factors.

Concerning the metabolic outcomes, before discussing the overall picture, it is important to note that most longitudinal clinical studies included in this review that investigated the effects of ELA on metabolism, report ELA effects in children and adolescents, while rodent studies most often study this aspect in adulthood. This is important because, for example, insulin insensitivity often develops with age or after prolonged high-fat diet exposure [201]. Effects of ELA on these parameters may be difficult to observe at a younger age and thus longitudinal human studies with follow-ups into adulthood are needed. Nonetheless, human studies suggest that girls might be more vulnerable to develop *overweight or altered waist-hip ratio* after ELA exposure. Similarly, rodent evidence indicates that ELA-exposed females, when exposed to an unhealthy diet later in life, gain more adiposity compared to their respective controls than males. An important aspect that will need more consideration in the future is that there is initial evidence for ELA to impact on food choice in both a human[242,243] and rodent setting[226], which will clearly also impact metabolic health.

Regarding the effects of ELA on *glucose metabolism and insulin sensitivity*, human studies are extremely sparse and inconsistent: both no association and a non-sex specific association between ELA and insulin sensitivity parameters are reported. Also within the relatively few rodent studies that investigate ELA effects on insulin sensitivity and glucose metabolism there are inconsistencies, but overall males more often showed decreased insulin sensitivity independent of later life diet, while females rather showed increased insulin sensitivity. While decreased insulin sensitivity has been associated with diabetes[244], the exact implication of increased sensitivity is not well understood. Thus, ELA-exposed females seem to be more vulnerable to develop adiposity, whereas males may be more susceptible for detrimental effects on insulin sensitivity. This is in line with the prevalence of the connected disorders in the general population where indeed obesity is also more prevalent in women[27,28], while diabetes is somewhat more prevalent in men, especially at middle age[29–31]. ELA could thus be at the origin of and contribute to these sex differences in disease prevalence.

7.2 Comorbidity cognitive and metabolic phenotype

Notable, the comorbidity between metabolic and brain disorders suggest converging or interacting underlying pathways might be at play[13–15,17–19]. Do these comorbidities have an early-life origin?

Human studies suggest a bidirectional relationship between depression and obesity: obesity increases risk for depression and depression is also predictive of obesity[13]. Within this review we found that PRS-exposed girls are more likely to develop depressive behaviours and girls seem more vulnerable to become overweight after ELA exposure. Also for rodent studies, females seem to be more vulnerable. Effects of ELA on depressive-like behaviours depend on the timing of both stress exposure and behaviour assessment but overall adult females exposed to PRS in late pregnancy are more likely to develop depressive(-like) behaviours in adulthood. This is in line with the metabolic vulnerability at this age since ELA mostly affects adiposity in adult females fed a high fat diet. Taken together, the ELA-induced adiposity and PRS-induced depressive-like phenotype are more pronounced in females/girls, supporting the hypothesis that ELA might be at the origin of this female specific vulnerability and that there might be converging pathways leading to the metabolic and mental disorders.

Moreover, metabolic diseases (both obesity and diabetes) are linked to the development of and often co-occur with cognitive impairments and Alzheimer's disease later in life[17-19,245]. We here report that cognitive deficits following ELA are more pronounced in males/boys, which is supported by a previous meta-analysis[246]. The ELA-induced sex-dependent vulnerability to metabolic alterations depended on the readout. As mentioned above, males seem more vulnerable with regard to glucose metabolism/insulin sensitivity in contrast to females being more vulnerable to increased adiposity. Obesity-induced inflammation and insulin resistance are suggested to be involved in mediating the effects on cognition[245,247]. We can thus conclude that ELA might be at the origin of the comorbidity of diabetes and cognitive decline in boys/males.

It is important to note that studies addressing simultaneously mental/behavioural and metabolic health outcomes are lacking, this is the case both for human and rodent literature. These will be needed in the future, to be able to more directly relate these two aspects and to further our understanding on their origin and sex-specific co-occurrence. Understanding more about the co-occurrence of various diseases, how they modulate one another's gravity and disease trajectory and how the treatment of one may impact the other, will give us new insights into possible converging pathways and joint targets.

While addressing the complex mechanisms at the basis of the programming by ELA is out of the scope of this review, we will discuss two potentially interesting substrates/mediators of the described effects: the placenta (Box 2) and the breast milk (Box 3), respectively key in mediating the maternal environment to the fetus/offspring during the pre- and the postnatal period. Of note, many other mechanisms might be at play, for example epigenetics and fetal-maternal sex steroid pathways, as well as the microbiome[248–252], which however will not be discussed in the current review.

8. Conclusion

Altogether, our findings show that ELA impacts later life mental and metabolic health differently in boys and girls, and suggests that ELA may play an important role in the origin of sex-specific prevalence of some of the most common disorders in our society, as well as their comorbidity. ELA-induced disease states may thus require specific strategies for prevention or intervention. Therefore, researchers should broaden their assessment in the context of population studies, intervention trials, treatments and diagnosis to more regularly include individuals early-life history, sex and comorbidities laying ultimately the basis for personalized treatment.

The high convergence of rodent and human findings across our comprehensive review further reinforces the validity of rodent models to study the biological substrates and exact mechanisms leading to the 'programming' of disease risk by ELA and to identify and test novel targets for preventive and intervention strategies.

Box 1: Factors impacting the outcome of ELA experiments

Throughout this review, it became clear that effects of ELA largely depend on the specific domain, the time of exposure, the specific ELA model, the age of testing and, importantly, the sex of the offspring. However, in particular for preclinical research, even when stratifying the outcomes into these sub-categories there is considerable discrepancy in the findings and, in addition, a considerable amount of experiments showed no effect of ELA on the various domains. Some of the aspects contributing to this variation are the breeding method and animal species/strain, early-life environmental factors such as diet composition and cage enrichments, and for behavioural research the animal facility and experience of the experimenter. While these are clearly important determinants they might be the hardest to fully standardize and control for, two specific aspects emerged while reviewing the literature that appear to have an important influence in the context of ELA-induced effects on behaviour and metabolism; a *methodological* aspect concerning the origin of the pregnant dams and a statistical aspect concerning the power of the current studies, which we will discuss here.

Origin of pregnant dams impacts on the offspring's later life outcome

Purchasing pregnant dams versus breeding within the own facility has been shown to be a modulating factor in preclinical ELA studies[246]. Indeed, transportation during pregnancy is stressful and thus acts as a stressor (first "hit"), both in controls as well as in the experimental animals prior to the actual ELA paradigm they will be exposed to, thereby altering the original design. This can affect the CTL dam and offspring as well as the response of the dam and offspring to ELA exposure, and thereby on the assessed outcome in the offspring possibly leading to incorrect conclusions. Nevertheless, several laboratories report ordering their dams pregnant as this saves time and can facilitate cross-fostering to assure large litters. Also within our literature search, we found some papers reporting ordering of pregnant dams. Within our review, the majority of the included research papers have reported in house breeding (breeding in house: 70.3%, ordering pregnant dams: 20.3%, not reported: 9.3%) and in particular, this aspect is mostly relevant for studies performed in rats, as ordering of pregnant mice happens only rarely (rats: 17.5%, mice: 1.5%).

Nevertheless, we saw that this was a modulating factor in the case of e.g. PRS effects on nsLearning and POS effects on anxiety-like behaviour, both when tested in adulthood. Indeed, when stratifying the experiments based on the origin of the dam, we found that PRS-exposure lead to increased memory impairment in females among studies where rodents were bred in house while there was no sex-difference in PRS-exposed offspring from dams that were ordered pregnant. Similarly, the increased male POS-induced vulnerability to anxiety-like behaviour was most pronounced in POS males that were born to dams that were ordered pregnant.

As this was impacting only a minority of the papers included in this review our overall conclusions concerning the effects of ELA remain reliable. Nevertheless, this is certainly

an element to pay attention to and to avoid such cofounder, we would recommend to standardize this aspect and choose to only breed in house when interested in studying the effects of ELA.

Limitation of rodent and human studies related to their experimental power

Concerning the human studies, a limitation in drawing overall conclusions when comparing studies based on different cohorts, is that the size of the various human cohorts varies largely. Low statistical power is an aspect that has been identified as a key problem across all neurosciences, and thus concerning ELA studies as well, as it reduces the chance of detecting a true effect and the probability that statistical significant results reflect true effects[253,254]. Next to large variations in human cohort studies with some likely having more or less statistical power than others, for rodent studies, despite the lack of a systematic check of the statistical power for each study, group sizes were mostly between 5-15 with often multiple experimental groups. Considering the well acknowledged individual variation when testing rodent behaviour, is plausible to assume that, at least part of the reviewed research papers might be underpowered. In fact it has been highlighted that a large proportion of the currently existing studies are underpowered, challenging the reliability and reproducibility of animal research across labs and experiments[246,253,255]. With the increasing complexity of experimental designs, often including second hits and possible interventions, it remains difficult to increase power and perform studies with sufficient animals per experimental group. Therefore, Bonapersona and colleagues have recently suggested an alternative solution for the problem of statistical power in preclinical research, called RePAIR, which includes previously obtained information in order to reduce the required number of animals and increase statistical power[255]. While this solution is applicable to tests or behavioural paradigms that are frequently used, other solutions concerning experimental design and statistical analysis have been suggested as well in order to maximise power[254] and improving the reliability of experiments.

Box 2. Role of the placenta in mediating sex differences during foetal development

The placenta is responsible for mediating the exchange of nutrients, respiratory gasses, waste products, and hormones between a mother and foetus during pregnancy[256]. Containing both maternal and foetal vascularisation, the placenta is the only organ composed of tissue from both mother and foetus. Throughout development, maternal blood interacts directly with foetal blood vessels allowing for the direct transfer of biological materials via diffusion, active transport, or selective absorption from mother to child and vice versa[256]. Due to the intimacy of this relationship the placenta is key for the foetal response to changes in the maternal environment. Importantly, there is evidence that the placenta is different and might respond differently to maternal adverse conditions depending on the sex of the developing offspring. We will describe this in relation to placental hormone levels, gene expression, and placental immune function[248,257,258]. For example, one important function of

the placenta is the placental transfer of maternal hormones that are essential for healthy growth and development of the foetus. Leptin is a hormone involved in energy homeostasis secreted by the adipose tissue, responsible for regulating energy homeostasis and fat storage. Interestingly, at birth females have higher leptin levels compared to males[259,260] and there is evidence that this might originate from a differential placental regulation of leptin. Indeed, placentas from male fetuses exhibit an increased amount of DNA methylation of their leptin promoters which might lead to reduced expression and thus transfer of leptin to males compared to females. Such sex differences also appear in response to glucocorticoids. Male placenta seems to be more susceptible to gestational exposure to cortisol, e.g. shown by cortisol induced placental growth[261] and an increased vasculature[262], while the female placenta seems to be more resilient to glucocorticoid exposure, possibly contributing to the sex-dependent effects of PRS described in this review. Next to hormonal transfer and sensitivity placenta also exhibits sex-dependent gene expression profile[257,258]. In general, gene expression levels related to immune function are higher in female as compared to male placentas[258], and also in response to PRS gene expression differs between male and female placentas[178]. When exposed to PRS, male mouse placentas show a higher expression of PPARα, IGFBP-1, GLUT4, and HIF3α while female placentas show a converse expression of PPARα and IGFBP-1[178]. These differential gene expression could contribute some of the sex-differences in the PRS effects described in this review. One hypothesis for these sex differences is related to maternal stress induced epigenetic changes. Indeed male mouse placentas have significantly lower baseline expression of DNMT1, the enzyme responsible for maintenance of DNA methylation, and no significant increase in DNMT1 expression in response to prenatal stress, while females do[178]. Finally an additional mechanism contributing to the sex-dependent placental gene expression is the process of placental X inactivation. During early embryogenesis, female embryos randomly inactivate one of their X chromosomes (either maternally or paternally derived) to prevent double transcription of X-linked gene products. However, during important steps of gestational development, the placenta is capable of reactivating the inactive X chromosome resulting in higher levels of their by-products in female offspring[257,263]. Indeed PRS has been shown to reactivate O-linked N-acetylglucosamine transferase (OGT), an enzyme responsible for nutrient-responsive regulation of cell signalling, a key part of glucose metabolism, again potentially contributing to the sex-dependent effects of PRS. There is thus increasing evidence that stress differently affects the male and female placenta, potentially contributing to sex differences in adult disease risk after PRS exposure, but more research is needed to further elucidate this.

Box 3. Sex-specificity in breastmilk composition?

Almost all new born mammals are initially fed with breastmilk, a rich bioactive liquid responsible for the transfer of nutrients, immune factors, hormones and microbiota from mother to offspring. Breastmilk composition is highly dynamic; within a single mother, large compositional differences exists within a single feed (foremilk/hindmilk), across the day

and over longer time periods/lactation stages. Breastmilk composition also varies greatly between different mothers, dependent on various environmental and maternal factors such as maternal diet and maternal BMI[264]. Interestingly, several experimental and clinical observations suggest that infant sex might be another driver of variability in maternal milk composition.

Evidence for sex-specific breastmilk composition has been collected in various animal species. The primate work of Hinde *et al*. in rhesus macaques (*Macaca mulatta*) has shown that milk produced for male singleton offspring is higher in energy and fat content, but lower in volume and calcium compared to milk produced for female offspring[265–267]. A cross-fostering study in bank voles (*Myodes glareolus*) demonstrated that all-female litters receive more milk than all-male litters, independent of maternal size or condition[268]. Similarly, a study analyzing numerous lactation records of >1.4 million Holstein dairy cows (Bos taurus) reports higher volume and energy content of milk produced for daughters compared to milk for sons 269. Since calves are removed from their mother on the day of birth and as milking occurs in a mechanical and standardized fashion, the sex-specific milk production is suggested to be the result of prenatal programming of the mammary gland by fetal sex. Under different conditions, maternal nursing behaviour could possibly regulate milk production in a different direction. Indeed, among captive red deers (*cervus elaphus hispanicus*), another ruminant species, milk volume and protein, fat and lactose content has been shown to be greater for sons than for daughters[270]. Also in marsupials, as well in kangaroos (*Macropus giganteus*) as in wallabies (*Macropus eugenii*), milk for sons is found to be higher in protein content but similar in volume and total energy[271].

Clinical observations that preterm boys respond differently to early nutritional interventions than girls[272] point towards sex-specific early-life nutritional requirements, in humans too. Interestingly, breastfed same-sex twins are found to be taller and heavier than breastfed opposite-sex twins[273], possibly because milk composition cannot be tailored to both sexes at the same time. This suggests that adaptation of milk composition to infant sex could be a potential mechanism via which optimal growth and development is achieved in both sexes.

So far, only few studies have addressed the relationship between infant sex and human milk composition and the results are somewhat conflicting. Some studies report no effect of infant sex on human milk macronutrient and energy content[274], or microbiota profiles[275]. Three studies, all with a modest sample size (25-61) report higher energy and fat content in milk for sons compared to daughters[276–278]. On the contrary, Hanh et al. report higher carbohydrate and energy content in milk for daughters[279]. In addition a study found that Kenyan mothers with a high social economic status produced milk with a higher fat content for sons, while mothers while poor mothers produced milk with a higher fat content for daughters[280]. Another study by Yahya et al. reports higher phosphor content and higher volume for sons, but higher calcium content in milk for daughters[281].

Altogether, the current evidence collected in various mammalian species, hints toward sex-specificity in breastmilk composition, and that a potentially differential adaptation of breastmilk composition to maternal stress depending on the sex of the infant might contribute to the sex-dependent effects of POS on offspring/children, but further studies are required to establish this.

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Supplementary table S1. Included ELA's for human and rodent literature

A. ELA exposures (human)	
PRS	Anxiety symptoms
	Depressive symptoms
	Stressful life events
	Perceived stress
	Job stress
	Parental cohabitation
	Exposure to flood
	Exposure to bereavement
	Exposure to ice storm
POS	Anxiety symptoms
	Depressive symptoms
	Stressful life events
	Post traumatic stress disorder
PRS+POS	Anxiety symptoms
	Depressive symptoms
	Intimate partner violence
B. ELA models (rodent)	
PRS	Restraint stress
	Unpredictable restraint stress
	Restraint and forced swim stress
	Chronic mild upredictable/variable stress
	Noise and/or swim stress
	Predator exposure
	Elevated platform stress
	Social stress
	Social defeat with aggresive lactating rat
	Psychological stress
	Footshock stress
POS	Maternal seperation (MS)
	MS + early weaning
	MS + social isolation
	MS + sham injection
	Unpredictable MS
	Maternal deprivation (MD)
	Limited nesting and bedding (LBN)
	LBN + substitute mother
	Neonatal isolation
	Feacal smell stress
	Forced swim stress
	Maternal immobilization stress

Supplementary table S2. Included readouts per domain for human and rodent literature

1. Questionnaires and tasks used per domain

Domain	Outcome	Questionnaire or task
Cognition	Attention	Eye tracking attention disengagment paradigm
		Continuous performance task
		Reaction time task
	Executive functions / inhibitory control	The Hearts and Flowers task
		Flanker task
	IQ	Wechsler Abbreviated Scale of Intelligence
		Wechsler Preschool Primary Scale of Intelligence
	Verbal IQ	Pebody Picture Vocabulary Test-Revised
	School performance	General Certificate of Secundary Education
	Visuospatial memory	Sequential memory test
	Cognitive functions	Cambridge Neuropsychological Test Automated Battery
Externalizing behaviour & disorders	Total externalizing behaviour	Strength and difficulties questionnaire (SDQ)
		Child behaviour checklist (CBCL)
		Infant-Toddler Social Emotional Assessment
		Brief Infant-Toddler Social Emotional Assessment
		Caregiver-Teacher Report Form
		Youth Self Report
		Rutter Revised Preschool Scales
		The Preschool Behaviour Checklist
		The Temperament Assessment Battery
	ADHD & CD symptoms	First time ADHD medication/hospitalization
		Index of psychiatric problems
		Diagnostic Interview Schedule according to DSM-IV
		Child and Adolescent Psychiatric Assessment
		Preschool Age Psychiatric Assessment
		The Development and Well-Being Assessment (DAWBA)
	Aggresive behaviour	Peer play
	Criminality	Number of criminal offences
Internalizing behaviour & disorders	Total internalizing behaviour	Strength and difficulties questionnaire (SDQ)
		Child behaviour checklist (CBCL)
		Brief Infant-Toddler Social and Emotional Assessment
		Infant-Toddler Social Emotional Assessment
		Rutter Revised Preschool Scales
	Depression and anxiety symptoms	Dimensions of Depression Profile for Children and Adolescents, CES-D
		Depression Anxiety Stress Scale
		Kiddie Schedule for Affective Disorders and Schizophrenia
		Index of psychiatric symptoms in children
		Revised Clinical Interview Schedule
		Diagnostic Interview Schedule according to DSM-IV
		Spence Preschool Anxiety Scale
		Children's Depression Symptoms Inventory
		Mood and Feelings Questionnaire-Short Version (MFQ)
		Preschool Age Psychiatric Assessment
		Child and Adolescent Psychiatric Assessment (CAPA)
		The Development and Well-Being Assessment (DAWBA)

B. Behavioural paradigms per domain

Domain	Behaviour paradigm
nsLearning	Object Location Task
	Object Recognition Task
	Morris Water Maze *
	Barnes-maze
	Y-maze
	T-maze
	Water T-maze
	Attention-Set-Shifting-Task
sLearning	Fear conditioning
	Active avoidance
	Passive avoidance
	Morris Water Maze **
	Emotional learning
Social behaviour	Social interaction
	Social investigation/approach
	Social preference
	Social play/fighting
	Social recognition/memory
	Competitie behaviour
	Aggression
Anxiety-like behaviour	Open Field Test
	Elevated Plus Maze
	Plus Maze
	Elevated Zero Maze
	Dark-light box
	Novelty-suppressed feeding/drinking
	Thigmotaxis
	Novelty seeking
	Continuous video monitoring
	Acoustic startle response test
	Hole-board
	Emergence test
	Free exploratory paradigm
	Defensive burying
Depressive-like behaviour	Forced Swimming Test
	Swim Escape Test
	Open Space Swimming Test
	Tail Suspension Test
	Learned Helplessness
	Sucrose Preference Test
	Sucrose consumption
	Sucrose Negative Contrast Test

CHAPTER 2

2

Maternal postpartum stress in mice affects the fatty acid composition in maternal milk and in the offspring brain and erythrocytes in a preclinical mouse model

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Abstract

Maternal postpartum stress (PPS) not only affects the mother adversely, but also the offspring during a sensitive period of development (referred to as early-life stress, ES). ES programs several aspects of the offspring, often for life, and increases among others the risk to develop both metabolic and mental disorders. Fatty acids (FAs) are critical for proper (brain) development, and alterations in the dietary FA composition during the early-life period can, similar to ES, alter the risk for later-life obesity and cognitive dysfunction. We have previously shown that ES in a preclinical mouse model changes the central and peripheral FA composition of offspring, and that a diet provided to pups enriched in omega-3 (n-3) FAs provided during early development, could prevent ES-induced cognitive decline at a later age in rodents. As maternal milk is the exclusive source of FAs early in life, we hypothesized that maternal PPS affects the milk FA status, and thereby alters offspring FA composition. For this, we use a validated mouse model in which mice are provided with limited nesting and bedding material from postpartum/postnatal day (P)2-P9, extracted milk from the lactating dam at P9, and analysed the FA status of the milk, erythrocytes (dam and offspring), and brain (offspring only). PSS affected the FA concentration and composition in mouse milk as well as the erythrocyte FA composition in dams. In the offspring, ES resulted in alterations in brain (both cortex and hippocampus) and erythrocyte FA composition. Moreover, we observed sex-specific effects of ES mostly in hippocampal FA composition, with ES-exposed females more often showing alterations in FA composition compared to males. Finally, we show that the milk and offspring FA composition correlates. In conclusion, we provide preclinical evidence that stress alters both the maternal milk and offspring FA status, suggesting that stress-induced changes in maternal milk composition could contribute to the effects of ES on the offspring.

1. Introduction

Exposure to postpartum stress (PPS) not only affects the mother, but also her infants (referred to as early-life stress, ES), notably during a critical developmental period with potential consequences for health outcomes later in life (i.e. 'programming'). In fact, there is ample of clinical evidence that ES exposure increases the risk to develop a wide range of disorders, including metabolic diseases (Danese and Tan, 2014), mental disorders (Herbison et al., 2017; Quarini et al., 2016) and cognitive dysfunction (Hay et al., 2008; Kersten-Alvarez et al., 2012). The early life environment encompasses many essential elements, including exogenous factors provided by the mother, such as tactile stimulation and nutrition, as well as endogenous modulators including stress hormones (Hoeijmakers et al., 2015; Lucassen et al., 2013). For example, preclinical studies show that variations in maternal care (e.g. high versus low licking and grooming) programs the stress response and cognitive function of offspring in adulthood (Francis and Meaney, 1999; Liu et al., 2000, 1997). It is however likely that different aspects of the early environment interact and together contribute to ES-induced programming and later-life disease risk (Hoeijmakers et al., 2015; Lucassen et al., 2013).

The importance of nutrition during early life stages is widely recognized. Especially during the first 1000 days of human life, the quality of nutrition has considerable impact on later life health. Importantly, human milk is the main and optimal nutrition during this critical developmental period (Cusick and Georgieff, 2016; Hicks et al., 2007; Horta et al., 2015; Lee et al., 2016; Mameli et al., 2016; Mayer-Davis et al., 2006). In particular lipid quality during early life seems critical for proper brain development as lipids are important building blocks for the brain (Schipper et al., 2020). Indeed, lipids constitute nearly 60% of the human brain dry weight (O'Brien and Sampson, 1965). Approximately 51% of the FAs in the human neonatal brain are saturated fatty acids (SFA), ±17% are mono-unsaturated fatty acids (MUFAs) and ±31% are poly-unsaturated fatty acids (PUFAs) (Makrides et al., 1994). In neonatal mice (postnatal day 5), ±49% of FAs are SFA, ±17% are MUFA, and ±30% are PUFA (Schipper et al., 2016). While in the developing brain, SFAs and MUFAs are important for myelin formation and neuronal differentiation (Martínez and Mougan, 1998; Tabernero et al., 2001), PUFAs have received most attention regarding brain development. PUFAs support critical physical and functional membrane properties and thereby influence membrane receptors, enzymatic activities and neuronal growth and plasticity, as well as affect neuroinflammation (Dyall, 2015; Youdim et al., 2000).

Specifically omega-3 (n-3) and omega-6 (n-6) long chain PUFAs are known for their role in both metabolic and cognitive development (Innis, 2008; Oosting et al., 2010; Schipper et al., 2020). For example, providing mice a diet rich in n-3 (long chain) PUFAs during development prevented excessive body fat accumulation later in life (Oosting et al., 2010), while (developmental) n-3 deficiency resulted in learning impairments in rats, which could be reversed by supplementation of the long chain n-3PUFA docosahexaenoic acid (DHA)

during lactation (García-Calatayud et al., 2005; Moriguchi et al., 2000). Furthermore, in humans, high dietary intake of n-6 PUFAs and a diet with a high n-6/n-3 ratio are associated with excessive adipose tissue development (Ailhaud et al., 2006; Simopoulos, 2016; Torres-Castillo et al., 2018) and higher risk on cognitive decline and dementia (Loef and Walach, 2013). Thus, while n-3 PUFAs such as DHA have been associated to an improved metabolic and cognitive outcome, excessive levels of certain n-6 PUFAs (e.g. linoleic acid, LA) or a high n-6/n-3 ratio appear detrimental for metabolic and cognitive health. Of note, since n-3 and n-6 PUFAs alpha-linolenic acid (ALA) and LA compete for endogenous conversion to longer chain (>20C) n-3 and n-6 PUFAs, the n-6/n-3 ratio is important for homeostasis and development (Simopoulos, 2011). Next to these effects of PUFAs, also inadequate SFAs early in life are detrimental for offspring development, while less is known about MUFAs. For example, a high-fat diet rich in SFAs early in life increased adiposity (Maejima et al., 2020) and reduced cognitive function (Noble and Kanoski, 2016) in rodents.

Thus, a suboptimal nutritional fatty acid (FA) composition during early-life leads to a similar later-life disease vulnerability as ES exposure does, i.e. to an increased risk of metabolic and cognitive disorders. It is thus intriguing to explore if and how early-life nutrition and FA status are affected by ES, and (eventually) if this could be involved in ES-induced programming. There is initial evidence that ES alters plasma and brain PUFA status in rat and mouse offspring, respectively (Clarke et al., 2009; Yam et al., 2019). Importantly, the n6/n3 ratio of the maternal diet during lactation is reflected in milk (Oosting et al., 2015), and we previously showed that reducing the dietary LA/ALA ratio, while providing similar absolute amounts of PUFAs in the maternal and early-life diet fully prevented ES-induced cognitive dysfunction in mice (Yam et al., 2019).

With mammalian milk being the sole dietary FA source early in life, we therefore hypothesized that PPS alters the maternal milk FA composition leading to alterations in the peripheral and central FA status in the offspring. As it has been suggested that ES affects the offspring in a sexually dimorphic manner (Araya et al., 2009; Boynton-Jarrett et al., 2010; Hay et al., 2008; Park et al., 2018), we here specifically focused on possible sex-differences as well.

To investigate effects of PPS and ES on the maternal and offspring FA status, we used an established mouse model that involves housing the dam and her offspring together, yet in an impoverished environment, i.e. with limited amounts of nesting and bedding material (Naninck et al., 2015), which constitutes a stressor for both dam and her offspring, and extracted milk from the lactating dams. We assessed the FA profile (absolute concentration and relative composition) of the milk and erythrocytes (relative composition only) of the lactating dam, as well as the relative composition in the brain and erythrocytes of her offspring and studied the relationship between these FA profiles. We show that i) PPS alters the FA profile in milk and erythrocytes of dams, ii) ES results in alterations in brain and erythrocyte FA profile in a sex-specific manner, and iii) that milk and offspring FA composition correlate. Further research is needed to understand if and how these effects of stress on the

milk and offspring FA composition are involved in the ES-induced metabolic and cognitive phenotype.

2. Materials and methods

2.1 Mice and breeding

For these studies, 16 mouse dams (control (CTL): 13, of which 9 for milk and erythrocyte FA analysis; ES: 11, of which 7 for milk FA analysis/8 for erythrocyte FA analysis) and 74 pups (CTL male (M): 20; ES M: 18; CTL female (F): 20; ES F:16) were used. Mice were kept under standard housing conditions (temperature 20–22C, 40–60% humidity, 12/12h light/dark schedule (lights on at 08:00 AM), standard chow (CRM (P), 801722, Standard Diets Services, Essex, United Kingdom, 3.585 kcal/g, where: 22% protein, 9% fat, and 69% carbohydrates) and water *ad libitum*). This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of the University of Amsterdam, and all animal procedures related to the purpose of the research were approved under the Ethical license of the national competent authority, securing full compliance to the European Directive 2010/63/EU for the use of animals for scientific purposes.

Experimental animals were bred in house to standardize the perinatal environment. 8 weeks old primiparous C57Bl/6J female and male mice were purchased from Envigo Laboratories B.V. (Venray, The Netherlands) and group-housed in type II or III conventional cages. After habituation for 1-2 weeks, 2 females were housed with a male to allow for mating in a clean type III conventional cage with standard sawdust bedding material and paper straw cage enrichment. After 1 week, the male was removed and females were housed together for another week in a clean type II conventional cage with nest material (square piece of cotton, 5x5 cm, Techninlab-BMI, Someren, The Netherlands) and paper straw cage enrichment). Females were not checked for pregnancy. Next (14 days after the start of the breeding procedure), females were housed individually in a type II cage with nesting material and a filtertop (to minimize noise and smell disturbances), and checked for the birth of pups each morning before 09:00 AM (light phase) from day 18 of breeding onwards without disturbing the cage. When a litter was born, the previous day was determined as postnatal day 0 and left undisturbed until P2.

2.2 Stress paradigm for dam and offspring

Stress (postpartum stress (PPS) (when referred to for the mother), and early-life stress (ES) (when referred to for the offspring) was induced by limiting the nesting and bedding material from postpartum/postnatal day (P)2 to P9, as described previously (Naninck et al., 2015; Yam et al., 2019). Briefly, at P2, litters were culled to 6 pups (containing both sexes) and randomly assigned to the control (CTL) or ES condition. Litters with less than 5

pups or only one sex were excluded. CTL cages consisted of a standard amount of sawdust and one square piece of cotton nesting material (5x5 cm, Techninlab-BMI, Someren, The Netherlands), while ES cages only had a little amount of sawdust on the bottom, covered with a fine-gauge stainless steel mesh, and half a square piece of cotton nesting material (2.5x5 cm). All cages were covered with a filtertop. Pups, dams, food, and water were weighted at both P2 and P9. To calculate bodyweight (BW) gain from P2 to P9 for the pups, the average BW for the male and female pups of one nest was calculated at P2, and subtracted from the average BW for that sex at P9.

2.3 Milk collection

Milk collection always happened at the same time of day (between 10:00 and 12:00 AM, in the inactive phase of the mice) to minimize potential circadian variation in milk composition. A maximum of 2 dams were milked per day, when possible 1 ES and 1 CTL nest, and the order (first CTL or first ES) of milking was alternated. Milking was performed with a human breast pump adjusted to fit the mouse nipple as previously described (Oosting et al., 2015). For milk to accumulate, pups were removed from the dam 2 hours prior to the start of the milking procedure and immediately sacrificed (see section 2.4). The dam was anesthetized with an intraperitoneal injection of ketamine/xylazine (100 mg/kg ketamine, 10 mg/kg xylazine), and placed on a heating pad. The dams were next injected with 2 IU oxytocin between the 4th and 5th nipple (1 IU on each side) to stimulate milk release. Within five minutes after the injection of oxytocin, milk collection started. Nipples were wetted with a brush with sterile saline for proper vacuum, and milk was collected from the 4th and 5th nipple (these are most easily accessible with the pump, maximal 5 minutes per nipple). The whole procedure lasted approximately 20-30 minutes per mouse. Directly after the milking procedure ended, the dams were sacrificed and milk was stored at -80 °C.

2.4 Tissue collection

At the morning of P9, between 08:00 and 09:00 AM, all pups from the respective nest were sacrificed by rapid decapitation (no anaesthesia). Trunk blood was collected in EDTA-coated tubes (Sarstedt, The Netherlands), centrifuged, and the plasma and erythrocytes were separately stored at -40 °C. Pup hippocampus and cortex were dissected, snap frozen and stored at -80 °C. Maternal blood samples were collected in EDTA-coated tubes (Sarstedt, The Netherlands) between 08:00 and 09:00 AM by a tail cut (without anaesthesia), and the plasma and erythrocytes were stored separately at -40 °C.

2.5 Corticosterone measurements

Corticosterone (CORT) in plasma was measured using an ELISA kit (RE52211, IBL International GmbH), according to manufacturer's instructions.

2.6 Fatty acid (FA) measurements

Hippocampus and cortex samples were pooled from 2 pups of the same sex and litter, homogenized, and 50x diluted in Milli-Q. Milk and erythrocyte samples could directly be used for the extraction of lipids. Lipids were extracted as previously described (Bligh and Dyer, 1959) and membrane FA composition was assessed by gas chromatography. Samples were transferred to glass tubes containing 1 ml EDTA (1%), and 2.2 ml methanol and 1 ml dichloromethane were added. Tubes were vortexed for at least 5 min. 1 ml EDTA and 1 ml dichloromethane were added, and samples were vortexed again (5 min). Subsequently, tubes were centrifuged at 2000 g for 10 min, and 400 µl of the bottom layer was to a new glass tube and evaporated. 2 ml methanol and 40 µl concentrated H2SO4 were added, and tubes were placed in a heating block at 100 °C for 1h. Tubes were then cooled, 2 ml hexane and 0.5ml 2.5 m-NaOH were added, and samples were vortexed. The top layer was transferred to a clean tube, evaporated, and residues were reconstituted in 200 µl iso-octane. FA composition/concentration was analysed on a gas chromatograph equipped with a flame ion-solation detector.

Values were expressed either as a percentage of total FA (FA composition; brain, erythrocytes and milk) and in absolute values (FA concentration; milk only). This was done as for some questions the FA composition is most relevant (e.g. when studying membrane compositions), but with regard to the milk FA status, both concentration (how much FAs do the offspring get) and composition (what is the relative abundance of the individual FAs) are meaningful. FAs that were detected in less than 65% of the samples were excluded from the analysis.

2.7 Statistical analysis

Data were analysed with SPSS 25.0 (IBM software), Graphpad Prism 6 (Graphpad software), and R studio version 1.2.1335. All data are presented as mean ± standard error of the mean (SEM). To analyse the bodyweight, food intake, glucocorticoid and absolute FA data (FA concentration), data was tested for outliers in SPSS, and outliers were removed for the specific measure they were outlier for (0-1 outliers were identified and removed per analysis, depending on the specific measurement). For relative FA composition (%FA) data, because of their dependent nature, the dataset was checked for outliers, and samples that were a significant outlier in more than 50% of the individual FAs were removed from the *entire* dataset. This occurred once: in the erythrocyte FA data of the pups 1 sample was removed from the entire dataset. Next, data with only condition as predictor variable (related to the dams) was analysed with an independent t-test or nonparametric alternative. Data with both condition and sex as predictors (related to the offspring) was analysed with a 2-way ANOVA. Multiple mice from one litter were included in these experiments, resulting in nested data. We always tested for contributing effects of litter to the outcome variable, and corrected when needed by performing mixed model analysis with litter as random factor. We used the Benjamini Hochberg procedure with a false discovery rate of 0.15 to

correct for multiple testing for all individually measured FAs. For expression of sum FAs and FA ratios, no multiple testing correction was performed.

3. Results

3.1 Effects of PPS and ES on BW gain, food intake and CORT levels

At P2, BW was not different between CTL and PPS dams (data not shown), and PPS exposure did not affect BW gain from P2 to P9 in dams ($t(22)=1.354$, $p=0.19$) (Fig. 1A), while food intake during the stress exposure was increased ($t(21)=-2.406$, $p=0.02$) (Fig. 1B). Water intake from P2-P9 ($t(19)=0.537$, $p=0.598$) (Fig. 1C) and plasma basal CORT levels of dams at P9 ($t(21)=0.907$, $p=0.375$) (Fig 1D) were unaffected by stress exposure. In the offspring, there was a significant difference between the BW at P2 of CTL and ES litters, with pups designated to the ES group having a higher BW compared to CTL litters *before* the start of the ES paradigm ($F_{condition}(1,34.464)=6.721$, $p=0.014$) (E). Following exposure to ES, ES decreased BW gain from P2 to P9 in both males and females ($F(1)=58.082$, $p<0.001$) (Fig. 1F). Moreover, CORT levels were higher in female offspring ($F(1, 80.241)=4.995$, $p=0.029$), and lower in ES-exposed males and females ($F(1, 57.628)=5.873$, $p=0.019$) (fig. 1G).

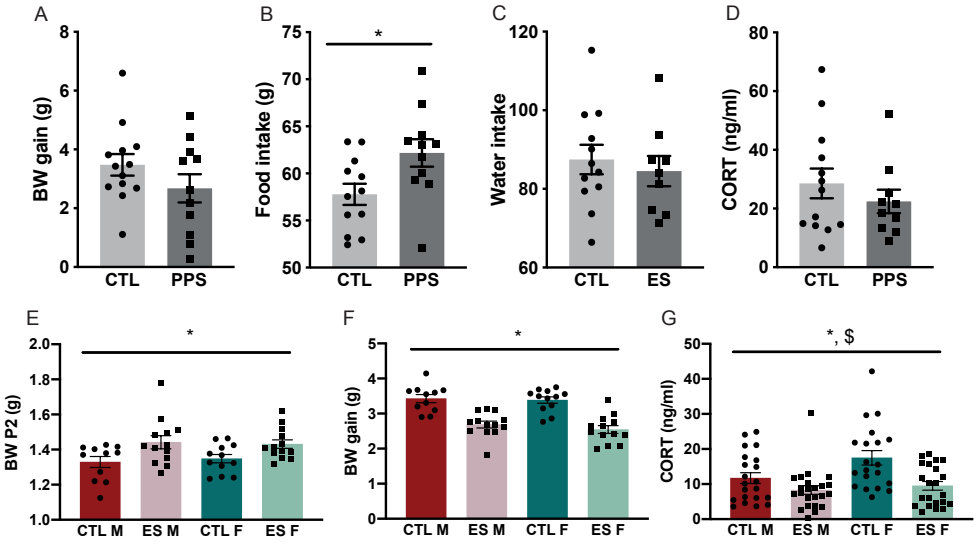

Figure 1. Effects of stress from postpartum/postnatal day (P)2-P9 on bodyweight (BW) gain, food intake and glucocorticoid (CORT) levels in dams and offspring. A) BW gain in dams (CTL: n=13, ES: n=11) was not affected by postpartum stress (PPS). B) Food intake of dams exposed to PPS was increased. C) PPS did not affect water intake. D) PPS did not affect maternal CORT levels. E) BW at P2 was higher in pups designated to the ES condition (taken per litter, CTL M: 11; ES M: 13; CTL F: 12; ES F: 13). F) BW gain in pups (taken per litter, CTL M: 11; ES M: 13; CTL F: 12; ES F: 13) was lower in mice exposed to early-life stress (ES). G) Female offspring had higher CORT levels compared to males, and ES decreased CORT levels in both sexes (CTL M: 21; ES M: 23; CTL F: 20; ES F: 22). Indicated is mean ± SEM, $p<0.05$. * main effect of condition, $ main effect of sex.

3.2 PPS alters FA concentration and composition in milk and erythrocytes

Quantitate analysis of the FAs in milk revealed that PPS increased the concentration of total (∑)FAs ($t(13)=-2.305$, $p=0.038$), as well as of total saturated fatty acids (∑SFAs) ($t(13)=-2.729$, $p=0.017$), but not the concentration of total mono-unsaturated fatty acids (∑MUFAs) or ∑PUFAs (table 1). When looking at the individual SFAs, PPS increased the concentration of C12:0 ($t(13)=-2.351$, $p=0.035$), C14:0 ($t(13)=-2.817$, $p=0.015$), C16:0 ($t(13)=-2.879$, $p=0.013$), C20:0 ($t(13)=-2.762$, $p=0.016$), C22:0 ($t(13)=-2.286$, $p=0.04$), and C24:0 ($t(13)=-3.682$, $p=0.003$). In addition, PPS increased the concentration of the MUFA C16:1n-7 ($t(13)=-3.397$, $p=0.005$), and the PUFAs C18:3n-6 ($t(13)=-2.224$, $p=0.044$), C18:4n-3 ($t(13)=-2.328$, $p=0.037$), and C22:3n-3 ($t(13)=-2.406$, $p=0.032$).

However, when looking at the relative FA composition (as a percentage of total FA), PPS did not affect the %∑SFA or %∑MUFA, while the %∑PUFA was decreased ($t(14)=2.408$, $p=0.03$) (table 1). This was caused by a decrease in both %∑n-6 PUFAs ($t(14)=2.343$, $p=0.034$), and %∑n-3 PUFAs ($t(14)=2.216$, $p=0.044$). The differences between PSS and CTL in relative concentration of the individual FAs were not significant after correction for multiple testing. Moreover, in line with the higher SFA concentrations in milk, the %∑SFA in the erythrocytes of dams exposed to PPS was increased ($t(15)=-2.182$, $p=0.045$), whereas the %∑MUFA was decreased ($t(15)=2.665$, $p=0.02$) and PPS did not affect %∑PUFA or any of the individual FAs in erythrocytes of dams after correction for multiple testing (table 1).

3.3 ES affects FA composition in the brain and erythrocytes of offspring in a sex-dependent manner

In the cortex of male and female offspring, ES did not affect %∑SFA, %∑MUFA or %∑PUFA, neither did it affect the %∑n-6 PUFA and %∑n-3 PUFA (table 3). However, when looking at the specific FAs, ES decreased the %C14:0 ($F_{condition}(1, 32)=31.552$, $p<0.001$) and %C16:0 ($F_{condition}(1, 32)=6.552$, $p=0.015$), while it increased %C22:0 ($F_{condition}(1, 32)=4.365$, $p=0.045$). For the MUFAs, ES increased %C16:1n-7 ($F_{condition}(1, 32)=16.645$, $p<0.001$), while it decreased %C18:1n-7 ($F_{condition}(1, 35.145)=8.049$, $p=0.008$), %C20:1n-9 ($F_{condition}(1, 35.101)=4.588$, $p=0.039$), and %C20:3n-9 ($F_{condition}(1, 32)=4.527$, $p=0.041$) similarly in both sexes. ES also increased the PUFAs %C18:3n-6 ($F_{condition}(1, 33.13)=6.301$, $p=0.017$), %C20:3n-6 ($F_{condition}(1, 32.922)=29.019$, $p<0.001$) and %C22:5n-3 ($F_{condition}(1, 34.532)=10.246$, $p=0.003$) independent of offspring sex. Finally, %C18:4n-3 ($F_{condition}(1, 32)=4.175$, $p=0.049$) was lower in ES-exposed animals.

Table 1. Effects of PPS on milk fatty acid (FA) concentration (mg/L) and composition (as percentage of total FA levels) (CTL: n=9; ES: n=7). Indicated are means ± SEM, p values and significance after Benjamini Hochberg (BH) correction. Blue indicates significantly higher in stressed dams, red indicates significantly lower in stressed dams. *: significantly different after BH correction, ns: not significant.

		FA concentration (mg/l) milk						%FA milk					
		CTL		PPS		P value		CTL		PPS		P value	
		Mean	*SEM*	*Mean*	*SEM*	*ES vs CTL*	*BH*	*Mean*	*SEM*	*Mean*	*SEM*	*ES vs CTL*	*BH*
SFA	C8:0	287.6	39.2	366.7	40.0	0.197	ns	0.259	0.020	0.234	0.018	0.299	ns
	C10:0	5731.3	646.5	7669.9	749.5	0.075	ns	5.198	0.140	5.063	0.311	0.758	ns
	C12:0	11038.9	1205.8	15435.4	1402.6	0.035	*	10.054	0.260	10.163	0.554	0.851	ns
	C14:0	15002.1	1602.1	21925.0	1805.8	0.015	*	13.746	0.461	14.413	0.669	0.411	ns
	C15:0	72.8	12.4	93.3	17.6	0.345	ns	0.065	0.009	0.059	0.010	0.660	ns
	C16:0	30939.9	3466.5	45085.9	2970.0	0.013	*	28.204	0.690	29.599	0.569	0.157	ns
	C17:0	67.2	9.7	92.5	7.2	0.08	ns	0.060	0.004	0.060	0.002	0.758	ns
	C18:0	1602.9	187.6	2136.9	150.4	0.062	ns	1.452	0.025	1.439	0.042	0.772	ns
	C20:0	44.7	4.9	64.4	4.8	0.016	*	0.041	0.001	0.043	0.001	0.142	ns
	C22:0	34.1	3.2	44.8	3.1	0.04	*	0.031	0.001	0.030	0.001	0.351	ns
	C23:0	10.5	1.7	9.1	3.1	0.665	ns	NA		NA		NA	
	C24:0	47.6	4.3	73.7	6.0	0.003	*	0.044	0.002	0.049	0.002	0.042	ns
MUFA	C16:1n-7	3473.6	417.3	5539.9	399.1	0.005	*	3.147	0.098	3.547	0.158	0.041	ns
	C18:1n-9	20654.8	2979.2	25626.1	2776.0	0.27	ns	18.132	1.025	16.777	1.260	0.413	ns
	C18:1n-7	2658.6	362.8	3602.8	359.2	0.101	ns	2.354	0.090	2.341	0.164	0.942	ns
	C20:1n-9	807.1	132.2	947.3	121.6	0.475	ns	0.697	0.059	0.617	0.061	0.367	ns
	C20:3n-9	81.1	11.3	86.4	7.7	0.735	ns	0.072	0.004	0.056	0.003	0.011	ns
	C22:1n-9	105.3	16.8	126.1	15.6	0.406	ns	0.091	0.007	0.082	0.008	0.403	ns
	C24:1n-9	154.1	20.3	198.9	22.3	0.17	ns	0.138	0.008	0.130	0.009	0.535	ns

PUFA	C18:2n-6 LA	12836.7	1612.7	16645.1	984.0	0.1	ns	11.484	0.122	10.939	0.189	0.024	ns
	C18:3n-6	144.8	16.0	195.9	14.6	0.044	*	0.132	0.004	0.128	0.003	0.434	ns
	C20:2n-6	1147.0	163.7	1307.7	111.0	0.481	ns	1.009	0.049	0.868	0.048	0.062	ns
	C20:3n-6	863.5	108.3	1114.7	90.0	0.123	ns	0.777	0.033	0.730	0.031	0.338	ns
	C20:4n-6 ARA	765.1	93.8	936.0	87.3	0.23	ns	0.692	0.033	0.613	0.038	0.140	ns
	C22:4n-6 AdrA	349.8	50.0	397.5	42.9	0.513	ns	0.310	0.023	0.260	0.023	0.148	ns
	C22:5n-6 n6DPA	49.6	7.2	69.0	7.5	0.094	ns	0.045	0.003	0.046	0.004	0.792	ns
	C18:3n-3 ALA	766.9	102.3	968.6	56.5	0.158	ns	0.683	0.017	0.641	0.008	0.031	ns
	C18:4n-3	32.3	3.7	44.9	3.5	0.037	*	0.029	0.001	0.029	0.001	0.918	ns
	C20:3n-3	90.4	13.3	107.5	7.6	0.348	ns	0.080	0.003	0.071	0.002	0.042	ns
	C20:5n-3 EPA	134.0	15.6	180.8	13.5	0.055	ns	0.121	0.002	0.118	0.002	0.252	ns
	C22:3n-3	14.2	1.9	21.2	2.3	0.032	*	0.013	0.001	0.013	0.001	0.837	ns
	C22:5n-3	175.9	24.0	208.7	19.7	0.349	ns	0.156	0.007	0.136	0.008	0.086	ns
	C22:6n-3 DHA	124.1	15.9	173.8	21.3	0.079	ns	0.111	0.004	0.115	0.009	0.918	ns
	∑FA	110924.6	13043.7	152388.5	9811.2	0.038		NA		NA		NA	NA
	∑SFA	64879.6	7120.5	92997.6	6626.3	0.017		59.163	1.355	61.163	1.779	0.377	NA
	∑MUFA	27934.6	3912.8	36127.5	3599.0	0.17		24.629	1.137	23.551	1.561	0.576	NA
	∑PUFA	17494.4	2201.0	22371.4	1386.1	0.122		15.641	0.242	14.707	0.311	0.03	NA
	∑n6	16156.7	2030.0	20666.7	1273.9	0.121		14.449	0.231	13.587	0.294	0.034	NA
	∑n3	1337.9	173.7	1705.5	112.7	0.14		1.193	0.023	1.123	0.020	0.044	NA
	n6/n3	12.134	0.215	12.140	0.142	0.985		12.134	0.216	12.090	0.129	0.872	NA
	DHA/n6DPA	2.573	0.154	2.555	0.210	0.945		2.573	0.154	2.540	0.178	0.893	NA

Table 2. Effects of stress on maternal erythrocyte FA composition (as percentage of total FA levels) (CTL: n=9; ES: n=8). Indicated are means ± SEM, p values and significance after BH correction. Blue indicates significantly higher in stressed dams, red indicates significantly lower in stressed dams. *: significantly different after BH correction, ns: not significant.

		%FA Erythrocytes					
		CTL		PPS		P value	
		Mean	*SEM*	*Mean*	*SEM*	*ES vs CTL*	*BH*
SFA	C14:0	0.429	0.007	0.421	0.015	0.277	ns
	C15:0	0.264	0.010	0.246	0.015	0.327	ns
	C16:0	42.997	1.069	45.299	0.569	0.087	ns
	C17:0	0.477	0.017	0.480	0.014	0.899	ns
	C18:0	14.351	0.271	14.311	0.325	0.963	ns
	C20:0	0.423	0.014	0.479	0.018	0.024	ns
	C22:0	1.438	0.054	1.538	0.062	0.243	ns
	C24:0	2.754	0.124	2.995	0.154	0.237	ns
MUFA	C16:1n-7	0.758	0.045	0.701	0.041	0.370	ns
	C18:1n-9	14.057	0.524	12.590	0.298	0.033	ns
	C18:1n-7	2.757	0.039	2.641	0.059	0.118	ns
	C20:1n-9	0.339	0.017	0.301	0.008	0.066	ns
	C20:3n-9	0.054	0.005	0.044	0.005	0.123	ns
	C22:1n-9	0.067	0.007	0.071	0.004	0.683	ns
	C24:1n-9	1.523	0.047	1.540	0.057	0.823	ns
PUFA	C18:2n-6 LA	9.080	0.420	8.089	0.539	0.163	ns
	C18:3n-6	0.070	0.005	0.061	0.006	0.255	ns
	C20:2n-6	0.184	0.011	0.159	0.007	0.432	ns
	C20:3n-6	0.492	0.035	0.430	0.030	0.208	ns
	C20:4n-6 ARA	4.000	0.119	4.071	0.199	0.757	ns
	C22:4n-6 AdrA	0.366	0.018	0.423	0.025	0.081	ns
	C22:5n-6 n6DPA	0.251	0.011	0.248	0.020	0.898	ns
	C18:3n-3 ALA	0.269	0.020	0.226	0.019	0.046	ns
	C20:3n-3	0.023	0.005	0.037	0.006	0.114	ns
	C20:5n-3 EPA	0.060	0.005	0.062	0.007	0.786	ns
	C22:5n-3	0.172	0.011	0.190	0.011	0.285	ns
	C22:6n-3 DHA	0.750	0.029	0.726	0.057	0.700	ns
	∑SFA	63.139	1.009	65.769	0.583	0.045	NA
	∑MUFA	19.554	0.547	17.890	0.301	0.020	NA
	∑PUFA	15.716	0.544	14.721	0.634	0.250	NA
	∑n6	14.442	0.517	13.481	0.567	0.229	NA
	∑n3	1.276	0.041	1.238	0.078	0.663	NA
	n6/n3	11.364	0.369	11.021	0.422	0.548	NA
	DHA/n6DPA	3.047	0.184	3.002	0.254	0.885	NA

In the hippocampus, ES did not affect the %∑SFA, %∑MUFA or %∑PUFA. However, when looking at the individual FAs, ES increased %C14:0 ($F_{condition}(1, 33)=68.798$, $p<0.001$), %C16:1n-7 ($F_{condition}(1, 33)=23.776$, $p<0.001$), %C18:2n-6 (LA) ($F_{condition}(1, 33)=7.601$, $p=0.009$), %C20:3n-6 ($F_{condition}(1, 32.767)=7.423$, $p=0.01$) and %C22:5n-3 ($F_{condition}(1, 36.371)=7.893$, $p=0.008$) similarly in both sexes. In contrast, ES decreased %C20:1n-9 ($F_{condition}(1, 33)=12.212$, $p=0.001$), and %C20:3n-9 ($F_{condition}(1, 33)=13.41$, $p=0.001$) in males and females. Moreover, %C16:0 ($F_{condition*sex}(1, 33)=4.632$, $p=0.039$), %C18:0 ($F_{condition*sex}(1, 18.357)=9.442$, $p=0.006$), %C24:1n-9 ($F_{condition*sex}(1, 33)=4.743$, $p=0.037$), %C20:4n-6 (ARA) ($F_{condition*sex}(1, 16.114)=8.767$,

p=0.036) and %C22:4n-6 (AdrA) ($F_{condition*sex}$(1)=, p=0.009) were affected by ES in a sex-dependent manner. *Post hoc* analysis revealed that ES females had lower %C16:0 (p<0.001) and %C24:1n-9 (p=0.002) compared to their controls, while this was not true for males. In contrast, %C20:4n-6 was higher in ES females compared to CTL females (p=0.005). *Post hoc* analysis of %C18:0 and %C22:4n-6 revealed no significant differences between CTL and ES groups for either males or females. In addition, independent of postnatal treatment, females had lower %∑SFA (F_{sex}(1)=, p=0.013) and higher %∑PUFA (F_{sex}(1)=, p=0.036), as well as higher %∑n-6 PUFA (F_{sex}(1)=, p=0.012) compared to males.

In erythrocytes, and in line with the higher SFA concentration in milk of stressed dams, ES increased the %∑SFA ($F_{condition}$(1, 48.431)=6.042, p=0.018), while the %∑PUFA ($F_{condition}$(1, 46.168)=4.98, p=0.031) and %∑n-6 PUFA ($F_{condition}$(1, 46.12)=5.142, p=0.028) were reduced in ES offspring (table 5). In addition, when looking at the specific FAs, the %C16:0 ($F_{condition}$(1, 44.049)=8.694, p=0.005) and %C22:1n-9 ($F_{condition}$(1, 65)=8.121, p=0.006) were increased in ES males and females, whereas ES reduced the %C20:3n-9 ($F_{condition}$(1, 44.77)=9.232, p=0.004), %C18:2n-6 (LA) ($F_{condition}$(1, 65)=9.021, p=0.003), %C18:3n-6 ($F_{condition}$(1, 65)=5.361, p=0.024), %C20:2n-6 ($F_{condition}$(1, 48.225)=6.101, p=0.017) and C18:3n-3 (ALA) ($F_{condition}$(1, 65)=11.786, p=0.001) similarly in both sexes. Finally, the n6/n3 ratio was lower in females, but unaffected by early-life condition (F_{sex}(1, 65)=4.804, p=0.032).

3.4 Milk FA composition correlates to offspring FA composition

Table 4 shows an overview of the correlations between %∑SFAs, %∑MUFAs and %∑PUFAs, as well as (specific) n-6 and n-3 FAs and ratios, between milk and offspring brain and erythrocytes. For the %LA in the cortex, there was a negative correlation (r=-0.495, p-0.04) with the %LA in milk. Moreover, the DHA/n6DPA ratio (r=0.536, p=0.02) correlated significantly between milk and cortex. For the hippocampus, there was a significant correlation between hippocampal and milk %n6DPA levels (r=0.497, p=0.04), between hippocampal and milk n6/n3 ratio (r=0.631, p<0.001), and between hippocampal and milk DHA/n6DPA ratio (r=0.613, p=0.01). Finally, for erythrocytes, there were significant positive correlations between milk and erythrocyte ∑SFA (r=0.666, p<0.001), ∑MUFA (r=0.769, p<0.001), ∑PUFA (r=0.329, p=0.03), and ∑n-6 PUFA (r=0.403, p=0.02). When looking at the specific FAs, %C20:2n-6 correlated positively between milk and erythrocytes (r=0.803, p<0.001), whereas %C22:6n-3 (DHA) correlated negatively (r=-0.476, p=0.01). In addition, the DHA/n6DPA (r=0.449, p=0.01) ratio correlated between milk and offspring erythrocytes.

Table 3. Effects of ES on the FA composition (as percentage of total FA levels) in the cortex of male and female offspring at P9 (CTL M: 10; ES M: 9; CTL F: 10; ES F: 8). Indicated are means ± SEM, p values and significance after BH correction. For condition effects: blue indicates significantly higher in ES, red indicates significantly lower in ES; for sex effects: blue indicates higher in females, red indicates lower in females; interaction effects are indicated in green. *: significantly different after BH correction, ns: not significant.

		%FA cortex													
		CTL M		ES M		CTL F		ES F		P value					
		Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*ES vs CTL*	*BH*	*F vs M*	*BH*	*Int*	*BH*
SFA	C14:0	2.719	0.058	3.004	0.078	2.666	0.074	3.271	0.107	0	*	0.185	ns	0.052	ns
	C16:0	33.102	0.106	32.891	0.097	33.115	0.082	32.849	0.077	0.015	*	0.875	ns	0.768	ns
	C18:0	16.389	0.064	16.318	0.059	16.416	0.087	16.208	0.113	0.101	ns	0.62	ns	0.415	ns
	C20:0	0.050	0.003	0.047	0.003	0.051	0.003	0.046	0.003	0.255	ns	0.983	ns	0.855	ns
	C22:0	0.025	0.006	0.026	0.008	0.015	0.006	0.038	0.002	0.045	*	0.882	ns	0.076	ns
MUFA	C16:1n-7	2.446	0.020	2.548	0.050	2.421	0.036	2.624	0.043	0	*	0.497	ns	0.184	ns
	C18:1n-9	10.058	0.040	10.040	0.144	10.013	0.049	9.828	0.085	0.225	ns	0.128	ns	0.317	ns
	C18:1n-7	2.731	0.012	2.681	0.021	2.721	0.024	2.618	0.018	0.008	*	0.056	ns	0.857	ns
	C20:1n-9	0.131	0.005	0.115	0.008	0.130	0.003	0.112	0.004	0.039	*	0.538	ns	0.758	ns
	C20:3n-9	0.052	0.004	0.042	0.003	0.052	0.003	0.047	0.004	0.041	*	0.486	ns	0.431	ns
	C24:1n-9	0.033	0.004	0.037	0.007	0.033	0.003	0.038	0.003	0.376	ns	0.86	ns	0.931	ns
PUFA	C18:2n-6 LA	0.885	0.024	0.901	0.025	0.842	0.022	0.951	0.031	0.946	ns	0.081	ns	0.016	ns
	C18:3n-6	0.068	0.002	0.085	0.004	0.070	0.005	0.097	0.007	0.017	*	0.767	ns	0.247	ns
	C20:2n-6	0.209	0.010	0.218	0.013	0.221	0.007	0.215	0.008	0.853	ns	0.675	ns	0.441	ns
	C20:3n-6	0.499	0.008	0.550	0.006	0.489	0.005	0.555	0.008	0	*	0.207	ns	0.287	ns
	C20:4n-6 ARA	13.321	0.055	13.346	0.086	13.373	0.034	13.406	0.052	0.616	ns	0.34	ns	0.945	ns
	C22:4n-6 AdrA	2.404	0.025	2.425	0.011	2.479	0.019	2.394	0.035	0.184	ns	0.362	ns	0.032	ns
	C22:5n-6 n6DPA	1.341	0.050	1.255	0.045	1.431	0.043	1.265	0.057	0.097	ns	0.101	ns	0.4	ns
	C18:4n-3	0.027	0.004	0.028	0.002	0.033	0.002	0.023	0.001	0.049	*	0.9		0.033	ns
	C22:5n-3	0.242	0.008	0.284	0.006	0.245	0.006	0.290	0.014	0.003	*	0.947	ns	0.635	ns
	C22:6n-3 DHA	10.810	0.075	10.831	0.095	10.701	0.078	10.698	0.136	0.927	ns	0.213	ns	0.898	ns
	∑SFA	52.286	0.050	52.285	0.151	52.261	0.075	52.408	0.046	0.405	NA	0.576	NA	0.399	NA
	∑MUFA	15.461	0.049	15.468	0.139	15.375	0.069	15.285	0.122	0.842	NA	0.208	NA	0.818	NA
	∑PUFA	29.813	0.050	29.934	0.106	29.898	0.090	29.901	0.140	0.527	NA	0.788	NA	0.549	NA
	∑n6	18.726	0.111	18.786	0.127	18.904	0.056	18.884	0.108	0.96	NA	0.51	NA	0.846	NA
	∑n3	11.087	0.075	11.150	0.093	10.993	0.076	11.019	0.125	0.631	NA	0.227	NA	0.84	NA
	n6/n3	1.690	0.021	1.686	0.022	1.721	0.014	1.716	0.024	0.822	NA	0.151	NA	0.986	NA
	DHA/n6DPA	8.170	0.345	8.721	0.392	7.541	0.247	8.572	0.393	0.081	NA	0.213	NA	0.873	NA

Table 4. Effects of ES on the FA composition (as percentage of total FA levels) in the hippocampus of male and female offspring at P9 (CTL M: 10; ES M: 9; CTL F: 10; ES F: 8). Indicated are means ± SEM, p values and significance after BH correction. For condition effects: blue indicates significantly higher in ES, red indicates significantly lower in ES; for sex effects: blue indicates higher in females, red indicates lower in females; interaction effects are indicated in green. *: significantly different after BH correction, ns: not significant.

		%FA hippocampus													
		CTL M		ES M		CTL F		ES F		P value					
		Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*ES vs CTL*	*BH*	*F vs M*	*BH*	*Int*	*BH*
SFA	C14:0	1.914	0.035	2.242	0.062	1.856	0.026	2.193	0.027	0	*	0.188	ns	0.918	ns
	C16:0	32.496	0.047	32.381	0.044	32.445	0.137	31.923	0.111	0.002	*	0.011	*	0.039	*
	C18:0	17.279	0.042	17.176	0.036	17.224	0.034	17.331	0.104	0.061	ns	0.101	ns	0.006	*
	C20:0	0.06898	0.00572	0.0613	0.00564	0.06848	0.00341	0.0512	0.00907	0.263	ns	0.89	ns	0.552	ns
	C22:0	0.11206	0.03578	0.05805	0.02417	0.06108	0.0194	0.13771	0.03636	0.705	ns	0.631	ns	0.034	*
MUFA	C16:1n-7	1.618	0.009	1.749	0.034	1.610	0.017	1.761	0.049	0	*	0.94	ns	0.727	ns
	C18:1n-9	10.873	0.050	10.761	0.050	10.850	0.027	10.801	0.078	0.129	ns	0.869	ns	0.545	ns
	C18:1n-7	2.547	0.020	2.533	0.022	2.566	0.016	2.541	0.030	0.383	ns	0.54	ns	0.8	ns
	C20:1n-9	0.16365	0.00408	0.15216	0.00615	0.16341	0.00318	0.14113	0.00588	0.001	*	0.252	ns	0.272	ns
	C20:3n-9	0.03754	0.00139	0.03205	0.00264	0.03747	0.00093	0.02288	0.00521	0.001	*	0.101	ns	0.106	ns
	C24:1n-9	0.0334	0.00202	0.03219	0.00485	0.03219	0.0042	0.01217	0.00598	0.019	*	0.019	*	0.037	*
PUFA	C18:2n-6 LA	0.65252	0.01292	0.67897	0.0141	0.65869	0.01222	0.71384	0.02047	0.009	*	0.175	ns	0.339	ns
	C18:3n-6	0.03374	0.003	0.04494	0.0022	0.03609	0.00222	0.03563	0.00543	0.111	ns	0.296	ns	0.085	ns
	C20:2n-6	0.14669	0.02647	0.15214	0.02566	0.16867	0.01865	0.18887	0.0104	0.566	ns	0.193	ns	0.741	ns
	C20:3n-6	0.52148	0.00786	0.55266	0.00489	0.51015	0.00764	0.57248	0.0133	0.01	*	0.259	ns	0.068	ns
	C20:4n-6 ARA	13.825	0.049	13.821	0.041	13.859	0.052	14.091	0.073	0.042	*	0.008	*	0.036	*
	C22:4n-6 AdrA	2.514	0.019	2.513	0.019	2.582	0.031	2.533	0.030	0.591	ns	0.001	*	0.009	*
	C22:5n-6 n6DPA	1.120	0.048	1.063	0.028	1.178	0.033	1.030	0.049	0.099	ns	0.744		0.921	ns
	C18:4n-3	0.02362	0.00527	0.02044	0.00427	0.02311	0.00289	0.02107	0.00542	0.569	ns	0.989	ns	0.901	ns
	C22:5n-3	0.1981	0.0032	0.22759	0.00684	0.20519	0.0052	0.22752	0.01168	0.008	*	0.381	ns	0.687	ns
	C22:6n-3 DHA	11.337	0.072	11.303	0.067	11.330	0.065	11.234	0.093	0.385	ns	0.607	ns	0.674	ns
	∑SFA	51.870	0.057	51.920	0.052	51.654	0.134	51.636	0.107	0.866	NA	0.013	NA	0.724	NA
	∑MUFA	15.276	0.070	15.261	0.084	15.258	0.033	15.279	0.122	0.971	NA	0.998	NA	0.823	NA
	∑PUFA	30.372	0.101	30.379	0.084	30.548	0.104	30.650	0.117	0.598	NA	0.036	NA	0.645	NA
	∑n6	18.814	0.103	18.827	0.062	18.990	0.096	19.165	0.114	0.336	NA	0.012	NA	0.405	NA
	∑n3	11.557	0.074	11.551	0.067	11.558	0.065	11.485	0.095	0.603	NA	0.668	NA	0.658	NA
	n6/n3	1.629	0.017	1.630	0.012	1.644	0.014	1.670	0.020	0.383	NA	0.088	NA	0.428	NA
	DHA/n6DPA	10.297	0.462	10.703	0.290	9.696	0.317	11.062	0.503	0.055	NA	0.826	NA	0.921	NA

Table 5. Effects of ES on the erythrocyte FA composition (as percentage of total FA levels) of male and female offspring at P9 (CTL M: 20; ES M: 18; CTL F: 20; ES F: 16). Indicated are means ± SEM, p values and significance after BH correction. For condition effects: blue indicates significantly higher in ES, red indicates significantly lower in ES; for sex effects: blue indicates higher in females, red indicates lower in females; interaction effects are indicated in green. *: significantly different after BH correction, ns: not significant.

		%FA erythrocytes													
		CTL M		ES M		CTL F		ES F		P value					
		Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*ES vs CTL*	*BH*	*F vs M*	*BH*	*Int*	*BH*
SFA	C14:0	8.287	0.358	7.643	0.369	8.407	0.339	7.103	0.458	0.183	ns	0.925	ns	0.511	ns
	C15:0	0.237	0.022	0.257	0.030	0.213	0.017	0.260	0.022	0.155	ns	0.654	ns	0.559	ns
	C16:0	44.628	0.517	47.580	0.664	43.915	0.592	47.891	0.778	0.005	*	0.672	ns	0.61	ns
	C17:0	0.214	0.009	0.230	0.011	0.191	0.008	0.246	0.011	0.088	ns	0.269	ns	0.13	ns
	C18:0	6.942	0.249	6.731	0.269	6.486	0.258	7.367	0.275	0.646	ns	0.608	ns	0.26	ns
	C20:0	0.251	0.016	0.265	0.021	0.255	0.014	0.283	0.026	0.277	ns	0.582	ns	0.712	ns
	C22:0	0.715	0.039	0.808	0.061	0.647	0.042	0.875	0.064	0.234	ns	0.432	ns	0.269	ns
	C24:0	1.070	0.056	1.271	0.097	0.935	0.065	1.352	0.105	0.094	ns	0.304	ns	0.268	ns
MUFA	C16:1n-7	2.163	0.105	2.168	0.133	2.173	0.115	1.793	0.106	0.588	ns	0.609	ns	0.377	ns
	C18:1n-9	12.747	0.304	12.532	0.463	13.421	0.498	10.625	0.441	0.123	ns	0.577	ns	0.479	ns
	C18:1n-7	3.064	0.056	3.188	0.066	3.104	0.044	3.061	0.110	0.406	ns	0.661	ns	0.643	ns
	C20:1n-9	0.430	0.008	0.433	0.016	0.465	0.015	0.380	0.019	0.3	ns	0.743	ns	0.286	ns
	C20:3n-9	0.099	0.007	0.069	0.006	0.108	0.004	0.078	0.007	0.004	*	0.243	ns	0.86	ns
	C22:1n-9	0.097	0.004	0.122	0.007	0.106	0.005	0.112	0.005	0.006	*	0.925	ns	0.065	ns
	C24:1n-9	1.165	0.063	1.298	0.069	1.062	0.064	1.396	0.096	0.17	ns	0.543	ns	0.286	ns
PUFA	C18:2n-6 LA	9.231	0.273	7.893	0.344	9.097	0.221	8.468	0.468	0.003	*	0.498	ns	0.279	ns
	C18:3n-6	0.116	0.006	0.097	0.005	0.123	0.005	0.111	0.010	0.024	*	0.118	ns	0.646	ns
	C20:2n-6	0.623	0.015	0.554	0.023	0.691	0.016	0.523	0.020	0.017	*	0.073	ns	0.068	ns
	C20:3n-6	0.719	0.025	0.684	0.027	0.761	0.025	0.786	0.046	0.795	ns	0.254	ns	0.69	ns
	C20:4n-6 ARA	2.768	0.167	2.307	0.148	3.123	0.181	3.155	0.236	0.514	ns	0.089	ns	0.766	ns
	C22:4n-6 AdrA	0.300	0.019	0.283	0.018	0.371	0.021	0.309	0.028	0.888	ns	0.015	ns	0.126	ns
	C22:5n-6 n6DPA	0.125	0.011	0.099	0.007	0.157	0.013	0.145	0.018	0.304	ns	0.083	ns	0.928	ns
	C18:3n-3 ALA	0.380	0.013	0.349	0.013	0.379	0.016	0.303	0.021	0.001	*	0.14	ns	0.144	ns
	C20:3n-3	0.077	0.004	0.081	0.004	0.079	0.003	0.084	0.007	0.352	ns	0.576	ns	0.919	ns
	C20:5n-3 EPA	0.062	0.005	0.056	0.005	0.066	0.004	0.070	0.008	0.826	ns	0.108	ns	0.313	ns
	C22:5n-3	0.201	0.015	0.151	0.016	0.240	0.012	0.209	0.023	0.123	ns	0.115	ns	0.861	ns
	C22:6n-3 DHA	0.583	0.042	0.476	0.038	0.637	0.039	0.711	0.061	0.393	ns	0.275	ns	0.232	ns

∑SFA	62.344	0.521	64.783	0.739	61.050	0.695	65.371	0.755	0.018	NA	0.554	NA	0.506	NA
∑MUFA	19.764	0.359	19.808	0.572	20.437	0.534	17.444	0.529	0.195	NA	0.502	NA	0.561	NA
∑PUFA	15.185	0.423	13.029	0.467	15.726	0.334	14.871	0.759	0.031	NA	0.405	NA	0.457	NA
∑n6	13.883	0.381	11.916	0.436	14.323	0.303	13.495	0.684	0.028	NA	0.451	NA	0.463	NA
∑n3	1.303	0.053	1.113	0.048	1.401	0.040	1.375	0.081	0.099	NA	0.208	NA	0.401	NA
n6/n3	10.857	0.345	10.911	0.436	10.286	0.197	9.927	0.278	0.637	NA	0.019	NA	0.525	NA
DHA/n6DPA	4.926	0.360	4.894	0.322	4.365	0.298	5.433	0.455	0.639	NA	0.266	NA	0.337	NA

Table 6. Correlations between milk and offspring FA composition. Indicated are p-values. Blue indicates a significant positive correlation, red a significant negative correlation.

	FA	Milk-Cortex	Milk-Hippocampus	Milk-Erythrocytes
PUFA	C18:2n-6 LA	0.04	0.20	0.05
	C18:3n-6	0.57	0.95	0.08
	C20:2n-6	0.57	0.92	0.00
	C20:3n-6	0.23	0.26	0.31
	C20:4n-6 ARA	0.16	0.11	0.13
	C22:4n-6 AdrA	0.59	0.68	0.53
	C22:5n-6 n6DPA	0.10	0.04	0.07
	C18:3n-3 ALA	NA	NA	0.52
	C18:4n-3	0.15	0.96	NA
	C20:3n-3	NA	NA	0.49
	C20:5n-3 EPA	NA	NA	0.20
	C22:5n-3	0.10	0.33	0.76
	C22:6n-3 DHA	0.92	0.47	0.01
	∑SFA	0.49	0.62	0.00
	∑MUFA	0.06	0.28	0.00
	∑PUFA	0.19	0.32	0.03
	∑n6 PUFA	0.31	0.37	0.02
	∑n3 PUFA	0.42	0.15	0.74
	n6/n3 ratio	0.10	0.00	0.42
	DHA/n6DPA ratio	0.02	0.01	0.01

4. Discussion

Here, we set out to investigate whether PPS in mice alters; i) the milk FA composition and concentration, as well as erythrocyte FA composition of lactating dams; ii) if ES impacts on the brain and erythrocyte FA composition of male and female offspring; and iii) if and how milk and offspring FA status correlate. We show that PPS increased food intake in dams, without affecting bodyweight gain or glucocorticoid levels. Moreover, PPS increased the total FA content in milk, which seems to mostly be driven by an increase in SFAs, but also several n-7, n-3 and n-6 FAs were increased. When looking at the FA composition as a percentage of the total FA levels, PPS lowered the total %PUFA, %n-6PUFA, and %n-3PUFA in milk. In erythrocytes of the dams, similar as reflected in the absolute milk FA concentrations, we observed higher %SFA levels. This was accompanied by lower total %MUFA levels.

In the offspring, the general picture that emerges is that for the cortex, we found ES-induced alterations in the relative abundance of several SFAs and MUFAs, which were more often lower in ES-exposed animals (SFA: 2 down, 1 up; MUFA: 3 down, 1 up in ES), as well as in several PUFAs, which were more often higher in the ES groups (3 up, 1 down in ES). The cortical FA profile was not impacted by sex. For the hippocampus, similarly to the cortex, ES affected the FA composition (SFA: 1 down, 1 up; MUFA: 3 down, 1 up; PUFA: 0 down, 4 up in ES), but interesting sex differences emerged. In fact, ES-exposed females were more often affected compared to their respective controls than males. In addition, in erythrocytes of

offspring, we observed an opposite effect of ES, with a higher abundance of total SFAs, but a lower abundance of total PUFAs. Finally, several individual FAs as well as ratios correlated between milk and offspring brain and erythrocytes.

We will first elaborate on the effects of stress on maternal milk FA concentration and composition, followed by a discussion on the effects of ES in offspring, and the correlations between milk and offspring FA composition.

4.1 Effects of PPS on milk and maternal erythrocyte FA status

PPS did not affect maternal bodyweight gain, water intake or glucocorticoid levels, but increased food intake from P2 to P9. Most studies investigating PPS/ES in rodents focus on the offspring, and, with regard to the dam, only study maternal care behaviour (Naninck et al., 2015; Rice et al., 2008). To the best of our knowledge, only one study characterized the effect of the limited bedding and nesting model on dams at P9 (Ivy et al., 2008). Here it was shown that PPS did not affect bodyweight of the dam, which is in line with our study. However, in contrast to our observations, an increase in plasma glucocorticoid levels and higher adrenal weights were found, as well as increased anxiety-like behaviour in the open field, but not in the elevated plus maze, in PPS dams at P9 (Ivy et al., 2008). While the latter study was conducted in rats and ours in mice, it is unclear which factors contribute to these discrepancies in the effects of PPS on glucocorticoid levels between studies. With regard to food intake, stress exposure in adult mice is shown to either increase or reduce food intake, and this depends on the type and intensity of the stressor (Patterson and Abizaid, 2013). Whereas acute psychogenic and systemic stressors as well as chronic stressors often decrease food intake (Jeong et al., 2013; Martí et al., 1994; Patterson and Abizaid, 2013), repeated psychogenic stressors (e.g. social social defeat stress) may increase food intake (Bhatnagar et al., 2006; Patterson and Abizaid, 2013). PPS thus has comparable effects on food intake of the dam as other social stressors in adult rodents.

With regard to the FA status of the maternal milk, PPS increased the milk FA *concentration*, which was driven by an increase in SFAs, and also led to a lower relative abundance of PUFAs (lower %PUFAs, both %n-6 and %n-3). To the best of our knowledge, no other studies have so far reported on effects of stress on the milk FA (absolute) *concentration*, and only very few studies reported effects of a stress-related exposure on milk FA (relative) *composition* (as % of total FA levels), of which none in mice. While for some scientific questions, the FA composition is most interesting, for example when studying membrane compositions, when considering milk, both *concentration* and *composition* are meaningful, as both can have important implications for the FA intake by the offspring and their development. Due to the lack of rodent studies investigating the milk FA status, we will now mostly focus on studies involving human subjects to contextualize our findings. For example, it had been shown that depressive symptoms during pregnancy before 20 weeks, but not at 24-29 weeks, negatively correlated to milk DHA and total n-3 status, the latter being attenuated after adjustment for covariates including age, education, income, and n-3 intake (Keim et

al., 2012). In contrast, another study found no association between antenatal or postnatal depression and milk long chain PUFA composition (Urech et al., 2020). Unfortunately, these studies did not report on SFAs or MUFAs. The only other study attempted to asses this in mice was performed by our group (Yam et al., 2019), where milk was however, extracted from the stomach of offspring. While no effects of PPS were observed in the stomach-milk FA composition (total %SFA, %MUFA, %PUFA and individual %PUFAs), this partially digested milk might not reflect fresh milk accurately.

Although little is known about the effects of stress on the milk FA status, the influence of maternal diet and health as well as other environmental factors on milk FA status have been described more extensively (Álvarez et al., 2021; Karcz and Królak-Olejnik, 2021; Mäkelä et al., 2013; Miliku et al., 2019; Siziba et al., 2020). For example, it has been shown in human studies that maternal dietary intake affects the FA composition of their milk (Aumeistere et al., 2019; Koletzko et al., 1992; Liu et al., 2016), and that maternal obesity is associated with higher %SFAs, lower %n-3 PUFAs, and a higher n-6/n-3 ratio in milk (Álvarez et al., 2021; Mäkelä et al., 2013). Importantly, weight gain in the children of these mothers correlated with the %SFAs in their breastmilk (Mäkelä et al., 2013). We here find an increased FA *concentration*, driven by an increase in total SFAs, and a relative lower *percentage* of PUFAs (both %n-3 and %n-6, without affecting the n-6/n-3 ratio) in milk of PPS-exposed dams. Providing a high-fat diet rich in SFAs early in life was shown to increase later-life preference for fatty foods (Teegarden et al., 2009), to increase adiposity (Maejima et al., 2020) and to reduce cognition (Noble and Kanoski, 2016) in rodents. A higher SFA concentration and a lower %PUFAs in milk of stressed dams could thus be detrimental for offspring development. To better understand potential consequences of a high milk (S)FA concentration, it would be important to also measure the milk intake of the offspring in future studies.

In erythrocytes of PPS dams, we observed a higher total %SFAs, a lower total %MUFAs, but no effects on %PUFA levels. It has previously been shown in humans that antenatal depression is associated with a lower %n-3 PUFAs and a higher n-6/n-3 ratio in maternal erythrocytes during gestation, while for postpartum depression, no such association with long chain PUFA erythrocyte status was found (Urech et al., 2020). Moreover, another study investigated the plasma and erythrocyte FA *concentrations* in patients with recurrent depression, and while they found increased total FA, SFA, MUFA and PUFA concentrations in plasma of depressed patients, in erythrocytes the total FA and PUFA concentrations were lower, and the SFA and MUFA concentrations were unaffected (Assies et al., 2010). In human studies it is difficult to disentangle effects of stress from other related sociodemographic and environmental influences, such as food availability and dietary choices. Rodent studies can therefore help to better understand if and how PPS exactly alter the maternal erythrocyte FA composition.

Currently, the mechanisms behind the effects of postpartum stress on FA concentration/composition in milk and erythrocytes are poorly understood. Milk lipids can originate directly from dietary FAs in the circulation, from FAs released from the adipose tissue, and from FAs

obtained via de novo lipogenesis or lipid metabolism in the liver and/or mammary gland (Innis, 2007; Rudolph et al., 2007; Suburu et al., 2014). In our study, stressed dams ate more from P2-P9, and thus also consumed more FAs, which could explain the higher FA (absolute) *concentration* in their milk. However, this cannot explain effects of PPS on FA *composition*. In addition, since chronic stress exposure has been shown to affect lipid metabolism in the liver (in 8-week-old mice) (Ha et al., 2003), the option that PPS alters liver lipid metabolism in the dam should be explored. Similarly, whether PPS affects lipid synthesis in the mammary gland (Rudolph et al., 2007; Suburu et al., 2014) is important to investigate. An alternative explanation could be that stress influences only the types of FAs that are utilized. Coping with stress requires energy (Picard et al., 2014), which can be provided by FA oxidation, but not all FAs are oxidized at the same rate (DeLany et al., 2000; Leyton et al., 1987). Although speculative, it is possible that during stress, oxidation of some FAs is preferred over others, resulting in an altered FA status in the dam. This hypothesis, however, requires further investigation. Finally, another aspect that needs more investigation is whether PPS affects milk production/release. In humans, difficulty with breastfeeding is often attributed to stress (Lau, 2001). In a study where milk release was measured indirectly by measuring the weight gain of the pups during a refeeding period (after being separated from the mother first), it was shown that two different stressors (repeated maternal separation and the introduction of a male intruder) reduced milk release (Lau and Simpson, 2004). It is thus possible that the PPS model used in the current study led to reduced milk production.

4.2 ES alters the FA composition in the brain and erythrocytes of offspring

At P9, ES resulted in lower bodyweight gain and glucocorticoid levels in both males and females. A lower bodyweight gain is consistently found with this ES model (Naninck et al., 2015; Rice et al., 2008; Yam et al., 2017). With regard to the glucocorticoid levels, both lower (Moussaoui et al., 2016) and higher levels (Naninck et al., 2015; Rice et al., 2008) have been described directly after ES exposure (P9/P10). At this moment, it is unclear what factors contribute to this discrepancy. In addition, we found ES-induced changes in the brain (both cortex and hippocampus) and erythrocyte FA *composition*. We studied the hippocampus as well as the cortex, since both these regions have been shown to be affected by ES exposure (Naninck et al., 2015; Ohta et al., 2020; Pechtel and Pizzagalli, 2011). In hippocampus and cortex a roughly similar pattern was observed with ES affecting the relative abundance of several SFAs and MUFAs (mostly lower in ES), as well as PUFAs (both n-6 and n-3, mostly higher in ES). This, however, was restricted to individual FAs and not reflected in sum %SFA, %MUFA or %PUFA levels. Moreover, while there were little sex differences in the cortex, in the hippocampus there were more differences between males and females, as well as sex-specific effects of ES. In fact, females were more often affected by ES compared to males. Sex differences in brain FA composition have been described before in half brain homogenates, with females having lower SFA but higher n-6 PUFA levels, similar as to what we found in the

hippocampus (Rodriguez-Navas et al., 2016). However, to the best of our knowledge, we are the first to describe sex-specific effects of ES on the FA composition.

C16:0 is the most abundant SFA in the brain, and the %C16:0 was decreased in the cortex (males and females) and hippocampus (females only) of ES-exposed offspring. SFAs content in the brain decreases during development (Schipper et al., 2016). More specifically, C16:0 decreases during the first 3 weeks of life in mice, whereas C18:0 and its MUFA C18:1n-9 increase in this period (Schipper et al., 2016). Since we observe a decrease in C16:0 but no rise in %C18:0 nor %C18:1n-9, this suggest a FA disbalance at this developmental stage. In the developing brain, SFAs and MUFAs are important for myelin formation and neuronal differentiation (Martínez and Mougan, 1998; Tabernero et al., 2001). Indeed, ES exposure in rhesus monkeys has been shown to reduce white matter integrity (Howell et al., 2013). Brain SFA and MUFA levels were previously shown not to parallel changes in milk composition (Schipper et al., 2016). Indeed, C16:0, C18:0 and C18:1n-9 in the brain are not derived from the diet but rather seem to originate from local *de novo* lipogenesis (Edmond et al., 1998; Marbois et al., 1992). ES-induced changes in SFA and MUFA composition could thus derive from changes in *de novo* lipogenesis. Especially astrocytes are highly active in *de novo* lipogenesis (Camargo et al., 2017; Nieweg et al., 2009), and in line, astrocytes have been found to be affected by ES exposure (Abbink et al., 2020, 2019).

Regarding PUFAs in the brain, the long chain PUFAs arachidonic acid (ARA n-6) and DHA (n-3) are most abundant and rapidly accumulate in the brain in the first 1000 days of human life (Martínez and Mougan, 1998), where they support critical physical and functional membrane properties, among others (Dyall, 2015; Youdim et al., 2000). ARA and DHA can directly be obtained from the diet, or synthesized endogenously from the essential (i.e. need to be obtained from the diet) FAs LA and ALA via multiple elongation and desaturation steps (Rizzi et al., 2013). In fact, ARA and DHA requirements for the developing brain are so high that the endogenous synthesis from LA and ALA is likely not sufficient, and that thus a large portion comes from preformed ARA and DHA in the maternal milk (Brenna and Carlson, 2014; Decsi and Koletzko, 1995; Giovannini et al., 1995).

In the cortex, there were no effects of ES on the percentage of the highly abundant ARA, although the percentage of the intermediate forms C18:3n-6 and C20:3n-6 were increased by ES. In the hippocampus, the %LA and %C20:3n-6 were increased in ES-exposed males and females, and the %ARA was higher specifically in ES females. There were no ES effects on brain %DHA levels, although the low abundant intermediate %C22:5n-3 was higher in the cortex and hippocampus of ES-exposed offspring. The ES effects on %LA are in line with what we have shown previously in male mice at P9 (Yam et al., 2019). However, and in contrast to our current findings, in our earlier study, ES exposure also reduced %ARA, %n-6DPA and %DHA in ES-exposed males (females not investigated). Interestingly, while in our study we found hippocampal FA composition to be more often affected by ES in females, females may be less vulnerable to the effects of ES on hippocampus-based learning and memory deficits

(Bonapersona et al., 2019; Loi et al., 2017; Naninck et al., 2015). It would be interesting to explore whether such effects of ES on FA status in females could be protective.

Compared to the brain, in the erythrocytes of offspring, we observed opposite effects of ES, with higher total %SFAs, but lower total %PUFAs and %n-6PUFAs. Moreover, in line with our previous study, %LA and %ALA were lower in ES-exposed males and females (Yam et al., 2019). The lower erythrocyte %PUFA levels could potentially be explained by a higher brain uptake of these FAs. Since alterations in erythrocyte FA composition have been related to the metabolic syndrome (Kabagambe et al., 2008), psychopathology (Kim et al., 2016), and Alzheimer's disease (Goozee et al., 2017), it is important to further understand if such effects of ES are lasting, as well as if the erythrocyte FA composition could be implicated in the ES-induced disease risk.

4.3 Milk and offspring FA composition correlate

One of the aims of our study was to better understand if the milk FA status translates to alterations in offspring FA composition. For the cortex, %LA negatively correlated, while the DHA/n-6DPA ratio positively correlated with milk FA levels/ratios. Moreover, hippocampal %n-6DPA levels, as well as the n6/n3 ratio and DHA/n-6DPA ratio positively correlated with their levels in the milk. The DHA/n-6DPA ratio is of interest regarding the brain, since n-6DPA is the structural homologue of DHA and usually accumulates in the brain when DHA is low (Carrié et al., 2000). However, while the %n-6DPA levels correlated between milk and hippocampus, no effects of ES were observed on the brain the abundance of either %n-6DPA or %DHA.

Taken together, brain PUFA composition partly correlated to the milk PUFA composition, but no correlations between milk and brain total %SFA or %MUFA were found. This is in line with studies showing brain SFAs and MUFAs are not derived from the diet (Edmond et al., 1998; Marbois et al., 1992). Compared to the brain, erythrocyte FA status more often correlated to the milk FA composition. Total %SFA, %MUFA, %PUFA and %n-6PUFA, as well as several individual FAs and the DHA/n-6DPA ratio correlated with milk FA levels. Indeed, erythrocyte FA composition has been shown to reflect food intake (Hodson et al., 2014). Based on these correlations, it is suggested that stress-induced changes in milk FA status might, at least in part, impact offspring FA status.

As mentioned previously, we found several sex differences in offspring FA composition (predominantly in the hippocampus). It is interesting to note that sex differences in milk composition have also been reported in several species including rhesus macaques and humans (Hinde, 2007, 2009; Powe et al., 2010; Thakkar et al., 2013). In mice, sex differences in milk composition are difficult to study due to their large litters that include both sexes, and therefore this was not taken into account in the current study and correlation analyses. It would however be interesting to explore whether such sex differences in milk composition exist in mice, and if this is involved in the sex differences in offspring FA status.

Although in our study we did not correlate milk (FA) composition to offspring behavioural or metabolic outcome later in life, previous studies have shown such a relationship. For example, total caloric content of milk was associated with child BMI and adiposity (Prentice et al., 2016), milk fatty acid (FA) composition has been related to child temperament (Hahn-Holbrook et al., 2019), and milk chemokine levels affected spatial memory in mice (Liu et al., 2014). Thus, it is possible that stress-induced alterations in milk composition may contribute to the metabolic and cognitive phenotype in offspring (Di Benedetto et al., 2020). As milk FA status is influenced by the maternal diet (Aumeistere et al., 2019; Koletzko et al., 1992; Liu et al., 2016), this provides opportunity for nutritional strategies to supplement mothers and/or offspring to prevent such ES-induced programming. Indeed, we have previously shown in mice that manipulating the n-6/n-3 ratio throughout lactation and adolescence (P2-P42) altered the brain FA profile and prevented ES-induced cognitive deficits of offspring (Yam et al., 2019). These data encourage future testing of such dietary interventions in humans.

In summary, we present evidence for effects of PPS and ES on the milk and offspring FA status, respectively. These data provide avenues for future research on how ES programs offspring for life, as well as for the opportunity of nutritional interventions to prevent the detrimental effects of ES exposure on later-life health.

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CHAPTER 3

3

Effects of early-life stress, postnatal diet modulation and long-term western-style diet on peripheral and central inflammatory markers

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Abstract

Early-life stress (ES) exposure increases the risk of developing obesity. Breastfeeding can markedly decrease this risk, and it is thought that the physical properties of the lipid droplets in human milk contribute to this benefit. A concept infant milk formula (IMF) has been developed that mimics these physical properties of human milk (Nuturis®, N-IMF). Previously, we have shown that N-IMF reduces, while ES increases, western-style diet (WSD)-induced fat accumulation in mice. Peripheral and central inflammation are considered to be important for obesity development. We therefore set out to test the effects of ES, Nuturis® and WSD on adipose tissue inflammatory gene expression and microglia in the arcuate nucleus of the hypothalamus. ES was induced in mice by limiting the nesting and bedding material from postnatal day (P) 2 to P9. Mice were fed a standard IMF (S-IMF) or N-IMF from P16 to P42, followed by a standard diet (STD) or WSD until P230. ES modulated adipose tissue inflammatory gene expression early in life, while N-IMF had lasting effects into adulthood. Centrally, ES led to a higher microglia density and more amoeboid microglia at P9. In adulthood, WSD increased the number of amoeboid microglia, and while ES exposure increased microglia coverage, Nuturis® reduced the numbers of amoeboid microglia upon the WSD challenge. These results highlight the impact of the early environment on central and peripheral inflammatory profiles, which may be key in the vulnerability to develop metabolic derangements later in life.

1. Introduction

Exposure to adversity in the perinatal period increases one's risk of developing metabolic diseases, such as obesity, later in life. Metabolic diseases are increasingly prevalent in modern society. According to the World Health Organization, 50% of people in the European Union are overweight or obese. Moreover, the number of people adopting unhealthy "Western-style" diets has risen (Cordain et al., 2005). These diets are characterized among others by a higher intake of (saturated) fats, and known to contribute to obesity development (Mozaffarian et al., 2011). Understanding the (neuro)biological substrates that increase the risk of developing metabolic diseases is thus important.

There is emerging evidence that the perinatal environment defines the set-point around which bodyweight is regulated later in life [3,4]. Both the brain and adipose tissue rapidly develop in this period, making them sensitive to environmental disturbances [5,6]. Exposure to stress (e.g., physical, sexual or emotional abuse) during the early postnatal period increases the risk of developing obesity and metabolic syndrome later in life [7–9]. Importantly, the metabolic alterations induced by early-life stress (ES) are modulated by later-life diet: diets with a higher fat content during adolescence and adulthood can exacerbate adiposity in rodents exposed to ES induced by either limiting the nesting and bedding material or maternal separation in the early postnatal period [10,11].

Perinatal nutrition also modulates offspring risk of becoming obese, as shown in clinical and preclinical studies [12–15]. Adequate nutrition during development not only provides the necessary energy, but also the required building blocks for the developing offspring. Human milk offers optimal nutrition during development, and breastfeeding has been shown to have many health benefits including a lower risk for childhood obesity, diabetes type 2 and infection [16–20]. Importantly, the effects of breastfeeding on overweight and obesity last into adulthood (Horta et al., 2015), and early nutrition thus appears to "program" aspects of later metabolism.

Human milk contains nutrients as well as other bioactive substances, including hormones and cytokines that may contribute to these health benefits (Mosca and Giannì, 2017). Another important characteristic of human milk is the physical property of its lipid droplets (Spitsberg, 2005). The lipid droplet of human milk is large and covered by a phospholipid trilayer consisting of membrane proteins, phospholipids and cholesterol (milk fat globule membrane; MFGM). In contrast, the lipid droplets that make up regular infant formula (IMF) are much smaller and lack the complexity of the surface area characteristics (Michalski et al., 2005). To resemble these physical properties of human milk more closely, a novel concept IMF was developed (Nuturis®, N-IMF), which contains larger lipid droplets that are surrounded by phospholipids. Interestingly, an early-life nutritional intervention with Nuturis® in mice attenuated Western-style diet (WSD)-induced fat accumulation in adulthood up to 12 weeks of WSD exposure [23–25]. These protective effects disappeared after more prolonged exposure to WSD (27 weeks) (Abbink et al., 2020), as well as upon 18

weeks of exposure to a more severe high-fat diet (HFD, with a higher fat content compared to WSD) (Ronda et al., 2019).

How the early life environment determines energy homeostasis later in life is unknown. Maintenance of energy homeostasis is regulated by a complex interplay between the body and the brain. The orexigenic and anorexigenic neuronal populations in the arcuate nucleus of the hypothalamus are the main brain circuits involved in the regulation of energy intake and expenditure and its structural development continues into early postnatal life in rodents (Bouret and Simerly, 2006). ES and early-life dietary lipid quality have both been shown to alter the structural development of these hypothalamic feeding circuits [28,29], which may possibly contribute to the susceptibility to metabolic disease.

Next to these important hypothalamic neuronal populations and their neuropeptides, the role of peripheral and central inflammation has received increasing attention in the contexts of high-fat feeding and obesity. Obesity results in macrophage accumulation and inflammation in the adipose tissues, and causes a systemic low-grade inflammation, which may contribute to the health risks associated with obesity, like insulin resistance [30–32]. In addition, hypothalamic glia cells, including microglia, the brain's resident immune cells, are involved in body weight homeostasis and obesity [33–35]. Importantly, hypothalamic inflammation and gliosis occur as soon as three days after high-fat diet feeding, and before changes in bodyweight occur, suggesting that hypothalamic inflammation has an important role in diet induced obesity (DIO) (Thaler et al., 2012). Indeed, blocking one of the inflammatory pathways in the hypothalamus attenuated weight gain upon HFD feeding (Benzler et al., 2015), and specifically modifying microglia mitochondrial functions could protect against HFD-induced weight gain (Kim et al., 2019). Hypothalamic inflammation may thus be an essential mechanism contributing to the development of DIO.

Interestingly, there also seems to be a role for (neuro)inflammation in ES-induced programming. For example, systemic inflammation occurs in individuals exposed to early stress [39–42], and perinatal stress lastingly alters microglia in the hippocampus, as well as their response to challenges later in life [43–46]. Whether ES also affects hypothalamic microglia, however, is still unknown.

The mechanisms underlying how ES and postnatal nutrition modulate obesity risk, are currently poorly investigated. Given the reported detrimental effect of ES and the protective effects of Nuturis® on later life metabolic outcomes [10,23–25], we have previously investigated the effects of these environmental manipulations, both independently and in combination, on the metabolic phenotype (Abbink et al., 2020). We did so under standard dietary conditions later in life, as well as upon exposure to WSD. Instead of using the more commonly used HFD exposure for a few days or weeks, we specifically chose a prolonged exposure (27 weeks) to WSD, with a more moderate fat content, to better resemble human dietary exposure. We observed some interesting dynamics in body fat accumulation during (early) adulthood, which appeared to be transient: by the end of the prolonged exposure to WSD, ES and Nuturis® did no longer modulate fat accumulation (Abbink et al., 2020).

Considering the increasing interest in the role of peripheral and central inflammation in metabolic derangements and obesity, we here investigated short and long-term effects of ES and Nuturis® on the adipose tissue and hypothalamic immune profile: (1) directly after ES, i.e., at postnatal day (P)9, (2) in adolescence, i.e., at P42 (adipose tissue only), and (3) in adulthood, both (a) under standard adult diet conditions, as well as (b) in response to a prolonged WSD. We used the same cohort of adult mice as in our previous study (Abbink et al., 2020), which allowed us to relate our current findings to the available detailed characterization of their metabolic phenotype.

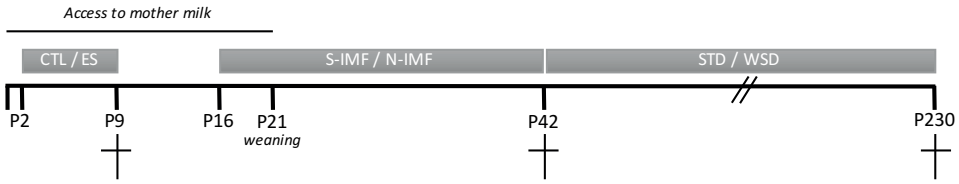

Figure 1. Experimental design. Animals were exposed to control (CTL) or early-life stress (ES) conditions from postnatal day (P)2-P9. Postnatal diet (standard infant milk formula (S-IMF) or Nuturis® IMF (N-IMF)) was provided from P16 to P42, and standard diet (STD) or western-style diet (WSD) from P42 to P230. Animals were sacrificed at either P9, P42, or P230.

2. Materials and Methods

2.1. Animals and breeding

8-week-old female and 6-week-old male C57Bl6j mice were ordered from Envigo (Venray, The Netherlands). Upon arrival, the mice were housed in groups of 3–5, and allowed to acclimatize for 2 weeks prior to breeding under standard housing conditions (type 3 conventional cage, sawdust bedding, paper straw cage enrichment, temperature: 20–22 °C, humidity: 40–60%, standard 12/12 h light/dark schedule (lights on 08:00 a.m.), food and water ad libitum). Breeding was performed in house to standardize the perinatal environment. To allow for mating, one male was housed with two nulliparous females for one week. Females were housed in pairs for another week in a clean cage, after which pregnant females were single-housed in a cage with filtertop equipped with standard bedding and nesting material (type 2 conventional cage, sawdust, one square piece of cotton nesting material (5 × 5 cm; Technilab-BMI, Someren, The Netherlands)). Birth of pups was monitored every 24 h before 09:00 a.m. When litters were born before 09:00 a.m., the previous day was designated as postnatal day (P) 0. Animals were weaned at P21 and housed with same-sex littermates (2–4 per cage). Only male offspring were used for these studies. Figure 1 shows an overview of the experimental groups and design. For P9 adipose tissue experiments, experimental groups were as follows: control (CTL): n = 9 (of 3 litters); ES: n = 7 (of 2 litters). For P9 microglia experiments, experimental groups were as follows: CTL: n = 7 (of 4 litters); ES n = 6 (of 3 litters). Experimental groups for P42 experiments were:

CTL S-IMF: *n* = 7 (of 2 litters); ES S-IMF: *n* = 8 (of 3 litters); CTL N-IMF: *n* = 8 (of 3 litters); ES N-IMF: *n* = 8 (of 3 litters). For studies at P230, experimental groups were as follows: CTL S-IMF standard diet (STD): *n* = 12 (of 4 litters); CTL N-IMF STD: *n* = 12 (of 4 litters); ES S-IMF STD: *n* = 13 (of 3 litters); ES N-IMF STD: *n* = 11 (of 5 litters); CTL S-IMF WSD: *n* = 13 (of 4 litters); CTL N-IMF WSD: *n* = 13 (of 4 litters); ES S-IMF WSD: *n* = 12 (of 4 litters); ES N-IMF WSD: *n* = 12 (of 3 litters). Mice were kept under standard housing conditions.

All experiments were approved by the Animal Experiment Committee of the University of Amsterdam and complied with national legislation and the principles of good laboratory animal care following the European Union (EU) directive 2010/63/EU for the protection of animals used for scientific purposes.

2.2. Early-life stress (ES) paradigm

From P2 to P9, animals were exposed to either control (CTL) or ES conditions. The limited nesting and bedding material model was used to induce ES, as previously described [10,47,48]. In short, on the morning of P2, litters were culled to 6 pups per litter (including both sexes, but when possible optimized for more males), weighted, and randomly assigned to CTL or ES condition. Litters with less than 5 pups or only containing one sex were excluded. CTL litters received standard amounts of sawdust bedding and a square piece of cotton nesting material (5 × 5 cm; Technilab-BMI, Someren, The Netherlands). ES cages were equipped with a fine-gauge stainless steel mesh, which was placed 1 cm above the cage floor. The cage floor was covered with a minimal amount of sawdust and dams received halve of the nesting material (2.5 × 5 cm). Cages were covered with filtertops. The litters were left undisturbed until the morning of P9, when the animals were weighted again and transferred to a clean cage with standard amounts of bedding and nesting material.

2.3. Experimental diets

For the short-term (P9) studies, to investigate acute effects of ES, the diet was standard rodent chow (SDS CRM (P), 801722, 9% energy from fat, 22% energy from protein, 69% energy from carbohydrates). For the adolescent (P42) and adult (P230) studies, diets were semisynthetic (Ssniff-Spezialdiäten GmbH, Soest, Germany) with a macro- and micronutrient composition according to AIN-93-purified diets for laboratory rodents (Reeves et al., 1993). Throughout breeding and until P16, dams were fed the AIN-93G diet (10% energy from fat). Between P16 and P42, litters either received standard infant milk formula diet (S-IMF) or Nuturis® IMF diet (N-IMF) (Oosting et al., 2014). Compared to the S-IMF diet, the lipid droplets in the N-IMF diet have altered physical characteristics (i.e., large, phospholipid-coated lipid droplets), due to altered processing and the addition of bovine MFGM-derived phospholipids. IMF diets consisted of 28.3% fat (fully derived from IMF) complemented with carbohydrates and protein to match the AIN-93G diet. To preserve lipid structure, IMF diets were freshly provided in the form of a dough ball on the cage floor on a daily basis. Until weaning (P21), pups were also able to drink milk from their mother. At P42, mice were switched to either

standard AIN-93M diet (standard diet (STD); 16.7% energy from fat, fully derived from soy) or WSD (39.8% energy from fat, derived from soy (15%) and lard (85%)) until the end of the study (P230). Absolute food intake of STD and WSD was not different across experimental groups, while WSD animals had a higher caloric intake compared to STD animals (Abbink et al., 2020).

2.4. Tissue collection

Adult (P230) animals were fasted for 4 h prior to sacrifice. Animals that were used to study the short-term effects of ES at P9 as well as the long-term effects at P230, were anesthetized via intraperitoneal injection of pentobarbital (120 mg/kg Euthasol®). Next, a piece of adipose tissue was rapidly dissected and immediately frozen on dry ice. More specifically, the inguinal depot at P9 and the gonadal depot at P42 and P230 were used for this study. In fact, at P9, only the subcutaneous inguinal adipose tissue is abundantly present, while visceral adipose tissue depots still need to develop. The gonadal adipose depot belongs to the group of visceral adipose tissues, which are considered more metabolically active and thought to be implicated in metabolic disease (Bjørndal et al., 2011). Afterwards, animals were transcardially perfused with 0.9% saline, followed by perfusion with 4% paraformaldehyde in phosphate buffer (PB 0.1M, pH 7.4). Dissected brains were post-fixed overnight and stored in PB with 0.01% sodium azide at 4 °C until further processing. Prior to slicing, brains were cryoprotected in 30% sucrose in 0.1M PB. Frozen brains were sliced in 40 um thick sections and stored in antifreeze solution (30% Ethylene glycol, 20% Glycerol, 50% 0.05M PBS) at −20 °C. For the adolescent (P42) studies, adipose tissue was quickly isolated after rapid decapitation (no anesthesia used) and immediately frozen on dry ice. Fresh frozen tissue was stored at −80 °C. Notably, the adult (P230) animals are the same animals as used in the study of Abbink et al., 2020, and underwent additional procedures that are not mentioned here (e.g., behavior tests and dual-energy X-ray absorptiometry scans) (Abbink et al., 2020).

2.5. Real-time PCR

RNA was extracted from either inguinal adipose tissue (P9) or gonadal adipose tissue (P42 and P230). In short, adipose tissue was homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA) and samples were centrifuged to remove excessive fat. Chloroform was added, samples were centrifuged and the aqueous phase containing RNA was removed. Clean RNA was obtained with an RNA clean and concentrator kit and DNAse I treatment (ZYMO, Irvine, CA, USA), and stored at −80 °C. Next, cDNA was made with SuperScript II Reverse Transcriptase (Invitrogen), and stored at −20 °C until further use.

Relative expression of genes involved in multiple inflammatory pathways was assessed by RT-PCR performed on an Applied Biosystems 7500 Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Hot FirePol EvaGreen Mastermix (Solis Biodyne, Tartu, Estonia) was used together with 150 nM of gene specific forward and reverse primers and a 0.135-ng/µL cDNA template. All primers (Eurogentec, Seraing, Belgium, Table 1) were tested for efficiency prior to experimental use and accepted when efficiency was between

90 and 110%. Cycling conditions were as follows: 15 min polymerase activation at 95 °C and 40 cycles of replication (15 s at 95 °C, 20 s at 65 °C, and 35 s at 72 °C). Qbase+ software (Biogazelle, Ghent, Belgium) was used for relative quantification of gene expression by the ΔΔCt method. Expression was normalized for two reference genes, which were not affected by experimental conditions and tested for stability in Qbase+.

Table 1. Primers used for RT-PCR.

Gene	Pathway/function	Forward primer (5'-3')	Reverse primer (5'-3')
CCL2	Immune cell infiltration	AGCTGTAGTTTTTGTCACCAAGC	GTGCTGAAGACCTTAGGGCA
CCR2	Immune cell infiltration	AGGGAGACAGCAGATCGAGTG	ACAACCCAACCGAGACCTCTT
F4/80	Macrophage marker	TGTGTCGTGCTGTTCAGAACC	AGGATTCCCGCAATGATGG
NLRP3	Inflammasome	CAGCCAGAGTGGAATGACACG	GCGCGTTCCTGTCCTTGATA
TNFa	Cytokine	GTAGCCCACGTCGTAGCAAAC	AGTTGGTTGTCTTTGAGATCCATG
RPS29	Reference gene	AGTCACCCACGGAAGTTCGG	GTCCAACTTAATGAAGCCTATGTCCTT
RPL19	Reference gene	TTGCCTCTAGTGTCCTCCGC	CTTCCTGATCTGCTGACGGG

2.6. Immunohistochemistry

Hypothalamic microglia were visualized with immunohistochemistry for ionized calcium binding adaptor molecule 1 (Iba1), a marker for microglia/macrophages. Free floating brain slices containing hypothalamus were washed (3 × 5 min) in 0.05 M tris buffered saline (TBS, pH 7.6) before a 15-min incubation in 0.3% H2O2 in TBS to block endogenous peroxidase activity. Next, sections were washed in TBS (3 × 5 min) and blocked in 1% bovine albumin serum (BSA) + 0,3% triton in TBS (blocking mix) for 30 min. Primary antibody incubation occurred in blocking mix at room temperature for 1 h, followed by an overnight incubation at 4 °C (rabbit anti-Iba1, Wako, 019-19741, 1:5000). The next day, sections were washed in TBS + 0.3% triton (3 × 5 min), and incubated in goat anti-rabbit biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA, 1:500) in blocking mix for two hours. After another washing step, sections were incubated in avidin–biotin complex in 0.05 M TBS for 90 min (Vectastain elite ABC- peroxidase kit, Brunschwig Chemie, Basel, Switzerland, 1:800). After washing sections in 0.05 M tris buffer (TB), Iba1 positive cells were visualized with 0.2 mg/mL diaminobenzidine (DAB) and 0.01% H2O2 in 0.05 M TB. Sections were thoroughly washed and mounted on pre-coated glass slides (Superfrost Plus slides, Menzel), dehydrated and cover slipped.

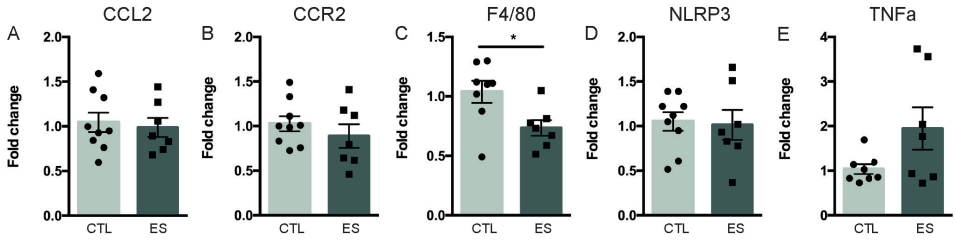

Figure 2. (A–E): effects of ES on adipose tissue inflammatory gene expression at P9. ES decreased expression of F4/80, a marker of macrophages. Data are presented as mean ± standard error of the mean (SEM); * effect of condition; $p < 0.05$.

2.7. Quantification of Iba1 coverage and cell density

Quantification of Iba1 staining was done by an experimenter blinded for condition and diet exposure. Only animals that had intact arcuate nucleus (ARH) after sectioning were used. This resulted in CTL: *n* = 7 and ES: *n* = 6 for P9 studies, and CTL S-IMF STD: *n* = 7; CTL N-IMF STD: *n* = 8; ES S-IMF STD: *n* = 8; ES N-IMF STD: *n* = 7; CTL S-IMF WSD: *n* = 8; CTL N-IMF WSD: *n* = 11; ES S-IMF WSD: *n* = 9; ES N-IMF WSD: *n* = 10 for the adult studies. Iba1 stained tissue was imaged using a 10x objective on a Nikon Eclipse Ni-E microscope using Nikon Elements software. Both sides of the ARH in 2–3 (P9) or 3–4 (P230) different sections per animal were imaged between bregma −1.055 and −1.955, based on the Allen Brain Atlas (2011). A region of interest (ROI) was defined at each side of the ARH, which, depending on the bregma, was placed with either one or two corners close to the third ventricle.

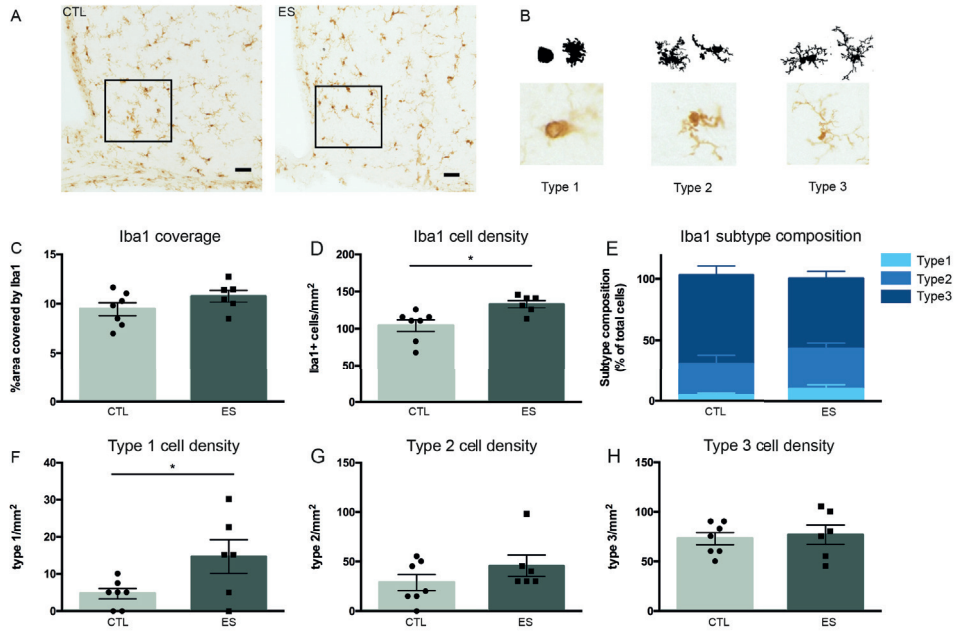

Figure 3. Microglia density is increased by ES at P9. (A): Representative images of Iba1 immunostaining and a defined region of interest (ROI; black square) in the arcuate nucleus of the hypothalamus (ARH). (B): Example illustrations of microglia subtypes. (C): Iba1 coverage is not affected by ES. (D): Iba1 cell density is increased directly after ES exposure. (E): ES does not alter Iba1 subtype composition at P9. (F–H): Iba1 subtype cell densities. ES increased type 1 cell density. Data are presented as mean ± SEM; scale bars 100 µm; * condition effect; $p < 0.05$.

For coverage analysis of both P9 and adult ARH, a fixed threshold was chosen to select stained Iba1 positive cells including their ramifications without selecting background. Next, the percentage of stained area within the ROI was calculated. Within the same ROI, cells were counted manually and divided into three categories based on their morphology (type 1–3). Type 1, amoeboid microglia present a rounded cell body which can have pseudopodia but no long ramifications. Type 2 ramified cells are intermediate forms; they have fewer

ramifications than type 3 cells and their ramifications can be thicker. Type 3 microglia present many thin ramifications (Karperien et al., 2013). The microglia were classified based on careful visual appreciation by a trained and blinded experimenter. In adulthood, microglia morphology is considered to be an indication of their activation: roughly, amoeboid microglia are considered activated while fully ramified microglia are considered surveying [51,52]. However, these morphology-based functional indications should be taken with caution as functional changes can be observed in the absence of detectable morphological alterations [51,52]. In contrast, during development, amoeboid microglia are indicative of a more immature state whereas ramified microglia are indicative of a more mature state [51,53].

2.8. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Data were analyzed using SPSS 25.0 (IBM software), Graphpad Prism 6 (Graphpad software), and considered significant when $p < 0.05$. For statistical analysis of RT-PCR results log transformed values were used. Firstly, the data were tested on outliers with SPSS, and significant outliers were removed from the dataset before proceeding with data analysis. Next, assumptions for parametric analysis were tested. Subsequently, the appropriate statistical test was performed. The general strategy was as follows: for comparisons between CTL and ES conditions at P9, independent t-tests (or a non-parametric alternative) was used. For analyzing the effects of both condition (CTL, ES) and postnatal diet (S-IMF, N-IMF) at P42, 2-way ANOVA was used. For the adult studies, we used the following approach: we first performed an independent t-test (or non-parametric alternative) on CTL S-IMF STD and CTL S-IMF WSD groups allowing us to assess the direct effects of the WSD on peripheral and central inflammation markers under control conditions and place our findings in the context of the current literature on DIO and inflammation [36,38,54–56]. Next, a 2-way ANOVA was performed on either STD or WSD groups (with condition and postnatal diet as predictor variables) to study modulating effects of ES and postnatal diet under standard (STD) and challenged (WSD) circumstances. Post-hoc comparisons were done with Tukey's post-hoc test. Because multiple animals from one litter were included in this study, we always tested for possible contributing effects of litter by performing mixed model analysis with litter as random factor. We did not find effects of litter to any of the assessed parameters.

3. Results

3.1. ES exposure alters the peripheral and central inflammatory profile at P9

We first investigated direct effects of ES exposure on the peripheral and central inflammatory profile. Expression of inflammatory genes in the inguinal adipose tissue was assessed at P9. Expression of CCL2 and CCR2 mRNA, genes important for immune cell infiltration, were unaffected by ES (CCL2: $t(14) = 0.313$, $p = 0.759$; CCR2: $t(14) = 1.153$, $p = 0.268$) (Figure 2A,B). ES reduced the mRNA expression of F4/80, indicative of lower

macrophage numbers (U = 9, Z = −2.199, p = 0.029) (Figure 2C), while NLRP3 expression, involved in the inflammasome, was not affected by ES (t(14) = 0.385, p = 0.706) (Figure 2D), nor was the expression of cytokine TNFα (t(7.7) = −1.753, p = 0.119) (Figure 2E).

Microglia coverage, cell density and subtype composition were determined in a defined ROI in the arcuate nucleus of the hypothalamus (ARH) of control and ES mice at P9 (Figure 3A,B). The area covered by Iba1-positive immunostaining was not affected by ES exposure (t(11) = −1.466, p = 0.171) (Figure 3C). Iba1 cell density however, was increased in the ARH of ES mice (t(11) = −2.969, p = 0.013) (Figure 3D). Moreover, while the relative composition of the different microglia subtypes was not affected by ES (%type1: t(11) = −1.518, p = 0.157; %type2: t(11) = −0.748, p = 0.47; %type3: t(11) = 1.553, p = 0.149) (Figure 3E), ES animals had a higher absolute density of type 1 microglia (t(11) = −2.263, p = 0.045), with the densities of type 2 and type 3 microglia being unaffected (type 2/mm2: U = 14.5, Z = −0.492, p = 0.639; type 3/mm2: t(11) = −0.376, p = 0.714) (Figure 3F–H).

3.2. Effects of early-life stress and postnatal diet on adolescent adipose tissue inflammatory markers

Next, we examined whether ES alters adipose tissue gene expression in adolescence (P42), and whether this is modulated by postnatal diet. Both condition and postnatal diet did not affect expression of CCL2 ($F_{condition}(1) = 0.001$, p = −0.976; $F_{diet}(1) = 0.237$, $p = 0.631$; $F_{condition*diet}(1) = 0.312$, $p = 0.581$), CCR2 ($F_{condition}(1) = 0.123$, $p = 0.728$; $F_{diet}(1) = 1.354$, $p = 0.255$; $F_{condition*diet}(1) = 1.179$, p = 0.288), and F4/80 ($F_{condition}(1) = 0.527$, $p = 0.474$; $F_{diet}(1) = 0.25$, $p = 0.621$; $F_{condition*diet}(1) = 0.011$, p = 0.918) (Figure 4A–C). Postnatal N-IMF diet increased the expression of NLRP3, a component of the inflammasome, with no further modulation by condition ($F_{condition}(1) = 1.367$, $p = 0.255$; $F_{diet}(1)$ = 4.759, $p = 0.04$; $F_{condition*diet}(1) = 0.022$, $p = 0.883$) (Figure 4D), while ES exposure, independent of postnatal diet, increased the expression of the proinflammatory cytokine TNFα ($F_{condition}(1)$ = 5.399, $p = 0.028$; $F_{diet}(1) = 0.201$, $p = 0.658$; $F_{condition*diet}(1) = 0.009$, $p = 0.924$) (Figure 4E).

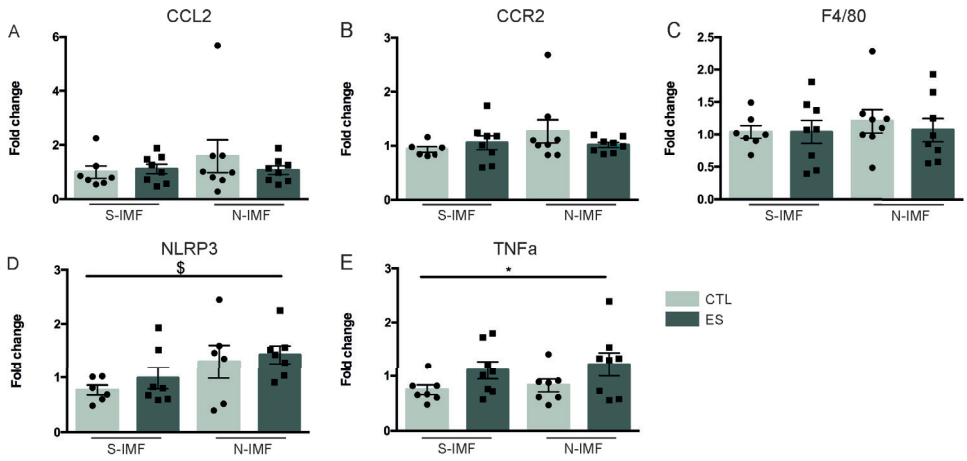

Figure 4. Effects of ES and postnatal diet on adipose tissue inflammatory gene expression at P42. (A–C): ES and postnatal diet do not affect CCL2, CCR2 and F4/80 expression. (D): N-IMF increased NLRP3 expression at P42. (E): ES increases TNFα expression levels. Data are presented as mean ± SEM; * condition effect; $ diet effect; $p < 0.05$.

3.3. Prolonged WSD increases inflammatory markers in adipose tissue and amoeboid microglia numbers in the hypothalamus.

We next questioned whether ES and postnatal Nuturis® feeding (1) have any effects on peripheral and central inflammation in adulthood, and (2) if this is modulated by a long-term western-style diet (WSD). Previous studies reporting inflammation in DIO often used high-fat diet (HFD; 45–60% energy from fat) for several days or weeks. Instead, in our study we chose a long-term WSD, which is more moderate in fat content (39.8% calories from fat) for ±27 weeks, to better mimic human diets. We therefore first studied if long-term exposure to such a diet results in a proinflammatory phenotype. Long-term WSD did not affect adipose tissue expression of CCL2 ($t(21) = -1.714$, $p = 0.101$), nor CCR2 ($t(22) = -1.37$, $p = 0.185$), while it increased the expression of F4/80 ($t(12,546) = -3.105$, $p = 0.009$) (Figure 5A–C). Moreover, NLRP3 expression was unaffected ($t(20) = -0.602$, $p = 0.554$) by WSD exposure, whereas TNFα expression ($t(20) = -2.379$, $p = 0.027$) was increased (Figure 5D,E).

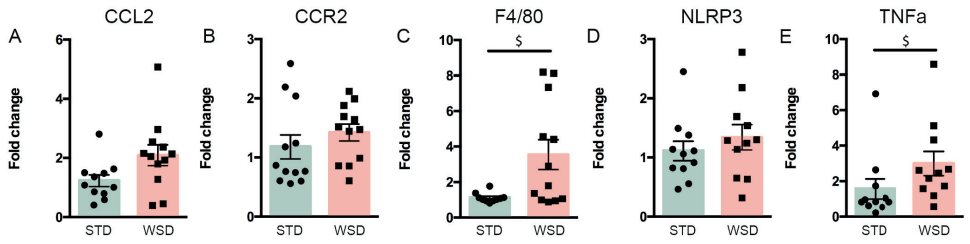

Figure 5. Effects of prolonged WSD exposure on adipose tissue inflammatory gene expression in CTL S-IMF animals at P230. (A,B,D): WSD exposure did not affect CCL2, CCR2 and NLRP3 expression levels. (C,E): WSD exposure increased F4/80 and TNFα expression in the adipose tissue. Data are presented as mean ± SEM; $ diet effect; $p < 0.05$.

In the ARH, the effect of WSD on microglia coverage, cell density and subtype composition was quantified in a defined ROI (Figure 6A,B). WSD did not affect the area covered by Iba1 positive immunostaining, neither did it affect Iba1 positive cell density (coverage: $U = 23$, $Z = -0.579$, $p = 0.613$; cell density $t(13) = -0.379$, $p = 0.711$) (Figure 6C,D). However, WSD did alter microglia subtype composition as shown by relatively higher numbers of type 1 and type 2 microglia, while lowering the percentage of type 3 microglia (%type1: $t(11) = -2.384$, $p = 0.036$; %type2: $t(11) = -3.274$, $p = 0.007$; %type3: $t(11) = 4.804$, $p = 0.001$) (Figure 6E). In line with these observations, type 1 cell density was also increased by WSD (type1/mm^2: $t(12) = -2.261$, $p = 0.043$; type2/mm^2: $t(12) = -2.126$, $p = 0.055$; type3/mm2: $t(12) = 0.996$, $p = 0.339$) (Figure 6F–H).

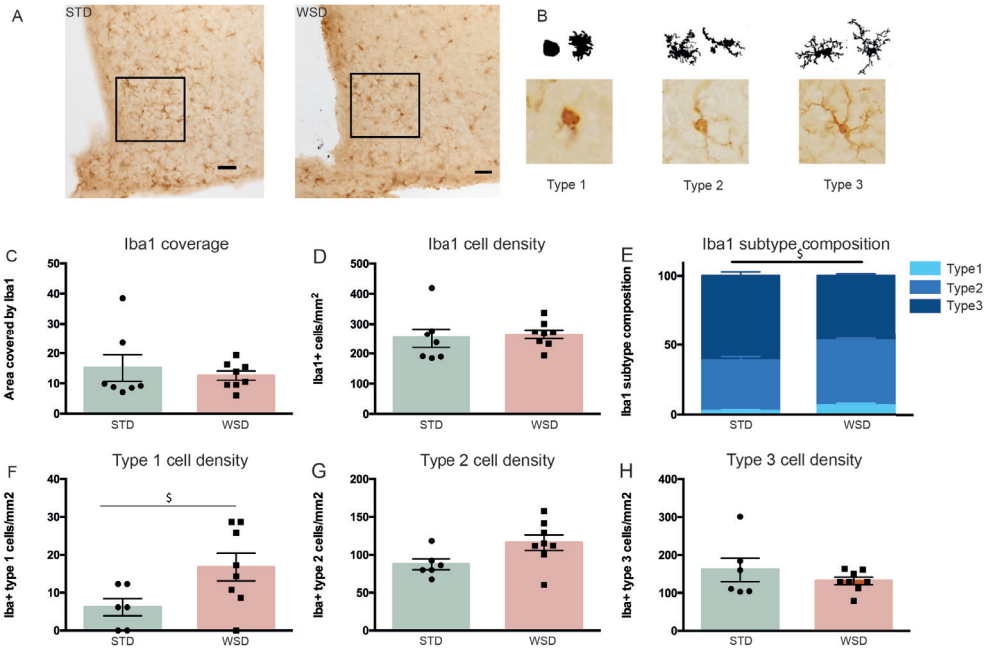

Figure 6. Effects of prolonged WSD exposure on hypothalamic microglia density and coverage. (A): Representative images of Iba1 immunostaining. Iba1 immunostaining was analyzed within a defined ROI (black square) in the ARH of STD- and WSD-exposed CTL S-IMF animals. (B): Example illustrations of microglia subtypes. (C): Iba1 coverage was not affected by WSD. (D): Iba1 cell density was not affected by WSD. (E): WSD altered Iba1 subtype composition at P230 by increasing the percentage of type 1 microglia. (F–H): Iba1 subtype cell densities. WSD increased type 1 cell density. Data are presented as mean ± SEM; scale bars 100 µm; $ diet effect; $p < 0.05$.

3.4. Effects of ES and postnatal diet on adult adipose tissue inflammatory markers and hypothalamic microglia under STD and WSD

Long-term WSD appeared to upregulate the expression of inflammatory markers in the adipose tissue and increase the density of amoeboid microglia in the hypothalamus. We next questioned if and how early-life stress exposure and/or postnatal Nuturis® diet modulate the inflammatory profile on the long-term when exposed to either standard diet (STD) or WSD. On STD, CCL2, CCR2, and F4/80 were not affected by either ES or postnatal Nuturis® diet (CCL2: $F_{condition}(1) = 0.769$, $p = 0.385$; $F_{diet}(1) = 2.773$, $p = 0.103$; $F_{condition*diet}(1) = 1.218$, $p = 0.276$; CCR2: $F_{condition}(1) = 3.703$, $p = 0.061$; $F_{diet}(1) = 2.278$, $p = 0.138$; $F_{condition*diet}(1) = 0.279$, $p = 0.60$; F4/80: $F_{condition}(1) = 0.532$, $p = 0.47$; $F_{diet}(1) = 0.882$, $p = 0.353$; $F_{condition*diet}(1) = 3.546$, $p = 0.067$) (Figure 7A–C). NLRP3 expression levels (inflammasome component) however, were lower in Nuturis® fed animals, without further modulation by condition ($F_{condition}(1) = 3.712$, $p = 0.062$; $F_{diet}(1) = 5.364$, $p = 0.026$; $F_{condition*diet}(1) = 0.619$, $p = 0.436$) (Figure 7D). Expression levels of TNFα were not modulated by either ES or Nuturis® when fed STD ($F_{condition}(1) = 0.48$, $p = 0.493$; $F_{diet}(1) = 0.11$, $p = 0.742$; $F_{condition*diet}(1) = 2.259$, $p = 0.142$) (Figure 7E).

On WSD, neither condition nor postnatal diet affected inflammatory gene expression in the adipose tissue (CCL2: $F_{condition}(1) = 0.611$, $p = 0.439$; $F_{diet}(1) = 0.634$, $p = 0.43$; $F_{condition*diet}(1) = 1.727$, $p = 0.196$; CCR2: $F_{condition}(1) = 0.008$, $p = 0.928$; $F_{diet}(1) = 1.928$, $p = 0.172$; $F_{condition*diet}(1) = 0.959$, $p = 0.333$; F4/80: $F_{condition}(1) = 0.567$, $p = 0.456$; $F_{diet}(1) = 0.995$, $p = 0.324$; $F_{condition*diet}(1) = 0.093$, $p = 0.762$; NLRP3: $F_{condition}(1) = 0.447$, $p = 0.507$; $F_{diet}(1) = 0.1399$, $p = 0.244$; $F_{condition*diet}(1) = 0.191$, p = 0.665; TNFα: $F_{condition}(1) = 0.028$, $p = 0.869$; $F_{diet}(1) = 0.314$, $p = 0.578$; $F_{condition*diet}(1) = 1.243$, $p = 0.271$) (Figure 7F–J).

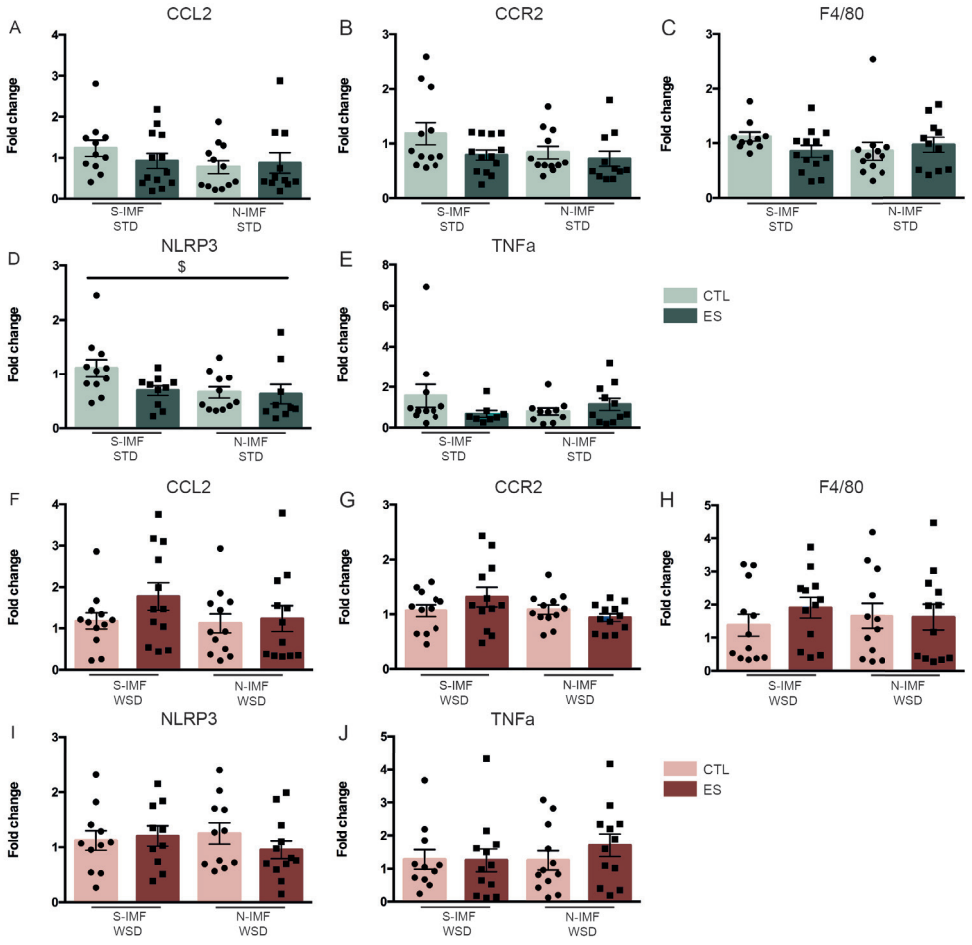

Figure 7. Effects of ES and postnatal diet on expression of inflammatory genes in adulthood, after either STD (A–E) or WSD (F–J). (A,B,C,E): ES nor postnatal diet affected adipose tissue gene expression of CCL2, CCR2, F4/80 or TNFα in adulthood when fed STD. (D): Postnatal N-IMF diet decreased NLRP3 expression at P230, when fed STD in adulthood. (F–J): ES and postnatal diet did not affect expression of CCL2, CCR2, F4/80, NLRP3 and TNFα in the adipose tissue when fed WSD in adulthood. Data are presented as mean ± SEM; $ diet effect; $p < 0.05$.

Finally, we investigated the effects of ES and postnatal diet on microglia coverage, cell density and subtype composition under either STD or WSD conditions, in a defined ROI in the ARH (Figure 8A). On STD, none of the studied hypothalamic microglia parameters were significantly affected by either early-life condition or postnatal diet. The area covered by Iba1 positive immunostaining ($F_{condition}(1) = 0.091$, $p = 0.765$; $F_{diet}(1) = 2.829$, $p = 0.105$; $F_{condition*diet}(1) = 0.19$, $p = 0.666$), nor Iba1-positive cell density ($F_{condition}(1) = 0.041$, $p = 0.841$; $F_{diet}(1) = 0.662$, $p = 0.423$; $F_{condition*diet}(1) = 0.318$, $p = 0.578$) were affected by condition or postnatal diet (Figure 8B,C). In addition, relative subtype composition (%type1: $F_{condition}(1) = 0.156$, $p = 0.696$; $F_{diet}(1) = 0.005$, $p = 0.943$; $F_{condition*diet}(1) = 0.017$, $p = 0.898$; %type2: $F_{condition}(1) = 0.003$, $p = 0.959$; $F_{diet}(1) = 1.791$, $p = 0.193$; $F_{condition*diet}(1) = 0.1941$, $p = 0.176$; %type3 $F_{condition}(1) = 0.038$, $p = 0.847$; $F_{diet}(1) = 1.553$, $p = 0.225$; $F_{condition*diet}(1) = 1.737$, $p = 0.2$) (Figure 8D), and absolute subtype cell densities (type1/mm2: $F_{condition}(1) = 0.177$, $p = 0.677$; $F_{diet}(1) = 0.029$, $p = 0.866$; $F_{condition*diet}(1) = 0.133$, $p = 0.719$; type2/mm2: $F_{condition}(1) = 0.355$, $p = 0.557$; $F_{diet}(1) = 0.091$, $p = 0.765$; $F_{condition*diet}(1) = 0.175$, $p = 0.679$; type3/mm^2 $F_{condition}(1) = 0.061$, $p = 0.808$; $F_{diet}(1) = 2.066$, $p = 0.163$; $F_{condition*diet}(1) = 1.154$, $p = 0.293$) (Figure 8E–G) were unaffected.

As described above (Figure 6), WSD increased type 1 (amoeboid) microglia density in control mice. Animals on WSD that were exposed to ES had increased Iba1 coverage compared to control animals on WSD, which was not further modulated by Nuturis® ($F_{condition}(1) = 7.824$, $p = 0.008$; $F_{diet}(1) = 1.178$, $p = 0.285$; $F_{condition*diet}(1) = 0.072$, $p = 0.79$) (Figure 8H). Total microglia density was not affected by ES or postnatal Nuturis® diet ($F_{condition}(1) = 0.757$, $p = 0.39$; $F_{diet}(1) = 0.153$, $p = 0.698$; $F_{condition*diet}(1) = 0.037$, $p = 0.849$) (Figure 8I), however, postnatal Nuturis® diet reduced the percentage of type 1 cells on WSD ($F_{condition}(1) = 0.208$, $p = 0.651$; $F_{diet}(1) = 6.119$, $p = 0.019$; $F_{condition*diet}(1) = 0.108$, p = 0.745) (Figure 8J). Furthermore, postnatal diet modulated ES-induced effects on relative microglia subtype composition, as shown by interaction effects for both %type2 and %type3 cells (%type2: $F_{condition}(1) = 1.234$, $p = 0.275$; $F_{diet}(1) = 0.926$, $p = 0.343$; $F_{condition*diet}(1) = 5.121$, $p = 0.03$; %type3 $F_{condition}(1) = 1.339$, $p = 0.256$; $F_{diet}(1) = 0.008$, $p = 0.931$; $F_{condition*diet}(1) = 4.531$, $p = 0.041$) (Figure 8J). Tukey post-hoc analysis however revealed no significant differences between individual groups for both the percentage of type 2 and type 3 cells. In accordance with the reduced percentage of type 1 cells in Nuturis® exposed animals, type 1 microglia density was affected by postnatal diet independent of condition ($F_{condition}(1) = 0.017$, $p = 0.897$; $F_{diet}(1) = 4.698$, $p = 0.037$; $F_{condition*diet}(1) = 0.13$, $p = 0.72$) (Figure 8K). Type 2 and type 3 microglia density were not affected by condition or postnatal diet (type2/mm2: $F_{condition}(1) = 0.007$, $p = 0.935$; $F_{diet}(1) = 0.619$, $p = 0.437$; $F_{condition*diet}(1) = 1.378$, $p = 0.249$; type3/mm^2 $F_{condition}(1) = 1.451$, $p = 0.237$; $F_{diet}(1) = 0.528$, $p = 0.472$; $F_{condition*diet}(1) = 2.291$, $p = 0.139$) (Figure 8L,M).

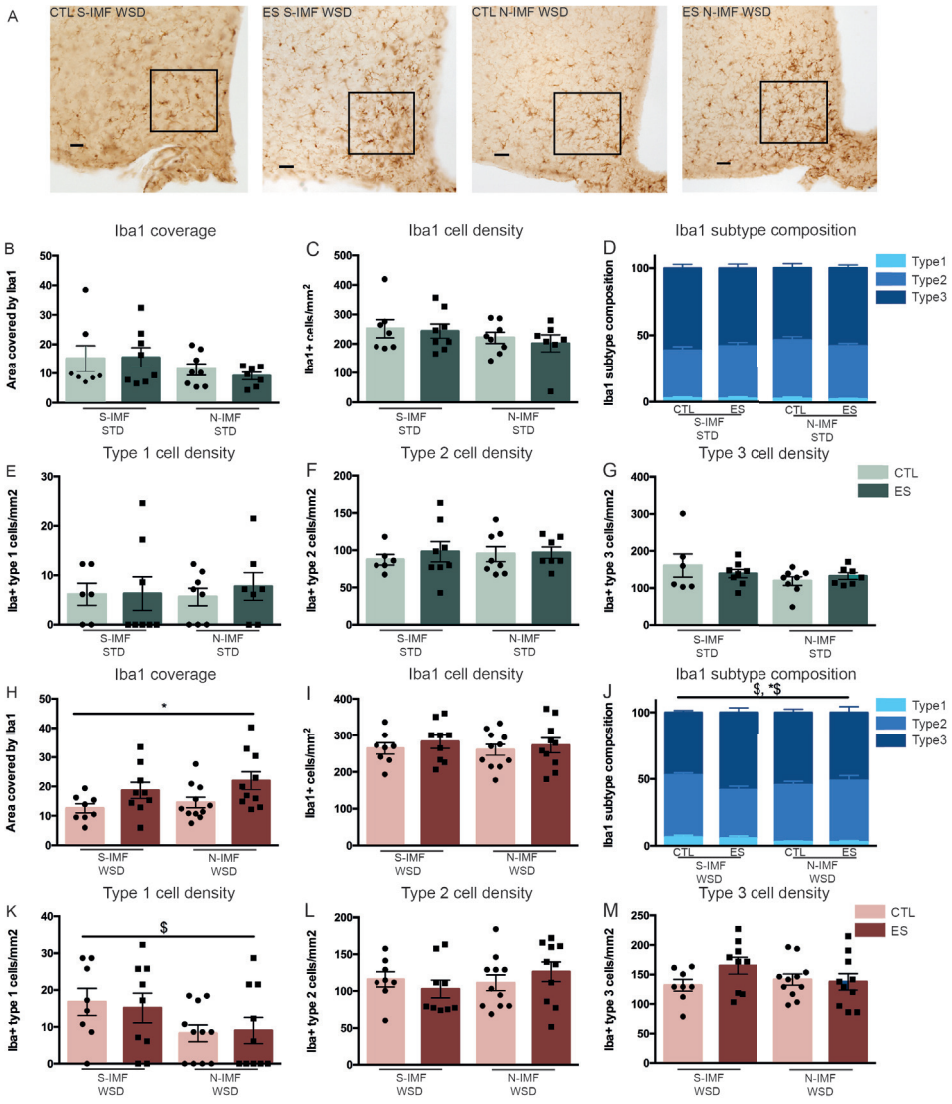

Figure 8. Effects of ES and postnatal diet on hypothalamic microglia at P230, when fed STD (B–G) or WSD (H–M) in adulthood. (A): representative images of Iba1 positive cells in the ARH of animals exposed to WSD. (B–G): On standard adult diet, ES nor postnatal diet affected hypothalamic microglia coverage, density or subtype composition (for different subtypes see Figures 3B and 6B). (H): On WSD, ES exposure increased Iba1 coverage (I): On WSD, ES and postnatal diet did not affect Iba1 cell density. (J): Microglia subtype composition was affected by postnatal diet and ES in animals on WSD (%type 1 diet effect; %type 2 and %type 3 diet*condition effect). (K–M): Type 1 density was decreased by postnatal N-IMF diet. Data are presented as mean ± SEM; scale bars 100 µm; * condition effect; $ postnatal diet effect; *$ condition by postnatal diet effect; $p < 0.05$.

4. Discussion

In the current study, we investigated effects of ES and postnatal diet on peripheral and central inflammatory markers, both in the short-term and into adulthood. We tested these parameters under standard dietary conditions as well as upon exposure to prolonged WSD in adulthood, the latter causing a mild peripheral and central inflammatory phenotype. We show that early in life, both directly after stress exposure as well as in adolescence, ES altered peripheral inflammatory gene expression, and that postnatal Nuturis® diet modulated adipose tissue inflammatory gene expression in an age-dependent manner, independent of ES exposure. In addition, both ES and postnatal Nuturis® diet had a lasting effect on the central inflammatory profile. We further observed increased microglia cell density and more amoeboid microglia in the hypothalamus directly after ES exposure. Interestingly, both ES and Nuturis® modulated the inflammatory profile in adulthood upon prolonged WSD exposure. More specifically, WSD induced an increase in the number of amoeboid microglia, whereas postnatal diet with Nuturis® reduced these numbers following WSD exposure. Furthermore, ES-exposed animals exhibited a different microglia subtype composition in response to WSD.

We will first discuss our findings on the effects of prolonged WSD on peripheral and central inflammation, and thereafter the modulatory effects of both ES and Nuturis®, upon STD and WSD exposure.

4.1. Prolonged WSD exposure leads to a mild inflammatory phenotype in control animals

We earlier reported that prolonged WSD exposure increases fat accumulation (Abbink et al., 2020). In the current study, we show in the same mice that prolonged WSD also affects the peripheral and central inflammatory phenotype. We are the first to describe the inflammatory effects of such a prolonged WSD. An important difference between our study and the available literature is the amount of fat in the diet and the duration of exposure. In DIO studies, HFD is commonly used which contains 45–60% calories from fat, while exposure is typically shorter, i.e., several days to weeks. In our design, however, we used a prolonged (27 week) exposure to WSD, consisting of a more moderate fat content (39.8% energy from fat), which we believe better resembles dietary patterns of obese individuals.

Due to the limited literature with a similar design, we compared our findings to existing literature on the effects of HFD exposure [36,38,54–57]. Concerning peripheral inflammation, we observed an increase in the expression of the macrophage marker F4/80 and the cytokine TNFα in the adipose tissue, with no effects on the expression of the other examined inflammatory genes (CCL2, CCR2 and NLRP3) after WSD. Indeed, obesity and the expansion of adipose tissue have been described to increase M1 “classically activated” macrophage infiltration as well as the production of pro-inflammatory cytokines [30,58,59], which is in line with the increased F4/80 and TNFα expression upon WSD in our current

study. In addition, in contrast to our observation, HFD has been described to increase the expression of the cytokine CCL2 [55,56]. CCL2 guides monocytes from the circulation to become tissue macrophages, which is the first step in the initiation of inflammation [60,61]. Although speculative, it is possible that due to the more prolonged nature of our (moderate) WSD exposure, after 27 weeks, new inflammatory cells are no longer recruited to the adipose tissue.

Concerning the central inflammatory profile, we observed a WSD-induced increase in amoeboid microglia numbers in the hypothalamus, with no effects on total microglia numbers or Iba1 coverage. Amoeboid types of microglia are considered to be activated, they secrete pro- and anti-inflammatory cytokines, and are thought to have a high phagocytic capacity for clearing debris and apoptotic cells (Fu et al., 2014). Thus, the WSD-induced increase in the numbers of these amoeboid/activated microglia in the ARH would suggest an inflammatory phenotype. However, these conclusions are for now only based on morphological analyses of microglia. To gain further understanding of the potential functional alterations in microglia and the inflammatory state within the brain in response to WSD, it will be important to also test cytokine levels in the hypothalamus in the future. While this is in line with the direction of effects in response to HFD, an exposure to HFD for several days up to 16 weeks, a shorter period compared to our prolonged WSD, appears to have more marked effects on hypothalamic inflammation, including increases in microglia density and size [36,38,57].

Thus, even though the WSD clearly had some inflammatory effects, the differences in study design (fat percentage in diet, length of exposure, specifics of the control diet (Morrison et al., 2020)) may explain some discrepancies with earlier literature. This highlights the importance of comparing different dietary fat percentages and durations of exposure with respect to the inflammatory processes they trigger, and how these may contribute to obesity.

4.2. Effects of ES and Nuturis® on peripheral inflammation under STD and prolonged WSD exposure

ES leads to a reduction in F4/80 expression in the adipose tissue at P9, indicative of lower macrophage numbers. As shown in our previous study with these same animals, ES at this age also leads to a decrease in bodyweight (Abbink et al., 2020), which was accompanied before by a reduction in fat mass (Yam et al., 2017a). It is intriguing to speculate about the biological mediators responsible for these changes in the adipose tissue inflammatory gene expression profile. Glucocorticoids (GCs) are a plausible candidate. In fact, ES leads to an increase in circulating GCs at P9 [47,48] and adipose tissue abundantly expresses the glucocorticoid receptor, which is not affected by ES (Yam et al., 2017a). GCs have been described to alter lipid metabolism, regulate adipogenesis, and increase central fat accumulation [64–67]. In addition, GCs are widely known for their anti-inflammatory capacity (Miyata et al., 2015). GCs inhibit adipocyte-induced macrophage recruitment in

vitro, and dexamethasone (a synthetic GC) can prevent high-fat diet induced macrophage infiltration (Patsouris et al., 2009), which is further in line with our findings.

In adolescence (P42), ES induced a transient increase in TNFα expression in adipose tissue, which normalized in adulthood (P230). Exposure to ES has been shown to also increase circulating TNFα levels in adolescence (do Prado et al., 2016), and we now show similar effects at the level of the adipose tissue. Moreover, regarding adipose tissue gene expression, prolonged WSD did not affect CTL and ES animals differently. As reported earlier, we neither observed effects of ES on adiposity, at either P42 or P230 after STD and WSD exposures (Abbink et al., 2020). One option is that the prolonged (±27 week) WSD challenge could possibly have overruled any subtle effects of ES in a similar way as it overruled ES effects on adiposity, which we observed after 8 weeks of WSD (Yam et al., 2017a). However, to the best of our knowledge, we are the first to study the effects of ES on adipose tissue inflammatory gene expression after a high-caloric diet, and there are no studies looking at shorter dietary interventions. As ES increases the risk of developing obesity, it will be key to understand in more detail how the adipose tissue responds to dietary challenges that contribute to adverse health risks.

Nuturis® caused an initial increase in the expression of the inflammasome sensor NLRP3 in the adipose tissue in adolescence (P42), while decreasing its expression in adulthood (P230) under STD conditions, suggesting an age dependent and long-lasting effect of the postnatal diet. No such lasting effects were found after prolonged WSD. Inflammasomes are cytosolic multiprotein complexes that are part of the innate immune system, and stimulate the cleavage and secretion of cytokines. NLRP3 is an inflammasome sensor molecule that triggers the assembly of inflammasomes and is critical for their activation (Mangan et al., 2018). Alterations in NLRP3 expression levels may directly affect inflammasome levels, potentially changing the levels of cytokine released (Bauernfeind et al., 2009). Importantly, NLRP3 expression in the adipose tissue has previously been associated with obesity-induced inflammation and insulin resistance in obese individuals, and diminishing NLRP3 levels can protect against insulin resistance in DIO mice (Vandanmagsar et al., 2011). This suggests that the Nuturis®-induced lower expression of NLRP3 in adulthood may provide some protective effects on insulin sensitivity on STD, which however now seems to be overruled by prolonged WSD. This is in line with earlier findings from our group showing that Nuturis® lowered insulin resistance when fed an STD, but not a WSD [24,25]. NLRP3 expression may thus provide a mechanistic link between Nuturis® and the earlier observed improved insulin sensitivity. How Nuturis® might program NLRP3 expression needs to be further investigated. A possible hypothesis is that lipid metabolism may play a role; fatty acids have been shown to provide a priming signal for NLRP3 in vitro and after HFD feeding in mice [73–75], and Nuturis® lastingly alters the plasma levels of certain lipids, including cholesterol and triglycerides (Oosting et al., 2012).

4.3. ES and Nuturis® lastingly affect hypothalamic microglia under STD and prolonged WSD exposure

We observed increased microglia cell density and an increase in amoeboid microglia in the arcuate nucleus of the hypothalamus directly after ES exposure at P9. Hypothalamic development continues into at least the first two weeks of postnatal life in rodents (Bouret and Simerly, 2006) and the microglia population expands and develops rapidly during this period [76–79]. Microglia change their morphology during development, which is commonly used to examine their maturity (Schwarz et al., 2012). The amoeboid morphology suggests a more immature state and is in line with findings by us and others on the effects of ES in the hippocampus [43,45]. Moreover, the increased microglia density at P9 might be explained by a GC-triggered increase in microglia proliferation. Indeed, it has been shown that elevated GC levels, induced by several days of restraint stress, can stimulate microglia proliferation directly after the stress exposure (Nair and Bonneau, 2006). However, opposite effects on microglia proliferation after only 24 h of in vitro GC exposure have also been described (Nakatani et al., 2012). It is thus possible that the length of exposure to elevated GCs determines the observed effects on microglia proliferation.

In adult animals, we did not observe any ES effects on microglia under basal (STD) circumstances. However, when exposed to WSD, ES-exposed animals showed increased microglia coverage in the ARH of the hypothalamus. Because of the absence of significant differences in microglia subtypes between experimental groups, understanding the source of this increased coverage as well as its functional implication needs further investigation. However, in line with our finding, such programming effects of perinatal stress on microglia in response to later-life inflammatory challenges (e.g., Alzheimer pathology and bacterial infection) have been observed before both in the hippocampus and prefrontal cortex [43,44,82]. The literature on the effects of early adversity on hypothalamic microglia is sparse, and to the best of our knowledge, we are the first to show that ES also primes the hypothalamic microglia response to a WSD. Programming of hypothalamic microglia by early adversity has been described in a study concerning overfeeding: neonatally overfed rats showed increased microglia cell numbers and coverage when challenged with a bacterial infection (Ziko et al., 2014). We here describe a similar altered hypothalamic microglia response to a WSD challenge after ES exposure.

Interestingly, while prolonged WSD increased the number of amoeboid microglia, postnatal diet with Nuturis® consistently reduced these numbers when fed WSD, independent of previous ES exposure. Moreover, Nuturis® seemed to, in interaction with ES, modulate relative microglia subtype composition, although post-hoc analysis revealed no significant differences between individual groups. Nonetheless, these findings suggest Nuturis® programs microglia and their response to later-life challenges. It is remarkable that a relatively subtle dietary intervention was able to modulate the microglial profile more than 6 months after the diet intervention was ended. Nuturis® may impact microglia in multiple ways. The altered physical structure of lipids in Nuturis® affects digestion and absorption

kinetics and, as a result, the bioavailability of lipids essential for brain development (Bourlieu and Michalski, 2015). Moreover, lipids are widely known for their capacity to modulate inflammation [85–87]. Both the type and quantity of fatty acids is known to affect microglia physiology directly as well as indirectly via hormones and the gut (Leyrolle et al., 2019). For example, gangliosides, which are present in Nuturis®, have been shown to have anti-inflammatory effects in microglia activated with lipopolysaccharide (Galleguillos et al., 2020).

It is intriguing to speculate that Nuturis® could modulate adipose tissue accumulation at least partly via microglia modulation. It is not clear yet how microglia could regulate fat accumulation, but one study showed that blocking microglia activation prevents HFD-induced fat accumulation and triggers the hypothalamic neurons that inhibit food intake, linking neuroinflammation to the neuronal populations that regulate energy homeostasis (Kim et al., 2019). Indeed, blocking microglia activation led to increased energy expenditure and decreased food intake, preventing HFD-induced weight gain. Nuturis® has been shown to modulate WSD-induced adiposity with WSD exposure up to 12 weeks [24,25], which was no longer present after a prolonged WSD exposure (27 weeks) (Abbink et al., 2020), nor after 18 weeks of HFD exposure (Ronda et al., 2019). Further studies need to elucidate the complex involvement of hypothalamic microglia in the modulation of adipose tissue accumulation, and their modulation by ES and Nuturis®.

A limitation of our study is the lack of inclusion of female mice. As mentioned earlier, this study is the follow up of a large investigation, which encompassed both cognitive and metabolic readouts (Abbink et al., 2020). This original study was designed to include males as we had shown previously that the used ES model affects cognitive function and neurogenesis primarily in males (Naninck et al., 2015), and also the reported effects of N-IMF on WSD-induced obesity have been studied in male mice [24,25], unfortunately limiting our possibility to include females in this study. However, there is increasing clinical and preclinical evidence that there are sex differences in the response to ES (Bonapersona et al., 2019; Boynton-Jarrett et al., 2010; Hay et al., 2008; Murphy et al., 2018, 2016), early-life nutritional challenges (Dearden et al., 2018), adipose tissue distribution and function (Fuente-Martín et al., 2013), as well as in microglia [97–99], indicating the importance of studying the differential effects of ES and dietary manipulations in both males and females in future experiments.

5. Conclusions

Both ES and postnatal diet with Nuturis® modulate peripheral and central inflammation. Even after prolonged WSD exposure that led to an obesogenic phenotype in all animals, both ES and Nuturis® modulated markers of central inflammation. While more research is needed for a complete understanding, it is tempting to hypothesize that such effects

might be involved in the earlier reported effects of both ES and Nuturis® on adiposity and metabolic derangements [10,24,25]. These findings highlight the importance of the early-life environment on later life (metabolic) health.

6. Patents

The Nuturis intervention tested is a concept that is protected by several patents, all filed before the start of the experiments reported in this study.

Conflicts of Interest: This study was co-funded by Danone Nutricia Research (DNR). L.S. is employed by DNR and E.M.v.d.B. was employed by DNR at the time of conducting of the study. L.S. contributed to the design of the study, the interpretation of the data and the writing of the manuscript. S.R.R., M.R.A., J.G., J.E.K., N.S., P.J.L. and A.K. declare no conflict of interest.

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CHAPTER 4

4

Adult food choices depend on sex and exposure to early-life stress: underlying brain circuitry, adipose tissue adaptations and metabolic responses

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Abstract

Exposure to early-life stress (ES) increases the risk to develop obesity later in life, and these effects may be sex-specific, but it is currently unknown what underlies the ES-induced metabolic vulnerability. We have previously shown that ES leads to a leaner phenotype under standard chow diet conditions, but to increased fat accumulation when exposed to an unhealthy obesogenic diet. However these diets were fed without a choice. An important, yet under investigated, element contributing to the development of obesity in humans is the choice of the food. There is initial evidence that ES leads to altered food choices but a thorough testing on how ES affects the choice of both the fat and sugar component, and if this is similar in males and females, is currently missing. We hypothesized that ES increases the choice for unhealthy foods, while it at the same time also affects the response to such a diet. In a mouse model for ES, in which mice are exposed to limited nesting and bedding material from postnatal day (P)2-P9, we investigated if ES exposure affected i) food choice with a free choice high-fat high-sugar diet (fcHFHS), ii) the response to such a diet, iii) the brain circuits that regulate food intake and food reward and iv) if such ES effects are sex-specific. We show that there are sex differences in food choice under basal circumstances, and that ES increases fat intake in females when exposed to a mild acute stressor. Moreover, ES impacts the physiologic response to the fcHFHS and the brain circuits regulating food intake in sex-specific manner. Our data highlight sex-specific effects of ES on metabolic functioning and food choice.

1. Introduction

Metabolic diseases are increasingly common in the modern society in which high caloric foods are abundant and readily available (Malik et al., 2013). In 2016, worldwide 39% of adults were overweight and 13% were obese (World Health Organization, 2018). Thus, it is key to understand which factors contribute to the development of obesity. The vulnerability to become overweight or obese is heavily influenced by the perinatal environment, during which a metabolic setpoint is likely established (Bouret, 2009; Bouret and Simerly, 2006; Levin, 2006). There is evidence that exposure to early-life adversity increases the risk to develop metabolic diseases, including obesity and diabetes, later in life (Alciati et al., 2013; Balsevich et al., 2019; Bellis et al., 2015; Danese and Tan, 2014) and these effects are potentially sex specific (Boynton-Jarrett et al., 2010; Kozyrskyj et al., 2011; Murphy et al., 2018; Park et al., 2018). Early-life stress (ES) exposure affects many children worldwide. For example, in the United States, it is estimated that 61% of adults experienced some form of ES (e.g. abuse, neglect, parental separation, poverty) (Merrick et al., 2018). It is therefore urgently needed to understand how ES impacts metabolic vulnerability in order to develop strategies to prevent and reduce the incidence of metabolic disorders later in life. Such an early-life-induced setpoint might determine how an organism will respond to its later life nutritional environment, but possibly also the food choices an individual makes in the first place.

To understand ES-induced metabolic vulnerability, it is important to include a metabolic challenge, like the exposure to an unhealthy diet later in life (Maniam et al., 2014; Maniam and Morris, 2010; Murphy et al., 2018; Panetta et al., 2017; Yam et al., 2017a). For example, we have previously reported that, while ES exposure followed by a (healthy) standard chow diet later in life resulted in a leaner phenotype, a western-style diet (WSD) exposure increased adipose tissue accumulation in ES animals compared to controls (Yam et al., 2017a), suggesting an increased vulnerability to such an unhealthy diet. Dietary exposure studies most often use a design with an *ad libitum* availability of the obesogenic diet, without any option for a choice. While such an experimental design allows to study the physiological response to an unhealthy diet, this deviates from the human condition where individuals can make food choices. In fact, a few studies suggest that ES may increase the preference for unhealthy foods, possibly further contributing to the increased metabolic vulnerability. For example, child maltreatment was shown to be a strong predictor of obesogenic food consumption in young children (Jackson and Vaughn, 2019), and prenatal exposure to the Dutch famine has been associated with increased preference for fatty foods in adulthood (Lussana et al., 2008). Moreover, in a study on female rats, ES exposure increased preference for palatable chow, containing both increased fat and sugar levels, over standard chow (Machado et al., 2013), giving a first indication of a direct relationship between ES exposure and food choice, rather than only being circumstantial (i.e. children exposed to ES may more often grow up in poverty situations and/or in environments with

higher availability to unhealthy foods (Conroy et al., 2010; Smoyer-Tomic et al., 2008)). Thus, a lot remains to be understood about how ES increases metabolic risk and whether there indeed is a role for food choice.

The regulation of energy homeostasis (e.g. energy intake and expenditure) and food choice is regulated by a complex interplay between the periphery and the brain (Timper and Brüning, 2017). Peripherally, the adipose tissue has a crucial role in energy homeostasis. Besides storing lipids, the white adipose tissue (WAT) is also an active endocrine organ, which secretes adipokines including leptin. In obesity, WAT expands by both increasing adipocyte numbers as well as cell size. Rapid expansion associated with weight gain is accompanied by local inflammation and insulin resistance (Mraz and Haluzik, 2014; Ouchi et al., 2011). Adipose tissue dysfunction is considered to be a determinant metabolic complications associated with obesity (Longo et al., 2019). Interestingly, we have shown before that ES induced by limiting the bedding and nesting material (LBN) affects WAT inflammatory and adipokine gene expression (Ruigrok et al., 2021; Yam et al., 2017a). Centrally, homeostatic as well as reward neural circuitry are at play. The hypothalamus is involved in the homeostatic regulation of food intake, and integrates signals from hormones (e.g. leptin, insulin, ghrelin) and nutrients (e.g. glucose, fatty acids) to determine energy availability (Lam et al., 2005; Timper and Brüning, 2017). The arcuate nucleus of the hypothalamus contains two main neuronal cell populations that do so: agouti-related protein (AgRP) and neuropeptide Y (NPY) expressing neurons which stimulate food intake, and pro-opiomelanocortin (POMC) expressing neurons that inhibit food intake. The mesolimbic pathway is involved in the rewarding aspects of food intake, with dopamine signalling in the ventral tegmental area (VTA) being central to this pathway. For food intake, the connection between the VTA and the striatum including the nucleus accumbens (NAcc) is most studied (Volkow et al., 2011), and it has been shown that palatable foods such as fat and sugar increase dopamine release within the NAcc (De Macedo et al., 2016). While the homeostatic and reward circuits are often studied separately, they are actually integrated at several levels including a connection between the hypothalamus and the VTA (Dietrich et al., 2012; Hsu et al., 2018). It has been shown that AgRP neurons innervate the VTA via GABAergic connections, and AgRP has been shown to function as a reward circuit setpoint by affecting dopamine cell excitability and dopamine levels (Dietrich et al., 2012). Indeed, ablating hypothalamic AgRP increases palatable food intake, and feeding responses in AgRP-ablated mice depended on dopamine tone (Denis et al., 2015). In fact, AgRP ablation has been proposed to create a context favourable of comfort eating in which feeding is less driven by energy demand and more sensitive to stress and reward (Denis et al., 2015). Interestingly, we have earlier reported that ES (LNB model) altered hypothalamic AgRP fiber density early in life (Yam et al., 2017b), and ES induced by maternal separation has been linked to exacerbated food-motivated behaviour, and blunted dopamine release in the NAcc upon consumption of palatable foods (Romaní-Pérez et al., 2016), suggesting that both these circuits might be at play in the effects of ES on food choice and metabolism.

When investigating food choice in rodents, it is important to mimic human dietary choices as much as possible. For such studies, often the fatty and sugar components are combined in the chow, however we have shown earlier that a free choice high-fat high-sugar diet (fcHFHS) consisting of a choice among a solid fatty component (100% beef tallow), a sugary drink (10% sucrose water), a nutritionally complete chow and water, causes prolonged hyperphagia, snacking behaviour and increased food-motivated behaviour in rodents, simulating more closely what is observed in human obesity (la Fleur et al., 2010; La Fleur et al., 2014). In the current study, we set out to test whether ES affects i) food choice using a fcHFHS paradigm, ii) the metabolic response (i.e. adipose tissue mass and gene expression) to such a diet, as well as iii) the underlying brain circuits (i.e. the hypothalamus and VTA), and iv) whether these measures are different in males and females. We show that food choice and the metabolic response to the fcHFHS are different for males and females, that ES exposure affects food choice upon a mild stressor (in the form of tail cuts and 4h fasting) as well as the metabolic response to the diet in a sex-specific way, and provide evidence that the brain circuits that regulate hedonic food intake are affected by previous ES exposure.

2. Materials and methods

2.1 Mice and breeding

For these studies, 96 experimental animals (CTL M STD: n=10; ES M STD: n=8; CTL F STD: n=10; ES F STD: n=13; CTL M HFHS: n=15; ES M HFHS: n=11; CTL F HFHS: n=13; and ES F HFHS: n=15) were used, originating from 18 litters (9 CTL, 9 ES), each containing five-six pups. Animals were kept under standard housing conditions (temperature 20–22C, 40–60% humidity, 12/12h light/dark schedule). Standard chow and water *ad libitum* were provided, unless noted otherwise. Animals were weaned at postnatal day 21, and group-housed until the dietary choice experiment started. All experimental procedures were conducted under national law and European Union directives on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam.

Experimental animals were bred in house to standardize the perinatal environment. Eight-ten week old C57Bl/6J female and male mice were purchased from Invigo Laboratories B.V. (Venray, The Netherlands). After habituation for one-two weeks, two primiparous females were housed together with one adult male to allow for mating, for one week. Females were housed together for another week and were given nesting material (square piece of cotton) to practice. Afterwards, females were housed individually in a standard cage with filtertop and new nesting material, and placed in a ventilated cabinet to provide a standardized and quiet environment. Starting from 18 days after the breeding, females were checked each morning before 09:00 A.M. When a litter was born, the previous day was determined as postnatal day 0.

2.2 Early-life stress paradigm

Early-life stress was induced by providing limited nesting and bedding material from postnatal day(P) 2 to P9, as described previously (Naninck et al., 2015; Rice et al., 2008). To avoid differences in maternal care due to litter size, large litters were culled to 6, and no litters of less than 5 pups were included. Control cages had a standard amount of sawdust and one square piece of cotton nesting material (5x3x5 cm, Techninlab-BMI, Someren, The Netherlands). ES cages consisted of a little amount of sawdust on the bottom, covered with a fine-gauge stainless steel mesh, and half a square piece of cotton nesting material (2.5x3x3 cm). All cages were covered with a filtertop. At P2 and P9 the pups, dams, and food were weighted. At P9, all litters were moved to new cages containing standard amounts of sawdust, and were left undisturbed until weaning at P21.

2.3 Bodyweight measurements

Bodyweight (BW) was measured throughout development and diet exposure. At P2, P9, P21, P35 BW was assessed in group-housed animals. BW gain from P2 to P9 was calculated per litter for each sex, by subtracting the average P2 BW from the average P9 BW of all male and female pups respectively. From P63 onwards, animals were individually housed and weighted weekly until the end of the experiment.

2.4 Food choice

To allow for adequate food intake measurements, animals were housed individually at 9 weeks of age. After one week of acclimatization, animals were randomly divided into the free choice high-fat high-sugar diet (fcHFHS) group, or control chow group. The control group had *ad libitum* access to regular chow (CRM (P), 801722, Standard Diets Services, Essex, United Kingdom, 3.585 kcal/g, where: 22% protein, 9% fat, and 69% carbohydrates) and tap water. The fcHFHS group had *ad libitum* access to four different components: regular chow; a bottle of tap water; pellets of beef fat (beef tallow, Vandemooretele, France, 9 kcal/g); 10% sugar water (0.4 kcal/mL) for 5 weeks (fig. 1). For basal measurements, food intake and body weight was measured on a weekly basis. In addition, food intake was measured in the 24h following a mild stressor composite of the following disturbances: a 4 hour fasting period at the start of the light period and two tail cuts (see below). This intrinsic aspect of our experimental design allowed us to assess the effects of such an exposure on food choice. Fasting is considered stressful and increases glucocorticoid levels in mice (Champy et al., 2004). While generally mice tend to eat mostly during the night, after several weeks of HFHS diet exposure, mice eat equal amounts of fat and sugar during the day and night (Blancas-Velazquez et al., 2018), thus the fasting during the light period as performed in our experiment does disrupt food intake patterns of animals on a HFHS diet and thereby can be considered a stressor in combination with the two tail cuts. We measured food intake in the 24 hours prior and following this event.

2.5 Blood collection and fasting

Blood (30-80 μl) was collected via tail cuts at multiple time points by making a small incision at the base of the tail to measure either corticosterone (CORT) or glucose levels (see fig. 1A). Blood was drawn (without restraint) within 1 minute after removing the mouse from its cage. For plasma CORT measurements, blood was collected between 08:00 and 09:00 A.M from *ad libitum* fed mice. After this first tail cut, animals were fasted for 4 hours to measure blood glucose levels (blood drawn between 12:00 and 01:00 P.M). In the 24 hours following the exposure to this fast and tail cuts, food intake was measured.

2.6 Glucose and corticosterone measurements

Blood was collected in EDTA-coated tubes (Sarstedt, The Netherlands), and centrifuged at 13000 rpm for 15 min. Plasma was stored at -40°C. CORT levels were measured with a radioimmunoassay kit (MP Biomedicals, The Netherlands). Glucose was measured using a FreeStyle Optium Neo meter (Abbott Laboratories, Abbott Park, IL, USA).

2.7 Tissue collection

After five weeks of diet exposure, at 15 weeks of age, animals were sacrificed to study the adipose tissue and brain. Mice were anaesthetized by an IP injection of pentobarbital (Euthasol 120 mg/kg), and gonadal white adipose tissue was quickly weighted, snap-frozen, and stored at -80°C. Afterwards, mice were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB, 0.1M, pH 7.4). Brains were post-fixed in 4% PFA overnight at 4°C, and stored in PB with 0.01% azide (at 4°C) until slicing. Brains were cryoprotected with sequentially 15% and 30% sucrose solutions, sliced in 40 μm thick coronal sections, and stored in antifreeze (30% ethylene glycol, 20% glycerol, 50% 0.05M PBS) at -20°C.

2.8 Real-time PCR

To obtain RNA, adipose tissue was homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA) and samples were centrifuged to remove excessive fat. After the addition of chloroform (Sigma Aldrich, Saint Louis, MO, USA) and more centrifuging, the RNA appeared in the upper (aqueous) phase. Next, a RNA clean and concentrator kit with DNAse I treatment (ZYMO Research, Irvine, CA, USA) was used to obtain clean RNA samples. RNA was stored at -80 ˚C until cDNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). cDNA was stored at -20 ˚C until further use. Relative gene expression was assessed by RT-PCR performed on a QuantStudio(TM) 6 Flex System (Thermo Fisher Scientific, Waltham, MA, USA). Hot FirePol EvaGreen Mastermix (Solis Biodyne, Tartu, Estonia), 150 nM of gene specific forward and reverse primers and 0.135 ng/μl cDNA template were added to the reaction mix. Primers (Eurogentec, Liege, Belgium, table 1) all had an efficiency between 90-110%. Cycling conditions were as follows: 15 min polymerase activation at 95°C and 40 cycles of replication (15 s at 95°C, 20 s at 65°C, and 35 s at 72°C). ΔΔCt method was used to

calculate relative gene expression, and was performed in Qbase+ software (Biogazelle, Gent, Belgium). Expression was normalized for two reference genes, which were not affected by experimental conditions and tested for stability in Qbase+.

Gene	*Pathway/function*	*Forward primer (5'-3')*	*Reverse primer (5'-3')*
CD36	Fatty acid uptake	GCAAAGAACAGCAGCAAAATC	CAGTGAAGGCTCAAAGATGG
FABP4	Fatty acid binding protein	GAAATCACCGCAGACGACAG	ATAACACATTCCACCACCAGC
FASN	Fatty acid synthesis	GCGCTCCTCGCTTGTCGTCT	TAGAGCCCAGCCTTCCATCTCCTG
Leptin	Anorexigenic adipokine	AGCTGCAAGGTGCAAGAAGAA	CCTGGACTTTCTGGATAGGCA
PPARg	Fatty acid storage and glucose metabolism	GTCTCACAATGCCATCAGGTT	CAAATGCTTTGCCAGGGCTC
CANX	Reference gene	AGAGCTCAGCCTGGATCAATTC	TTGTAGTCCTCTCCACACTTATCTG
RPL19	Reference gene	TTGCCTCTAGTGTCCTCCGC	CTTCCTGATCTGCTGACGGG

2.9 Fluorescent immunohistochemistry

To study the brain circuits relevant for food intake, brain slices were immuno-stained with agouti-related protein (AgRP), tyrosine hydroxylase (TH), and glutamate decarboxylase 65 (GAD65). First, free-floating brain slices were washed (3x10 min) with 0.05M tris-buffered saline (TBS, pH 7.6). Slices were incubated in 3% bovine serum albumin (BSA) and 0.3% Triton-X100 in 0.05M TBS (blocking buffer) for one hour, and subsequently incubated in a primary antibody solution containing goat anti-AgRP (Neuromics, Edina, MN, USA, GT15023, 1:1000), rabbit anti-TH (Pel-Freez, Rogers, AR, USA, P40101-150, 1:1000), and mouse anti-GAD65 (Abcam, Cambridge, UK, 26113, 1:2500) in blocking buffer, for 24 hours at room temperature (RT). Following another series of washes in TBS (3x10 min), sections were again incubated in blocking buffer for two hours, followed by an incubation with secondary antibodies overnight at 4°C (donkey-α-rabbit 488, Invitrogen A21206; donkey-α-goat 568, Invitrogen A11057; donkey-α-mouse 647, Invitrogen A31571, all 1:500, in blocking buffer). Sections were washed and mounted with a DAPI-containing mounting solution. Negative controls containing no primary antibody in the blocking buffer (but a similar treatment otherwise) were taken along to confirm the specificity of the labelling.

2.10 Confocal microscopy and analysis

Analysis was done by a researcher blind to experimental conditions. All pictures were taken using a Nikon A1 confocal microscope. For analysis of hypothalamic AgRP, 4 pictures with a 20x objective were taken throughout the arcuate nucleus of the hypothalamus (ARH), between bregma -1.055 and -1.955 (based on Allen Brain Atlas, 2011). For analysis of AgRP in the ventral tegmental area (VTA), also four pictures were obtained with a 20x objective, between bregma -2.48 and -3.68. To obtain a proper representation of the staining throughout each brain section, each picture consisted of a Z-stack with 20 individual photos with a distance of 1 μm, and to achieve a correct representation of the whole brain region (either ARH or VTA), the imaged brain sections had an approximate similar intersection

distance. AgRP rapidly diffuses from the cell body into the fibers, and therefore only AgRP fibers were quantified. Fiber density was determined with a thresholding method within a defined region of interest using ImageJ software (National Institutes of Health, Bethesda, MD, USA). First, Z-stacks were collapsed with the sum projection method. Subsequently, a threshold was determined selecting only AgRP positive staining. Finally, for each animal, the obtained area covered by AgRP positive staining was averaged over the different sections, for either the ARH or VTA.

For analysis of TH^+ cells in the VTA, 4 pictures (Z-stacks) were taken with a 20x objective, similar as described above, and TH^+ cell bodies were counted in a defined ROI within the VTA. Next, the average number of TH^+ cells per μm^2 over the different pictures was calculated for each animal.

To study presynaptic GABA terminals (GAD65) on dopaminergic cells in the VTA, 4 pictures were taken between bregma -2.88 and -3.38 with a 60x oil objective. Each picture consisted of a Z-stack with 13 individual photo's, with a distance of 1 μm. In these pictures, both GAD65 punctae on TH^+ cell bodies as well as on TH^+ fibers were counted. For counting of GAD65 punctae on cell bodies, all cell bodies that were well visible and not touched by other cell bodies were counted (±13 cells per animal) on two different Z planes with 2 μm between them to not count the same punctae double. GAD65 punctae were counted when they were directly touching the cell bodies. The counts of both Z planes of each cell body were added together, and next, an average of all cell bodies was calculated for each animal. To quantify the GAD65 punctae on fibers, two ROIs in each picture were chosen that did contain TH^+ fibers, but no TH^+ cell bodies. Similar as for the cell bodies, this was done for 2 different Z planes, with 2 μm between them. In these ROIs, first the area covered by TH^+ staining was quantified using a set threshold. Next, all punctae touching or directly on top of fibers were counted, and the number of punctae per μm^2 fiber was calculated for each animal.

2.11 Estrous cycle determination

Estrous cycle sampling was performed by gently rubbing an öse along the ventral/rostral side of the vagina directly after a tail cut or when females were anesthetized (before sacrificing) to minimize extra handling. Vaginal swabs were then transferred to a glass slide (with a drop of water), and stained with Giemsa for 10 minutes followed by several washed with water. Determination of the estrous cycle stage was done with a light microscope and performed by a trained researcher blinded for experimental conditions.

2.12 Statistical analysis

Data were analysed with SPSS 25.0 (IBM software, Armonk, NY, USA), Graphpad Prism 6 (Graphpad software, San Diego, CA, USA) and R Studio 1.2.1335 (R Core Team, 2018). All data are presented as mean $\pm$ standard error of the mean (SEM). When $p<0.05$, data was considered statistically significant. For statistical analysis of RT-PCR results log transformed

values were used. Firstly, outlier analysis was performed in SPSS, and values that were outside the 1st quartile – 3*interquartile range or 3rd quartile + 3*interquartile range were excluded. Depending on the measure, this resulted in the exclusion of 0-5 outliers per analysis. For the intake of the different dietary components (chow, fat and sugar), outliers were identified and removed for each individual component (but not for the total caloric intake). If an animal was outlier for one of the components, it was also removed for the total caloric intake measure. For the relative caloric intake of each component (as a percentage of total intake), when an animal was outlier for one of the intakes, that animal was excluded from the analyses of the relative intake of all the other components as well. Because of a priori expectations of sex differences in metabolism as well as in response to ES (Fuente-Martín et al., 2013; Murphy et al., 2017; Naninck et al., 2015), all data were analysed with sex as independent variable. Data with condition (CTL/ES) and sex as predictor variables were analysed with a 2-way or repeated measures 2-way ANOVA. When a 2-way interaction between condition and sex was found, post hoc pairwise comparisons with Bonferroni correction were performed. Data with condition, sex and diet as predicted variables were analysed with a 3-way or repeated measures 3-way ANOVA. When a 3-way interaction between condition, diet and sex was found, the interaction was further explored with simple 2-way interactions, followed by pairwise comparisons with Bonferroni correction. When in a 3-ANOVA, a 2-way interaction was found, because this suggested no contribution of the third predictor to the interaction, we did not explore these 2-way interaction further with pairwise comparisons between each individual experimental group. As multiple mice from one litter were included in these experiments, data are considered nested. We therefore tested for contributing effects of litter and corrected when necessary by performing mixed model analysis with litter as random factor. To test if estrous cycle influenced the outcome variables across the groups including female mice, we tested for contributing effect of estrous cycle and corrected with mixed model analysis with estrous cycle as random factor when necessary, however estrous cycle did not influence any of the outcome variables.

Correlation plots exclusively showing the significant correlations were generated in R studio 1.2.1335 (R Core Team, 2018) for each individual experimental group using the ggcorrplot package. Pearson correlations were calculated based on complete pairwise cases, and correlation coefficients were tested against critical values on a two-tailed distribution (alpha=0.05).

3. Results

3.1 Effects of ES on bodyweight

Figure 1A depicts the experimental overview. Animals were exposed to either CTL or ES conditions between P2 and P9, and fed a STD or fcHFHS from P70 to P105. At P104 animals were exposed to a 4h period of fasting and 2 tail cuts (mild stress exposure). Bodyweight

(BW) was measured throughout the experiment, and during the dietary exposure, food intake was measured on a weekly basis as well as in the 24h after the mild stress exposure.

ES exposure reduced bodyweight (BW) gain from P2 to P9 in males and females ($F_{condition}(1, 30)=16.291$, $p<0.001$) (Fig. 1B). In addition, BW development depended on both condition ($F_{time*condition}(1.945, 175.075)=6.973$, $p=0.002$) and sex ($F_{sex}(1, 90)=532.483$, $p<0.001$; $F_{time*sex}(1.945, 175.075)=363.617$, $p<0.001$) (Fig. 1C). Further analysis revealed that at P21, ES reduced BW ($F_{condition}(1, 30.335)=4.404$, $p=0.044$) independent of sex. However, these ES effects normalized at P35. In addition, males had a higher BW compared to females at P35 ($F_{sex}(1, 92)=350.971$, $p<0.001$), P63 ($F_{sex}(1, 91)=608.652$, $p<0.001$), and P70 ($F_{sex}(1, 92)=607.92$, $p<0.001$).

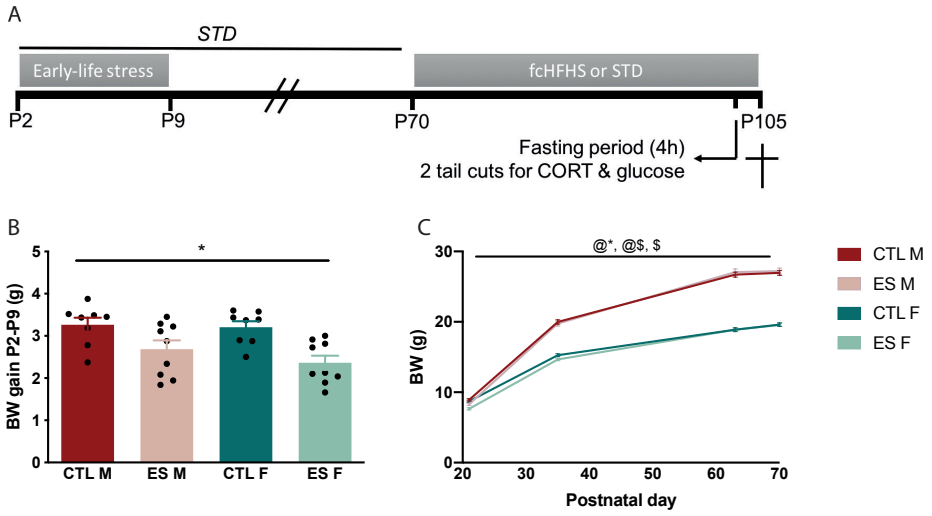

Figure 1. A) Experimental overview. Male and female mice were exposed to CTL and ES conditions from P2 to P9, and at 10 weeks of age either fed a standard diet (STD) or free choice high-fat high-sugar choice diet (fcHFHS) for 5 weeks. Body weight and food intake was measured weekly. At the end of the 5th week, blood was taken via a tail cut to measure basal CORT levels. Next, animals were fasted for 4 hours and blood was taken again to measure basal glucose levels. Food intake was measured after this manipulation. B) BW gain from P2-P9 is lower in ES exposed animals. C) BW development from P21 to P70. BW increases with age, and is affected by ES exposure (P21), and sex (P35-P70). Indicated is mean ± SEM, p<0.05. * main effect of condition; @ main effect of time; $ main effect of sex; @* time by condition interaction effect; @$ time by sex interaction effect.

3.2 Males and females have different food choice

To investigate whether ES and sex affect caloric intake and/or food choice, food intake was measured in CTL and ES-exposed male and female mice on a weekly basis during the 5-week dietary exposure. Food intake was affected in the following manner: *total* caloric intake was affected by time, diet and sex ($F_{sex}(1, 66)=6.999$, $p=0.01$; $F_{diet}(1, 66)=84.572$, $p<0.001$; $F_{time*diet*sex}(3.032, 200.132)=2.734$, $p<0.001$). Mice on STD had lower caloric intake compared to animals on fcHFHS, and fcHFHS fed mice exhibited increased intake over time with males having a higher intake compared to females (Fig 2A). Kcal *chow* intake over the

5-week period was higher in STD versus fcHFHS fed animals independent of sex (F_{diet}(1, 77)=2466.161, p<0.001; $F_{time*diet}$(3.022, 232.693)=4.456, p=0.004) (Fig. 2B). Moreover, across the fcHFHS fed groups, males ate more chow compared to females ($F_{sex*diet}$(1, 77)=27.507, p<0.001). Interestingly, while in both sexes, *fat* and *sugar* intake increased over time (fat: F_{time}(2.606, 130.295)=10.544, p<0.001; sugar: F_{time}(2.629, 107.728)=20.774, p<0.001), females had more kcal intake from fat compared to males (F_{sex}(1, 50)=23.167, p<0.001) (Fig. 2C), while males had a higher kcal sugar intake compared to females (F_{sex}(1, 41)=5.099, p=0.029) (Fig. 2D). ES exposure did not affect chow, fat or sugar intake over the 5-week period in either sex. Water intake over the 5 week period was affected by time, sex, diet and condition ($F_{time*diet*sex}$(3.023, 220.678)=3.333, p=0.02; $F_{condition*sex*diet}$(1, 73)=5.235, p=0.025) (Fig 2E). Water intake was higher in animals on STD diet compared to those on HFHS diet, and females drank more water compared to males, but this depended on ES exposure and diet.

In addition, we analysed the *percentage* kcal intake for each diet component based on their average total intake over the 5-week period, specifically for the animals exposed to the fcHFHS (Fig. 2F). Males took relatively more kcal from chow compared to females (F_{sex}(1, 51)=187.234, p<0.001), while females took relatively more kcal from fat compared to males (F_{sex}(1, 51)=42.647, p<0.001) (Fig. 2F). ES did not modulate choice for any of the components.

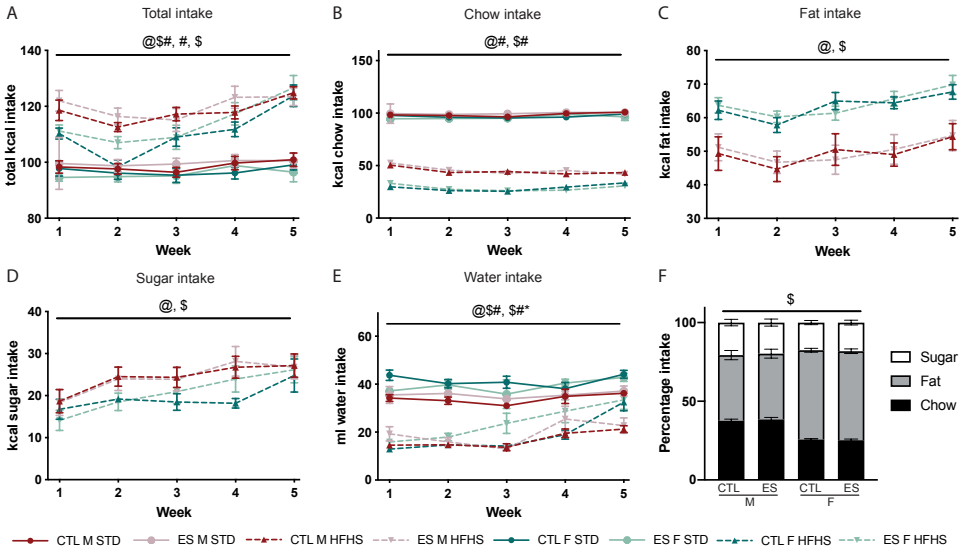

Figure 2. Food intake during the 5-week exposure to either STD or fcHFHS. Shown are kcal intake per week of each component. A) total kcal intake per week is affected by diet and sex. B) kcal intake from chow per week is affected by diet and sex. C) Kcal fat intake is higher in females. D) Kcal intake from sugar is higher in males. E) Water intake per week is higher affected by sex and diet. F) The relative intake of chow and fat as a percentage of total caloric intake is affected by sex. Indicated is mean ± SEM, p<0.05. @ main effect of time; # main effect of diet; $ main effect of sex; @# time by diet interaction effect; @$ time by sex interaction effect; #$ diet by sex interaction effect; @$# time by sex by diet interaction effect.

3.3 ES-exposed females increase fat intake after a mild stressor (4h fast and 2 tail cuts)

Palatable foods are considered comforting, and acute stress exposure alters the intake of such foods (Dallman et al., 2004; Rutters et al., 2009). Our experimental design offered the opportunity to test the effects of a mild stressor in the form of a 4h fast and 2 tail cuts during the 5th week of dietary exposure on food choice. First, as a baseline measurement, we analysed caloric intake in the 24 hours directly *before* the mild stressor (Fig. S1). In the 24h prior to the mild stressor, total kcal intake per day was higher in animals exposed to the fcHFHS ($F_{diet}(1, 78)=21.153$, $p<0.001$), and not affected by condition or sex (Fig. S1A). Kcal chow intake per day was lower in the fcHFHS groups, and lower in females when compared to males ($F_{diet}(1, 86)=498.061$, $p<0.001$; $F_{sex}(1, 86)=7.49$, $p=0.008$) (Fig. S1B). Fat intake was, as expected, higher in females but not affected by ES exposure ($F_{sex}(1, 50)=4.234$, $p=0.045$) (Fig. S1C). Kcal sugar intake per 24 hours was not affected by sex or condition (Fig S1D). Thus, ES exposure did not affect intake of any of the components, nor total intake, before the exposure to this mild stress. Subsequently, we analysed the caloric intake of the different components in the 24h *after* the mild stressor. Total caloric intake was higher in the fcHFHS groups ($F_{diet}(1, 82)=63.826$, $p=<0.001$) (Fig. 3A), while chow intake was lower in the fcHFHS groups ($F_{diet}(1, 83)=567.452$, $p<0.001$) (Fig. 3B). Both total and chow intake were unaffected by condition or sex or the interaction of these predictor variables. Fat intake was affected by sex depending on previous ES exposure ($F_{sex*condition}(1, 50)=4.043$, $p=0.05$) (Fig. 3C). Post hoc analysis revealed that specifically ES-exposed females showed increased fat intake compared to CTL females ($p=0.049$), whereas no such ES effects were observed in males ($p=0.39$). Moreover, ES females ate more fat than ES males ($p=0.005$). Sugar intake was not affected by sex or ES exposure (Fig. 3D).

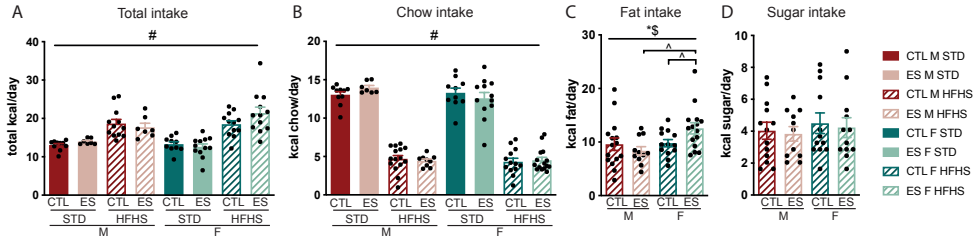

Figure 3. Food intake after mild stress exposure A) Total caloric intake is higher in animals exposed to fcHFHS. B) kcal chow intake is lower in the fcHFHS groups. C) Kcal fat intake is higher in ES exposed females. D) Kcal sugar intake is not affected by sex or condition. Indicated is mean ± SEM, p<0.05. # main effect of diet; $ main effect of sex; *$ condition by sex interaction effect; ^ significant Bonferroni post hoc comparison between experimental groups.

3.4 ES exposure affects bodyweight gain and adiposity in a sex-dependent manner

Next, we investigated the effects of STD and fcHFHS on BW gain and adiposity (Fig. 4). After 5 weeks of diet exposure, BW gain was higher in fcHFHS fed animals compared to STD fed animals, and while BW gain on STD was higher in females compared to males, BW gain on fcHFHS was higher in males than in females ($F_{diet*sex}(1, 86)=6.66$, p=0.012) (Fig. 4A). ES exposure also affected BW gain depending on sex, so that females exposed to ES had lower BW gain compared to CTL females, while this was not the case in males ($F_{condition*sex}(1, 86)=6.931$, p=0.01). When adjusting for sex differences in body size by taking the BW gain as a percentage of their BW at the start of diet exposure, it was shown that the fcHFHS increased %BW gain ($F_{diet}(1, 86)=40.138$, p<0.001), and that ES reduced the %BW gain in females independent of diet ($F_{condition*sex}(1, 86)=8.538$, p=0.004).

gWAT levels (as percentage of BW) were lower in females compared to males, but increased in fcHFHS fed mice in both sexes ($F_{sex*diet}(1, 84)=21.563$, p<0.001) (Fig. 4C). In addition, there was an interaction between ES exposure and sex in their effect on gWAT levels ($F_{condition*sex}(1, 84)=6.121$, p=0.015) indicating that ES decreased gWAT levels in females.

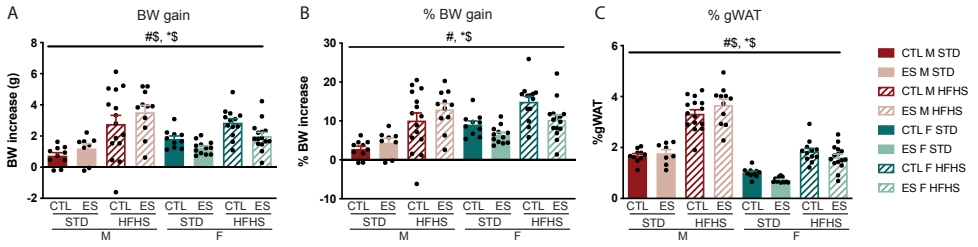

Figure 4. Effects of ES and fcHFHS on BW gain and gWAT in male and female mice. A) BW gain is affected by diet, ES exposure and sex. B) gWAT is affected by diet, ES exposure and sex. Indicated is mean ± SEM, p<0.05. # diet effect; $ sex effect; #$ diet by sex interaction effect; *$ condition by sex interaction effect.

3.5 The physiological response to the fcHFHS is dependent on sex

A 5-week exposure to the fcHFHS increased circulating glucose levels in males, but not females ($F_{diet*sex}(1, 84)=4.265$, p=0.042) (Fig. 5A). CORT levels were higher in animals fed fcHFHS ($F_{diet}(1, 77)=10.309$, p=0.002), and higher in females compared to males ($F_{sex}(1, 77)=15.278$, p<0.001) (Fig. 5B).

3.6 Adipose tissue metabolism-related gene expression is affected by ES exposure and sex

To further understand the effects of ES and sex on the adipose tissue after both STD and fcHFHS exposure, we studied the expression of genes involved in fatty acid metabolism and the adipokine leptin in the gonadal adipose tissue. Expression of CD36, a gene involved in fatty acid uptake, was elevated in animals on fcHFHS compared to those on STD ($F_{diet}(1,$

77)=42.85, p<0.001), and lower in females compared to males (F_{sex}(1, 77)=36.281, p<0.001) (Fig. 6A). FABP4, a fatty acid binding protein, was not affected by any of the predictor variables (Fig. 6B). Expression of FASN, involved in fatty acid synthesis, was higher in females fed STD ($F_{diet*sex}$(1, 75)=8.91, p=0.004) (Fig. 6C). In addition, ES exposure and sex interacted in their effect on FASN expression ($F_{condition*sex}$(1, 75)=5.54, p=0.021): ES increased FASN expression in females but not males. The gene expression of the adipokine leptin was higher in animals exposed to the fcHFHS (F_{diet}(1, 77)=71.508, p<0.001) and higher in males compared to females (F_{sex}(1, 77)=114.327, p<0.001) (Fig. 6D). Finally, PPARγ expression, involved in fatty acid storage and glucose metabolism, was unaffected by any of the predictor variables (Fig. 6E).

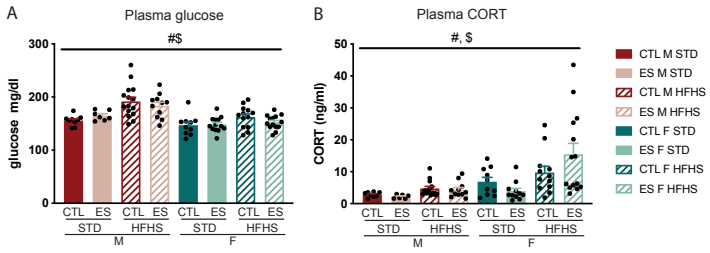

Figure 5. Effects of ES and fcHFHS on circulating glucose and hormones in males and females. A) Circulating glucose levels are higher in fcHFHS exposed animals depending on sex. D) CORT levels are higher in animals exposed to fcHFHS and higher in females. Indicated is mean ± SEM, p<0.05. # diet effect; $ sex effect; #$ diet by sex interaction effect.

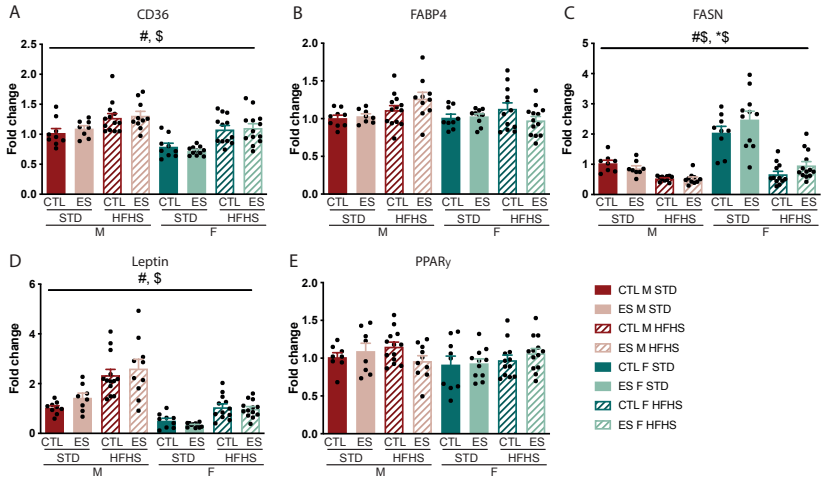

Figure 6. Effects of ES exposure and diet on adipose tissue gene expression in males and females. A) CD36 expression is higher in fcHFHS exposed animals and lower in females. B) ES, diet and sex do not affect FABP4 expression. C) FASN expression is affected by diet, sex and condition. D) Leptin expression is higher in males and in animals exposed to the fcHFHS E) PPARγ expression is not affected by diet, condition or sex. Indicated is mean ± SEM, p<0.05. # main effect of diet; $ main effect of sex; #$ diet by sex interaction effect; *$ condition by sex interaction effect.

3.7 ES affects the brain circuits regulating food intake in a sex-dependent manner

We next investigated if the brain circuits that regulate food intake are affected by ES, sex and/or diet. In the ARH, ES, diet, and sex did not affect AgRP fiber density (Fig. 7A,B). In the VTA however, AgRP fiber density was affected depending on ES exposure, diet and sex ($F_{condition*diet*sex}(1, 83)=5.434$, $p=0.022$) (Fig. 7C,D). When further exploring this 3-way interaction by stratifying on sex, we observed a condition by diet interaction effect in males ($F(1, 83)=7.547$, $p=0.007$), but not females. Pairwise comparisons between the male groups showed that on STD, ES males had lower AgRP fiber density in the VTA compared to CTL males ($p=0.004$). In addition, the fcHFHS lowered AgRP fiber density in CTL males ($p=0.006$) to a similar level as ES males on fcHFHS ($p=0.407$). Finally, even though AgRP in the ARH was not significantly affected by any of the predictor variables, AgRP in the ARH and VTA did correlate ($r=.27$, $p=0.009$) (Fig. 7E).

Next, we investigated the number of dopaminergic cells as indicated by TH^+ cell bodies in the VTA. TH^+ cell density was higher in ES exposed animals compared to CTL animals, but only when fed STD ($F_{condition*diet}(1, 83)=6.527$, $p=0.012$) (Fig. 8A,B). Moreover, we investigated the number of inhibitory pre-synapses indicated by GAD65 punctae on TH^+ fibers and TH^+ cell bodies. There were no effects of ES, diet or sex on GAD65 punctae on cell bodies (Fig. 8C,D). However, ES decreased the number of punctae on fibers ($F_{condition}(1, 54)=6.315$, $p=0.015$), with no further modulation by diet or sex (Fig. 8E,F).

3.8 Correlations between food intake, brain parameters and physiological response to the fcHFHS

Our experimental design enables us to study the correlations between the assessed parameters across the experimental groups in more detail. This may give insights in how the regulation of food intake and metabolism is affected by early-life condition, sex and diet. Figure 9 displays the correlation plots for each experimental group showing only the significant correlations: CTL M STD (Fig. 9A), ES M STD (Fig. 9B), CTL F STD (Fig. 9C), ES F STD (Fig. 9D), CTL M HFHS (Fig. 9E), ES M HFHS (Fig. 9F), CTL F HFHS (Fig. 9G), and ES F HFHS (Fig 9H). The different experimental groups show different correlation patterns between food intake, brain and metabolic measures.

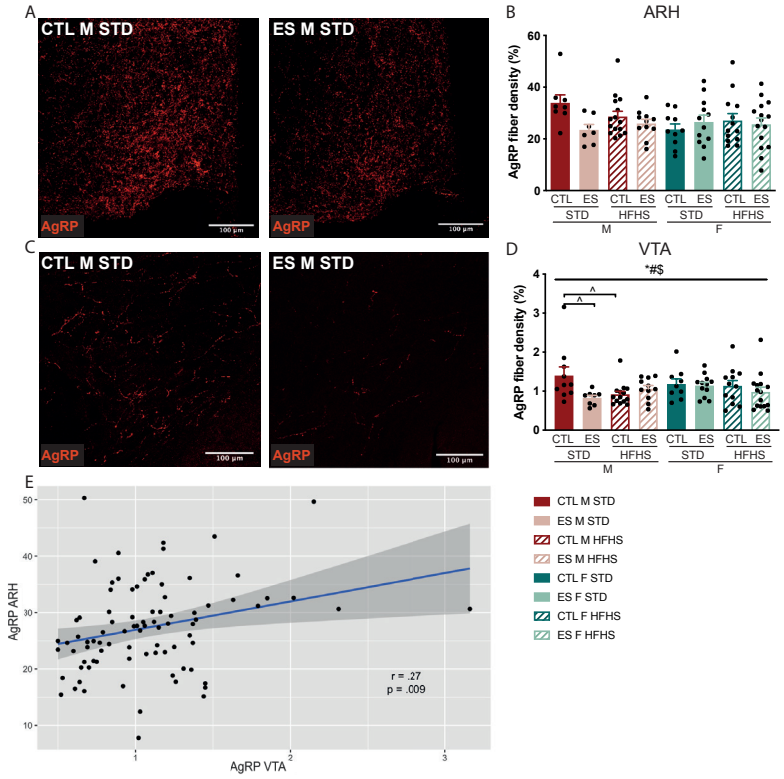

Figure 7. A) Representative pictures of AgRP staining in the ARH B) AgRP fiber density in the ARH was not affected by ES exposure or diet in males and females. C) Representative images of AgRP staining in the VTA. D) AgRP in the VTA was affected by the interaction between condition, diet and sex. E) AgRP in the ARH and VTA correlate. Indicated is mean +/- SEM, p<0.05. *#$ condition by diet by sex interaction effect; ^ significant Bonferroni post hoc comparison between experimental groups.

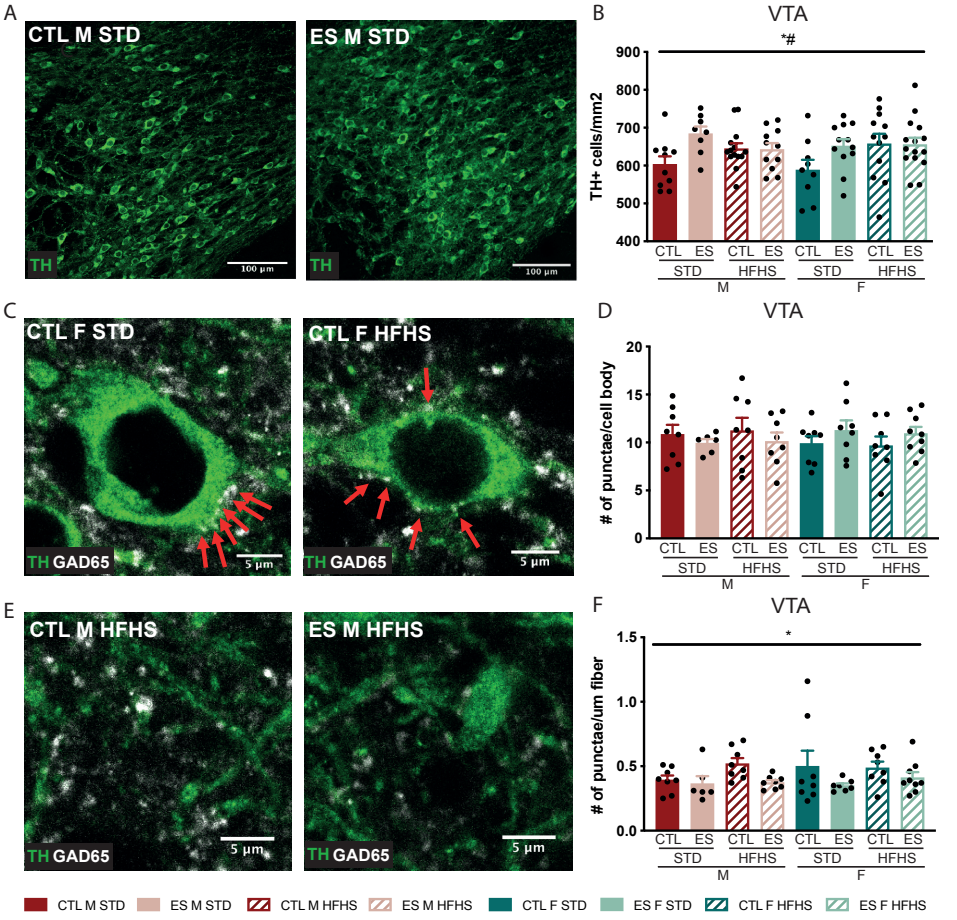

Figure 8. Effects of ES and fcHFHS on TH+ cells and inhibitory input in the VTA. A) Representative images of TH+ staining in the VTA. B) ES exposure affects TH+ cell numbers depending on diet. C) Representative images of GAD65 punctea on TH+ cell bodies. The red arrows indicate counted GAD65 punctae. D) GAD65 punctae on TH+ cell bodies was not affected by condition, diet or sex. E) Representative images of GAD65 punctae on TH+ fibers. F) GAD65 punctae on TH+ fibers was lower in ES-exposed animals. Indicated is mean ± SEM, $p<0.05$. * main effect of condition; *# condition by sex interaction effect.

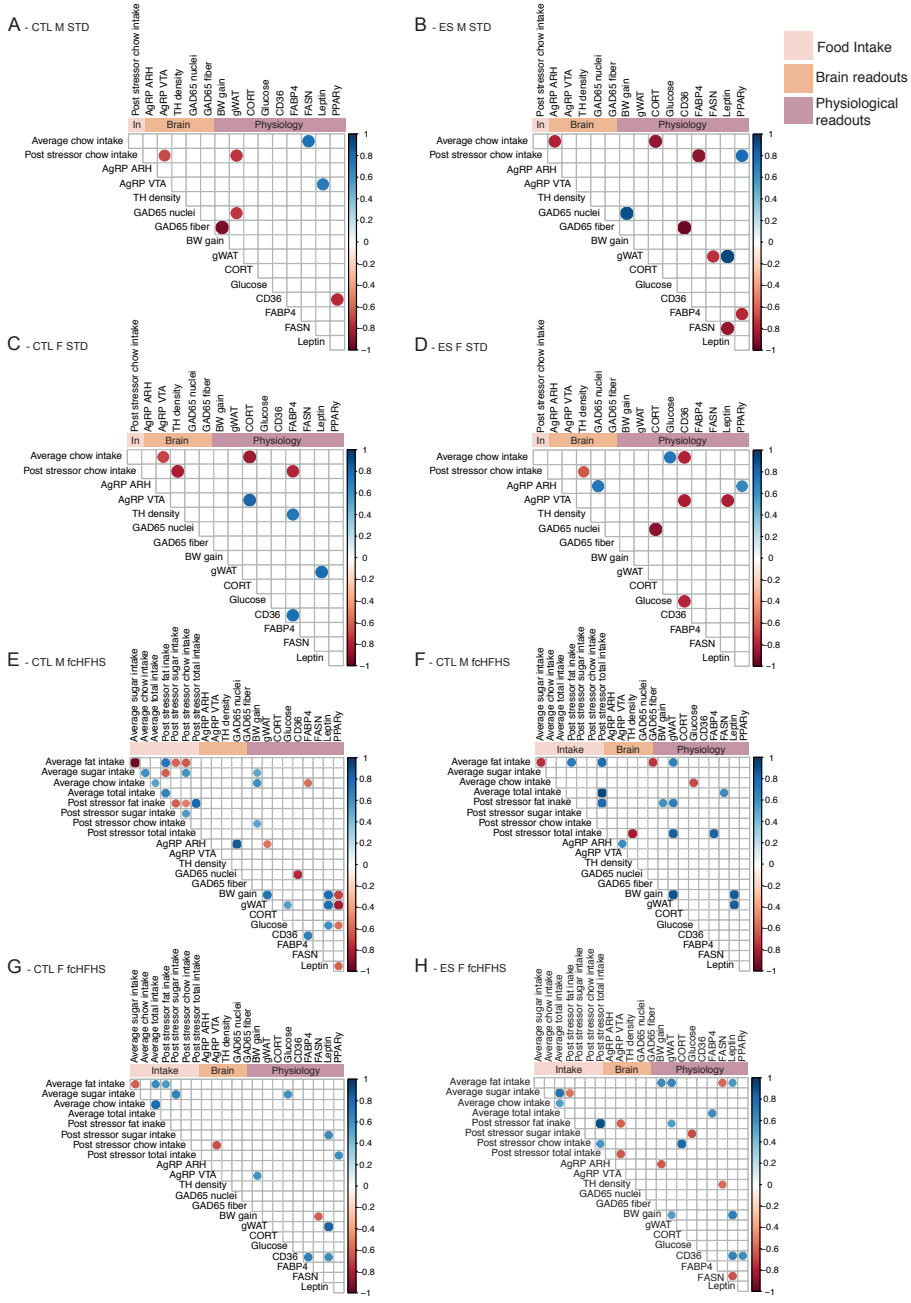

Figure 9. Correlation plots showing significant correlations in CTL M STD (A), ES M STD (B), CTL F STD (C), ES F STD (D), CTL M fcHFHS (E), ES M fcHFHS (F), CTL F fcHFHS (G) and ES F fcHFHS (H). Blue indicates a positive correlation, red indicates a negative correlation, and the larger/darker the dot the stronger the correlation.

4. Discussion

We investigated if and how ES exposure affects food choice on a fcHFHS, the physiological response to this diet, the feeding-related neuronal circuits, and if sex impacts this. We here show for the first time that i) males compared to females choose to eat more chow and sugar while females eat more fat. ii) ES does not modulate total intake and food choice for any of the components under basal state, however, after exposure to a stressful event (in the form of 4 h a fasting and 2 tail cuts) specifically ES-exposed females increased their fat intake. Moreover, iii) while as one would expect, the 5-week fcHFHS exposure increased BW gain across groups, ES-exposed females showed lower BW gain compared to CTL females with no such effects in males. iv) Expression of genes important for lipid metabolism in the adipose tissue were affected by the diet in a sex dependent manner. v) Finally, the brain circuits that regulate (palatable) food intake (encompassing AgRP, TH and the inhibitory inputs modulating TH in the VTA) were affected by both ES and diet exposure, partly in a sex-dependent manner. When taking all the data together and exploring the correlations between the various aspects, an interesting overall sexually dimorphic picture emerges (Fig. 9, Fig. 10). Below we will discuss our findings in more detail, starting with the sex differences in food choice, followed by sex-specific modulations by ES on food choice. Next, sex differences in the physiological response to the diet will be discussed, as well as sex-specific effects of ES on these parameters. We will finish by deliberating on the neural circuits that could be involved, and how sex and ES impact these.

4.1 Sex impacts on food choice

We observed a clear sex difference in food choice with males collecting a more carbohydrate-rich diet (chow and sugar) compared to females, and females choosing a more fat-rich diet. To the best of our knowledge, we are the first to address this question with our fcHFHS paradigm, unique in offering the choice between 4 separate components: standard chow, water, fat and sugar water. In fact, very few pre-clinical studies investigated sex differences in food choice, and none with our paradigm. Several studies investigated the effect of physical exercise on food choice in males and females, and showed that running induced avoidance of high-fat diet (HFD) only in males (Lee et al., 2017), or for a longer time period in males compared to females (Yang et al., 2020, 2019). In the sedentary controls in these studies, and in contrast to our findings, there was no sex difference in HFD preference (Yang et al., 2019). However, this diet also contained higher sucrose levels and sedentary control cages were enriched with a locked running wheel, thus a direct comparison of the findings is difficult. In addition, human studies also show sex differences in food intake and choice. For example, women have greater trust in healthy nutrition, and a higher fruit and vegetable intake, while men prefer fatty meals with a strong taste and fat-rich meat (Beardsworth et al., 2002; Grzymisławska et al., 2020; Spinelli et al., 2020). However, food choice in humans is confounded by sociocultural and psychological factors (Grzymisławska

et al., 2020). Thus, more research is needed to further increase our understanding in sex differences in food choice.

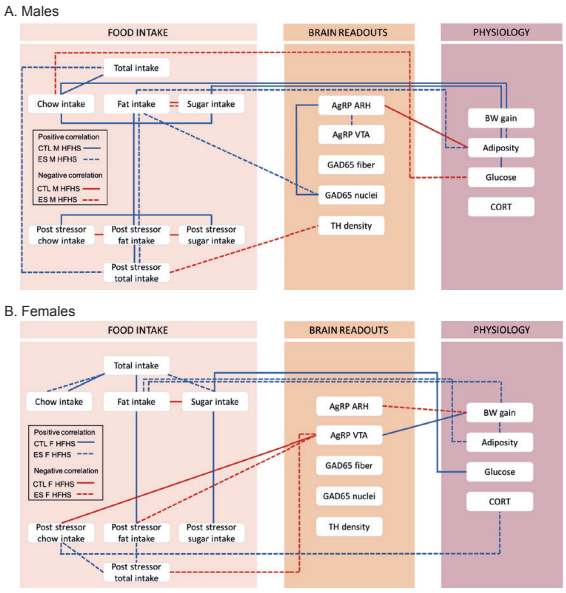

Figure 10. Representation of some of the salient correlations in the groups exposed to the fcHFHS in CTL and ES males (A), and CTL and ES females (B).

4.2 Effects of ES on food choice are sex dependent

We did not observe ES effects on food choice under basal circumstances, but importantly, after exposure to a mild acute stressor, ES-exposed females ate more fat, whereas sugar and chow intake were not affected. Previous studies addressing effects of ES on food choice have used different forms of palatable food and/or have not addressed this question in both sexes, making direct comparisons with our data difficult. However, Machado and colleagues showed that female rats exposed to the limited nesting and bedding (LBN) model had increased preference for the palatable food (combined high-fat high-sucrose pellet) during a 4 week choice paradigm, which they did not test in males (Machado et al., 2013). A recent study found that maternal separation (MS) in rats increased the intake of palatable food (consisting of condensed milk, sugar and milk powder) in both males and females during a 7 day exposure in adulthood, whereas exposure to maternal deprivation (MD) in rats had opposite effects in the two sexes (de Lima et al., 2020). In contrast, other studies showed that ES induced by repeated cross fostering or MS followed by social isolation decreased palatable food (chocolate) induced place preference in female mice, and that LBN decreased chocolate intake in male rats (not tested in females) (Bolton et al., 2018b; Sasagawa et al., 2017; Ventura et al., 2013). Moreover, studies in which the choice was given between normal water and sucrose water, next to their standard chow, have shown that ES

either reduced sucrose preference (Bolton et al., 2018a), increased sucrose intake but only in males (Michaels and Holtzman, 2007), or had no effect when exposed to a sucrose water option for a prolonged time (12 weeks) (Maniam et al., 2015). Thus, rodent studies show mixed results on the effects of ES on food choice. Diet composition (e.g. combined palatable chow versus multiple component choice diet) and form (solid versus liquid) affect food intake behaviour and should be taken into account in future studies (la Fleur et al., 2010; La Fleur et al., 2014). Importantly, human studies suggest that ES affects food preference (Jackson and Vaughn, 2019; Lussana et al., 2008), which however could be confounded by environmental factors (more exposure to unhealthy foods) and later life stress exposure.

We specifically observed increased fat intake in ES-exposed females after acute stress exposure. Indeed, palatable foods are considered comforting and affect the HPA-axis (Dallman et al., 2004): stress exposure increases the intake of comfort foods, which in turn can reduce the HPA-axis response to stress (Pecoraro et al., 2004), and CORT increases the intake of fat but not chow in a dose-response manner (La Fleur et al., 2004). In line, ES exposure increased anxiety-like behaviour in rats, which could be reduced by HFHS feeding (combined pellet with 43% fat and 40% sucrose) (Maniam et al., 2016), and high CORT responsive women eat more comfort foods compared to low responders (Epel et al., 2001) (not investigated in men). Indeed, women might be more prone to comfort feeding: when exposed to a stressful task, women increased chocolate candy intake as compared to women exposed to a non-stressful task, whereas men decreased their snack intake (Zellner et al., 2007, 2006). Our data are thus in line with such increased vulnerability in women/females.

4.3 Sex impacts the physiological response to fcHFHS exposure

Next to a strong sex effect in food choice, we also found a strong sex difference in the physiological responses to the fcHFHS: males had higher gWAT levels upon fcHFHS exposure compared to females, and only males showed fcHFHS-induced increased glucose levels. Increased glucose levels after 4 weeks of fcHFHS has been described before in male rats accompanied by increased insulin levels and decreased glucose tolerance (La Fleur et al., 2011). We now show a similar effect on glucose levels in male, but not female mice after 5 weeks of fcHFHS. Although this sexual dimorphic response to the fcHFHS could partly be mediated by their altered food choice and thus intake, such sex differences in the response to non-choice high-caloric diets have been described before. Compared to females, males fed a HFD have been shown to display higher weight gain, higher fat mass indexes, and more greatly impaired glucose tolerance (Estrany et al., 2013, 2011; Garg et al., 2011; Grove et al., 2010). Both the quantity and functioning of fat depots are different for males and females (Fuente-Martín et al., 2013; Power and Schulkin, 2008). Different fat depots vary in their adipokine production, free fatty acid release, inflammatory response and development of insulin sensitivity in a sex-specific manner (Macotela et al., 2009; Power and Schulkin, 2008; Wajchenberg et al., 2000). These differences in fat depot functioning, together with the fact that fat distribution is different between men and women, thus has functional

implications. Indeed, we observed sex differences in adipose tissue gene expression. CD36 and leptin expression, involved in fatty acid uptake and satiety respectively, were higher in males on both STD and fcHFHS, whereas females on STD had higher FASN (fatty acid synthase) expression. Sex differences in gene expression in the different adipose depots have been described before (Estrany et al., 2013; Grove et al., 2010). For example, males responded to a 12-week HFD exposure by upregulating inflammatory gene expression, while females were shown to have higher expression of genes related to insulin signaling and lipid synthesis, independent of diet (Grove et al., 2010). The sympathetic innervation, as well as the projection from the hypothalamus to WAT differ between males and females (Adler et al., 2012) and may underlie some of these differences in metabolic vulnerability between the sexes.

Finally, we found that 5 weeks of fcHFHS increased basal CORT levels in both males and females, and that females had remarkably higher CORT levels than males. Indeed, while comfort foods may reduce the stress response on the short-term (Pecoraro et al., 2004), long-term HFD feeding as well as obesity have been related to increased circulating CORT levels (Cano et al., 2008; van Rossum, 2017), and females have before been shown to have higher CORT levels compared to males in mice (Naninck et al., 2015). CORT is known to increase blood glucose levels (Kuo et al., 2015), however, while females had higher CORT levels compared to males, we observed an opposite sex effect on glucose levels with females having lower glucose levels and being protected from the HFHS-induced increase in glucose. This suggests other factors, such as the above mentioned adipose distribution and quantity, as well as sex hormones (Varlamov et al., 2014) might contribute to higher glucose levels in males.

4.4 The effect of ES on physiological readouts depends on diet and sex

ES exposed females had lower BW gain and gWAT levels compared to controls both on STD and fcHFHS, while for males no such ES effect was observed, and if any, ES seemed to increase BW gain in males. Previous studies showed that on standard chow diet, ES affected adiposity similarly in both sexes, although the directionality depends on the used ES model. For example, ES induced by MS has been found to be increase adiposity in both sexes (Jaimes-Hoy et al., 2016; Murphy et al., 2018), whereas LBN is shown to decrease adiposity in males and females (Yam et al., 2017a). In contrast, when fed a western-style diet with 39.8% fat content for 8 weeks, LBN exposure led to increased fat accumulation in males compared to controls (not performed in females) (Yam et al., 2017a). Studies using MS as ES paradigm did not observe effects of ES when males were fed a HFD, while female adiposity was increased upon 12 and 16 weeks of HFD respectively (60% fat) (Murphy et al., 2018, 2017). Differences in the used ES model as well as differences in diet composition and duration could contribute to these discrepancies. In our study, the lower BW gain and gWAT levels in ES-exposed females could not be explained by lower food intake, raising the question whether ES increases energy expenditure in females. Literature on the effect of ES

on energy expenditure is sparse. We have previously shown that ES increases the expression of a gene critical in heat production in both sexes at P9, but not in adulthood (Yam et al., 2017a). ES has also been reported to affect locomotor activity levels (Aya-Ramos et al., 2017; Hancock and Grant, 2009), as well as the hypothalamic-pituitary-thyroid axis (in both sexes), which is key in regulating metabolic rate (Jaimes-Hoy et al., 2019a; Mullur et al., 2014). However, to the best of our knowledge no studies measured effects of ES on metabolic rate itself. It thus remains to be determined what leads to the lower BW gain and gWAT levels in ES-exposed female offspring, and whether there is a role for energy expenditure.

Besides lower adiposity, we also observed higher adipose tissue FASN expression in ES-exposed females. Higher FASN expression has been observed in normoglycemic versus hyperglycemic individuals (Mayas et al., 2010), and dexamethasone (CORT analogue) increases adipose FASN expression *in vivo* (Wang et al., 2004). A higher FASN expression in females is thus in line with the observed lower glucose and higher CORT in females compared to males, but cannot explain the increased FASN in ES females. Interestingly, while in CTL fcHFHS fed females FASN was negatively associated with BW gain, in ES females on fcHFHS, FASN negatively correlated with fat intake but not with physiological measures such as BW gain or adiposity, suggesting FASN expression might be more sensitive to food intake in ES compared to CTL females.

We did not find effects of ES on glucose levels in both males and females, nor in interaction with the diet. Hyperglycemia is a sign of impaired insulin sensitivity, and previous studies have found ES to either have no effect, increase or decrease (various measures of) insulin sensitivity, partly depending on diet and sex of the animal, with males potentially being more vulnerable to develop insulin insensitivity (Jaimes-Hoy et al., 2019b; Maniam and Morris, 2010; Mela et al., 2012; Murphy et al., 2017). To better understand if ES affects insulin sensitivity it would be important to perform, next to basal glucose and insulin measurements, insulin and glucose tolerance tests.

In addition, we did not observe ES effects on adult basal CORT levels under any of the dietary exposures, in line with Naninck *et al*., 2015 (Naninck et al., 2015). Other studies did show effects of ES on basal CORT levels (Rice et al., 2008), as well as in response to an acute stressor or upon 12 weeks of HFD feeding (Machado et al., 2013; Murphy et al., 2017). As these studies were performed in rats, the possibility of interspecies differences should be kept in mind. Nonetheless, they also indicate that latent effects of ES on CORT could potentially be unmasked upon an acute challenge or more severe/prolonged HFD.

4.5 ES affects the brain circuits involved in hedonic driven food intake

Similar as previously reported, the fcHFHS did not affect *hypothalamic* AgRP levels, involved in homeostatic-driven food intake (la Fleur et al., 2010), nor was it different between males and females or affected by ES. However, we did find that AgRP in the *VTA* was decreased in ES-exposed males on STD compared to their respective controls, and that the fcHFHS also reduced AgRP in CTL males. In contrast, in females no such effects of ES or

diet were observed. Studies focusing on AgRP in the VTA are rare, and to the best of our knowledge we are the first to describe modulations of ES, diet and sex on VTA AgRP. It has been shown that hypothalamic AgRP neurons innervate the VTA and determine the reward circuit setpoint by affecting dopamine cell excitability and dopamine levels (Dietrich et al., 2012). Notably, ablating hypothalamic AgRP (thereby also ablating the AgRP projections to the VTA) increases palatable food intake, and has been proposed as a model for comfort feeding (Denis et al., 2015). Despite the absence of a significant effect of ES and/or sex on ARH AgRP, AgRP fiber density in VTA and ARH positively correlated, which might suggest that these alterations in the VTA might partly derive from the ARH. However, additional studies are needed to further understand the origin of the changes in the VTA. Moreover, some interesting sex dependent effects are worth noting. For example, despite a reduction in VTA AgRP specifically in males by ES and diet, we did not observe effects of ES on food choice in males, nor did AgRP levels in the VTA correlate with intake parameters. In contrast, in females, where none of the conditions impacted on VTA AgRP, there was a negative correlation between VTA AgRP and fat intake after acute stress specifically in ES-exposed females, while in CTL females VTA AgRP correlated negatively to chow intake after acute stress. The emerging picture seems thus to be that the sex-dependent modulation of AgRP by the various conditions (i.e. ES and diet) might be related to the differential food intake exhibited by males and females after a stress exposure, although this hypothesis needs further exploration.

We observed increased TH^+ cell numbers (indication of dopamine (DA) cell density) in the VTA of ES-exposed males and females, when fed STD but not when fed fcHFHS. Moreover, independent of diet exposure, we report decreased inhibitory input on TH^+ fibers in ES-exposed males and females. The mesocorticolimbic DA circuitry originates from VTA DA neurons that connect to limbic structures and the medial prefrontal cortex. This VTA network senses and links both the internal state and the appraisal of environmental stimuli, and in that way establishes emotional-motivational valuations (Douma and de Kloet, 2020). Palatable foods activate the reward circuitry (De Macedo et al., 2016) and both acute and chronic stress have been shown to affect DA release and DA neuron firing (Belujon and Grace, 2015; Chang and Grace, 2014; Holly and Miczek, 2016; Kaufling, 2019; Rincón-Cortés and Grace, 2017). The dopaminergic reward system continues to mature into adolescence (Kalsbeek et al., 1988; McCutcheon et al., 2012; Teicher et al., 1995; Voorn et al., 1988), and stress during this developmental period can affect its functioning later in life. In fact, ES alters multiple aspects of the reward circuitry. For example, ES exposure increased excitability of putative DA neurons in the VTA (Spyrka et al., 2020), blunted DA outflow in the prefrontal cortex (Ventura et al., 2013), lowered DA transporter sites in the striatum and NAcc (Brake et al., 2004), led to transcriptional changes in the VTA (Peña et al., 2017), and increased neuronal activity in the NAcc core (Bolton et al., 2018a). As most studies only included either males or females, it is unclear whether ES alters reward circuitry functioning in a sexually dimorphic manner.

In our study, the fcHFHS seems to overrule the more subtle ES effects on TH^+ cell numbers, although the ES effect on inhibitory input (GABAergic synapses) on TH^+ fibers remains. Approximately 30% of cells in the VTA are GABA neurons (the majority of non-DA cells in VTA) (Bouarab et al., 2019), which provide both local and long-range inhibition. The VTA GABA neurons synapse mostly on the proximal dendrites of DA neurons, while (inhibitory) inputs from other regions including the ventral pallidum and laterodorsal tegmentum synapse onto the cell body (Omelchenico and Sesack, 2005; Omelchenko and Sesack, 2009). Of note, these GABAergic VTA neurons are affected by stress, and (through their inhibition of DA neurons) also regulate reward. Acute stressors directly increase the firing rate of VTA GABA neurons thereby suppressing DA firing (Cohen et al., 2012; Tan et al., 2012), while over the days following the stress GABAergic plasticity is lost, removing VTA DA neuron inhibition (Niehaus et al., 2010; Polter et al., 2017, 2014). Although speculative, it is possible that the reduction in GABAergic input on TH^+ fibers in ES animals is at least partly derived from a reduction in VTA GABA input onto these neurons. An ES-induced reduction of inhibitory input on TH^+ fibers is in line with the previously described higher excitability of DA neurons (Spyrka et al., 2020). It however is important to note that the VTA also receives input from adrenergic and noradrenergic neurons located in e.g. the caudal medulla and locus ceruleus, which express TH as well (Mejías-Aponte et al., 2009). Therefore DA producing neurons will not be the only origin of the TH^+ fibers in the VTA.

Importantly, VTA DA neurons have been proposed to function differently between males and females. For example, females have higher VTA DA turnover and release (Becker and Chartoff, 2019) and the afferents to the VTA are also sexually dimorphic (Bale and Epperson, 2015). Moreover, CORT (which in our study was higher in females) directly affects the reward circuitry: adrenalectomy decreases DA release and DA transporters binding in the NAcc shell (Sarnyai et al., 1998). We did not observe sex differences in DA cell density or inhibitory input on DA neurons, but AgRP input in the VTA was affected by ES specifically in males, while it correlated with acute-stress induced food intake only in females. Thus, the circuits that regulate comfort feeding are sexually dimorphic, and besides the fact that ES impacts on aspects of this circuitry in both males and females, ES also has some sex-dependent effects. Sex differences in reward system functioning, together with sex differences in CORT, could be involved in the different vulnerability to comfort feeding between males and females.

4.6 General conclusion

We show, for the first time, that males and females make different food choices, and that ES exposure affects food choice after a mild acute stressful event only in females. Moreover, physiologically, males and females respond differently to the diet as well as to ES exposure. Finally, we provide evidence that ES alters the reward circuitry of both male and female mice, partly in a sex-specific manner. Although it remains to be understood how the different elements interact, a different picture emerges in males and females depending on previous ES exposure (Fig. 10). This suggests not only that food choice and metabolism are

differently regulated between sexes, but also that ES has a different impact on males and females. Our data highlights the importance of including both sexes in future studies.

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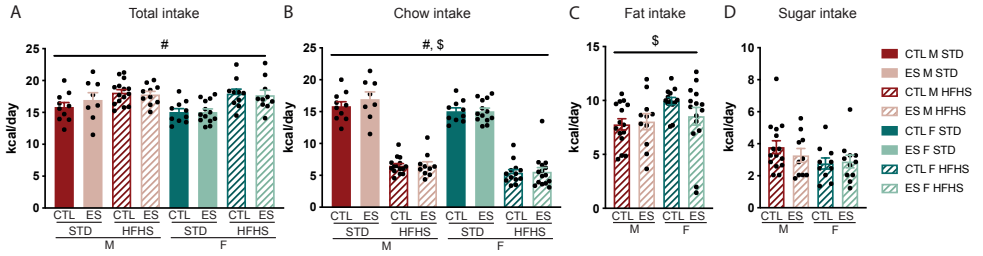

Figure S1. Kcal intake per day before the fasting period. A) Total caloric intake is higher in fcHFHS fed animals. B) Chow intake is lower in fcHFHS exposed animals, and higher in males. C) fat intake is higher in females. D) sugar intake is not affected by sex or condition. # main effect of diet; $ main effect of sex.

CHAPTER 5

5

Modulation of the hypothalamic nutrient sensing pathways by sex and early-life stress

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Abstract

There are sex differences in metabolic disease risk, and early-life stress (ES) increases the risk to develop such diseases, potentially in a sex-specific manner. It remains to be understood, however, how sex and ES affect such metabolic vulnerability. The hypothalamus regulates food intake and energy expenditure by sensing the organism's energy state via metabolic hormones (leptin, insulin, ghrelin) and nutrients (glucose, fatty acids). Here, we investigated if and how sex and ES alter hypothalamic nutrient sensing short and long-term. ES was induced in mice by limiting the bedding and nesting material from postnatal day (P)2-P9, and the expression of genes critical for hypothalamic nutrient sensing were studied in male and female offspring, both at P9 and in adulthood (P180). At P9, we observed a sex difference in both *Ppargc1a* and *Lepr* expression, while the latter was also increased in ES-exposed animals relative to controls. In adulthood, we found sex differences in *Acacb*, *Agrp* and *Npy* expression, whereas ES did not affect the expression of genes involved in hypothalamic nutrient sensing. Thus, we observe a pervasive sex difference in nutrient sensing pathways and a targeted modulation of this pathway by ES early in life. Future research is needed to address if the modulation of these pathways by sex and ES is involved in the differential vulnerability to metabolic diseases.

1. Introduction

The prevalence of obesity and the related chronic diseases rise, and postulate a global health concern (Malik et al., 2013). While both men and women are affected, there is a differential risk to develop metabolic diseases between sexes (Kanter and Caballero, 2012; Kautzky-Willer et al., 2016). Worldwide, more women than men are obese, although the prevalence of overweight and obesity among men and women vastly fluctuates between countries (Kanter and Caballero, 2012). Under healthy bodyweight conditions, men and women differ in fat quantity and distribution (Fuente-Martín et al., 2013; Power and Schulkin, 2008), and rodent studies show for example that males and females differently respond to high-fat diets (Estrany et al., 2013, 2011; Garg et al., 2011; Grove et al., 2010).

Next to sex as determinant of metabolic vulnerability, there is also evidence that the perinatal environment is critical for establishing a metabolic set point defining later-life metabolic health (Bouret, 2009; Bouret and Simerly, 2006; Levin, 2006; Yam et al., 2017a, 2015). Exposure to stress during this sensitive developmental period (e.g. neglect or abuse) increases the risk to develop metabolic diseases, including obesity and diabetes, later in life (Alciati et al., 2013; Basu et al., 2017; Danese and Tan, 2014). Indeed, we have previously shown in mice that early-life stress (ES)-exposed males and females have a leaner phenotype when fed a (healthy) standard chow diet, but that ES-exposed males accumulated more fat when later fed a western-style diet (WSD) with a moderate fat content, while how females respond to such a diet was not investigated in this study (Yam et al., 2017a). There is in fact increasing evidence that the effects of ES may be sexually dimorphic (Boynton-Jarrett et al., 2010; Kozyrskyj et al., 2011; Murphy et al., 2018; Park et al., 2018). However, the mechanisms contributing to the sex and ES-induced impacts on metabolic regulation are not well understood.

The arcuate nucleus of the hypothalamus (ARH) regulates energy homeostasis by controlling food intake, energy expenditure and glucose metabolism, and it has been proposed that its development is important for the establishment of the metabolic setpoint (Bouret, 2012, 2009; Bouret and Simerly, 2006; Dearden and Ozanne, 2015; Ramamoorthy et al., 2015). The ARH integrates a wide range of inputs, including those from metabolic hormones and peripheral nutrients (Lam et al., 2005; Timper and Brüning, 2017). The key neuropeptides regulating food intake in the ARH are agouti-related protein (AgRP) and neuropeptide Y (NPY), which stimulate food intake while inhibiting energy expenditure, and proopiomelanocortin (POMC), which has opposite regulatory effects (Barsh and Schwartz, 2002; Timper and Brüning, 2017). Metabolic hormones, including leptin, ghrelin and insulin, act on ARH neurons by binding to their respective receptors. Leptin is an adipokine secreted by the white adipose tissue, which, via binding to its receptor (LepR) inhibits food intake (Friedman, 2014). Insulin, via binding to the insulin receptor (InsR), has a comparable effect as leptin on food intake (Könner et al., 2009; Porte and Woods, 1981), whereas ghrelin (primarily secreted by the stomach) binds to the growth hormone secretagogue receptor

(GHSR) (Gil-Campos et al., 2006), and stimulates food intake and bodyweight gain (Wren et al., 2001). Importantly, we have shown before that ES reduced circulating leptin levels and leptin expression in the adipose tissue, while increasing *Lepr* expression in the choroid plexus, which is an important side of leptin entry into the brain (Zlokovic et al., 2000), in both males and females when fed standard chow diet (Yam et al., 2017a). ES furthermore affected circulating ghrelin levels in an age-dependent manner specifically in females, while altering the fiber density of NPY and AgRP in both males and females at postnatal day (P)14, i.e. when the hypothalamus is fully developed (Yam et al., 2017b).

As mentioned above, next to the hormonal regulation of food intake, circulating nutrients (e.g. glucose, fatty acids (FA)) are sensed in the hypothalamus and provide information about the energy state (Lam et al., 2005; Le Foll, 2019; Matzinger et al., 2000; Migrenne et al., 2006; Rijnsburger et al., 2016). More specifically, FA metabolism in the hypothalamus is suggested to function as sensor for nutrient/energy availability integrating both nutritional and hormonal signals. The intracellular pool of long-chain FAs depends on both biosynthetic and oxidative pathways (breakdown of FAs) (Lam et al., 2005) and thought to signal energy abundance (Dragano et al., 2020; Lam et al., 2005). Indeed, FA administration has been shown to suppress *Npy* and *Agrp* expression *in vitro* (Ma et al., 2017), and *in vivo* experiments showed that intracerebroventricular FA administration inhibits food intake and *Npy* expression (Obici et al., 2002). Furthermore, manipulating key proteins involved in both FA synthesis and oxidation (e.g. FASN, CPT1 and ACC) centrally affects food intake and bodyweight (Cesquini et al., 2008; Jo et al., 2009; Loftus et al., 2000; Obici et al., 2003; Shimokawa et al., 2002), and central peroxisome proliferator–activated receptors (PPARs), a family of transcription factors regulating lipid and glucose metabolism, have also been shown to modulate food intake and fat mass (Kocalis et al., 2012; Lu et al., 2011; Ryan et al., 2011). Importantly, glucose, leptin, ghrelin and insulin also affect FA metabolism pathways in the hypothalamus (Diéguez et al., 2011; Gao et al., 2007; Lage et al., 2010; Lam et al., 2005; López et al., 2008; Taïb et al., 2013). Thus, hypothalamic FA metabolism integrates nutrient and hormonal signals and affects hypothalamic neuropeptide expression and food intake.

Yet, it remains to be determined if and how sex and ES impact hypothalamic neuropeptide systems and nutrient sensing pathways. Therefore, we here investigated the gene expression of hypothalamic neuropeptides, hormone receptors and fatty acid metabolism at P9 and P180 in male and female control (CTL) and ES-exposed mice. We studied this in the cohort of mice that we used to report on the metabolic effects of ES in males and females (Yam et al., 2017a), giving us the opportunity to link these gene expression profiles to their metabolic phenotype. We show pervasive sex differences in nutrient sensing pathways and, early in life, a targeted modulation of these pathways by ES.

2. Materials and methods

2.1 Animals and breeding

In total 17 male and 14 female pups, and 17 male and 13 female adult mice were used for this study. Animals were kept under standard housing conditions (temperature 20-22 °C, 40-60% humidity, 12/12h light/dark schedule), and fed standard chow (CRM (P), 801722, Standard Diets Services, Essex, United Kingdom, 3.585 kcal/g, with 22% protein, 9% fat, and 69% carbohydrates) and water provided *ad libitum*. All experimental procedures were conducted under national law and European Union directives on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam.

To standardize the perinatal environment, experimental animals were bred in house. For breeding, 8-week-old C57Bl/6J female and male mice were purchased from Envigo Laboratories B.V. (Venray, The Netherlands), and allowed to habituate for 1-2 weeks. Next, two primiparous females were housed with one adult male to allow for mating. After one week, the male was removed and females were housed together for another week in a clean cage with nest material (square piece of cotton) to practice, and after another week, females were housed individually in a type II cage with nesting material and filtertop. Starting from 18 days after the start of the breeding, dams were checked for the birth of pups each morning before 09:00 AM. When a litter was born, the previous day was designated as postnatal day 0.

2.2 ES paradigm

The limited nesting and bedding material model was used to induce ES from P2 to P9, as described previously (Naninck et al., 2015; Rice et al., 2008). In short, litters were culled to 6 pups, including both males and females, and randomly allocated to the control (CTL) or ES condition at P2. Litters with less than 5 pups were excluded. CTL cages had a standard amount of sawdust and one square piece of cotton nesting material (5x5 cm, Tecnilab-BMI, Someren, The Netherlands), whereas ES cages had a thin layer of sawdust on the bottom, covered with a fine-gauge stainless steel mesh 1 cm above the bottom of the cage, and half a square piece of cotton nesting material (2.5x5 cm). All cages were covered with a filtertop. For the adult experiment, litters were moved to new cages containing standard amounts of sawdust at P9, and left undisturbed until weaning at P21, after which they were group-housed with same sex littermates.

2.3 Tissue preparation

To study the direct effects of ES on nutrient sensing pathways in male and female offspring, pups were sacrificed in the morning of P9 by rapid decapitation. For adult studies, animals were fasted for 4 hours and sacrificed by rapid decapitation at P180. The hypothalamus was rapidly dissected, snap frozen and stored at -80 °C. RNA was obtained using Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions and stored at -80

˚C until further use. Reverse transcription of RNA into cDNA was done with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), and cDNA was stored at -20 ˚C until further analysis.

2.4 Real-time PCR

Relative gene expression was measured using a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). In short, for all genes with exception of the leptin receptor (see below), hot FirePol EvaGreen Mastermix (Solis Biodyne, Tartu, Estonia) was used. Primers (Eurogentec, Liege, Belgium) of target and reference genes all had an efficiency between 90-110% (supplementary table 1). Gene specific forward and reverse primers together with cDNA template were added to the reaction mix according to manufacturer's instructions. Cycling conditions were as follows: 15 min polymerase activation at 95°C, followed by 40 cycles of replication (15 s at 95°C, 20 s at 65°C, and 35 s at 72°C) and a dissociation program. For the quantification of the leptin receptor, Taqman® probes were used. TaqMan® Gene Expression Master Mix (4369016, ThermoFisher Scientific, Waltham, MA, USA) was added to cDNA template and *Lepr* Taqman® probes (6749720_1, ThermoFisher Scientific, Waltham, MA, USA), and the following cycling conditions were used: 2 min 50°C and 10 min 95°C for activation, followed by 40 cycles of replication (15 s at 95°C, 1 min at 60°C) and a dissociation program. Gene expression was calculated with the ΔΔCt method in Qbase+ (Biogazelle, Zwijnaarde, Belgium) and normalized for two reference genes (*Rpl13a* and *Rplp0* for P9, *Tbp* and *Tuba1a* for P180) which were tested for stability.

2.5 Statistical analysis

Data were analysed with SPSS 25.0 (IBM software, Armonk, NY, USA), R studio 1.2.1335 (R Core Team, 2018) and Graphpad Prism 6 (Graphpad software, San Diego, CA, USA). All data are presented as mean ± standard error of the mean (SEM), and when $p<0.05$, data was considered statistically significant. Gene expression data was scaled against the CTL male group, and log transformed for statistical analysis. Outliers were identified in SPSS and removed, and data was analysed with a 2-way ANOVA with condition and sex as predictor variables. Multiple mice from the same litter were included in this study, and therefore data are nested. We verified if litter contributed to the outcome by performing mixed model analysis with litter as random factor. Litter did not affect any of the outcome variables. In addition, to test whether estrous cycle contributed to the variable outcomes, we performed mixed model with estrous cycle as random factor. Estrous cycle did not affect any of the outcomes. Total white adipose tissue levels (as percentage of bodyweight) and circulating leptin levels (previously published in Yam et al., 2017a) were correlated to hypothalamic gene expression levels with R studio software (version 1.2.1335) (R Core Team, 2018). Pearson correlations were calculated based on complete pairwise cases, and correlation coefficients were tested against critical values on a two-tailed distribution (alpha=0.05).

3. Results

3.1 Effects of ES on hypothalamic gene expression in male and female offspring at P9

Fig. 1A shows a full overview of the effects of ES and sex on hypothalamic gene expression. Among the genes that regulate fatty acid metabolism, *Ppargc1a* was higher in females compared to males (Fig. 1B). Moreover, *Lepr* expression was higher in females, and lower in ES-exposed males and females compared to their respective controls (Fig. 1C). The expression of other receptors and hypothalamic neuropeptides were not affected by sex or ES.

A

Function	Gene	Condition	Sex	Interaction
Fatty acid metabolism	*Acacb*	F(1,25)=0.005, p=0.943	F(1,25)=0085, p=0.772	F(1,25)=1.655, p=0.21
	Cpt1a	F(1,26)=2.10, p=0.1.59	F(1,26)=0.574, p=0.455	F(1,26)=0.009, p=0.927
	Cpt1c	F(1,26)=0.248, p=0.623	F(1,26)=0.125, p=0.726	F(1,26)=0.114, p=0.738
	Fasn	F(1,26)=1.376, p=0.251	F(1,26)=0.017, p=0.898	F(1,26)=1.617, p=0.215
	Ppara	F(1,26)=0.076 p=0.785	F(1,26)=1.21 p=0.281	F(1,26)=0.215, p=0.647
	Ppard	F(1,21)=2.261, p=0.148	F(1,21)=0.702, p=0.412	F(1,21)=0.35, p=0.575
	Pparg	F(1,24)=0.237, p=0.631	F(1,24)=1.077, p=0.31	F(1,24)=0.05, p=0.824
	Ppargc1a	F(1,25)=0.128, p=0.724	F(1,25)=5.268,p=0.03*	F(1,25)=0.014, p=0.906
Receptors	*Ghsr*	F(1,27)=1.087, p=0.306	F(1,27)=2.104, p=0.158	F(1,27)=0.521, p=0.476
	Insr	F(1,27)=0.054, p=0.818	F(1,27)=2.325, p=0.139	F(1,27)=1.94, p=0.175
	Lepr	F(1,27)=4.782, p=0.038*	F(1,27)=5.272, p=0.03*	F(1,27)=0.287, p=0.597
Neuropeptides	*Npy*	F(1,27)=0.428, p=0.518	F(1,27)=0.794, p=0.381	F(1,27)=0.033, p=0.857
	Agrp	F(1,27)=0.048, p=0.829	F(1,27)=2.206, p=0.149	F(1,27)=0.508, p=0.482
	Pomc	F(1,27)=0.00, p=0.996	F(1,27)=3.749, p=0.063	F(1,27)=0.402, p=0.531

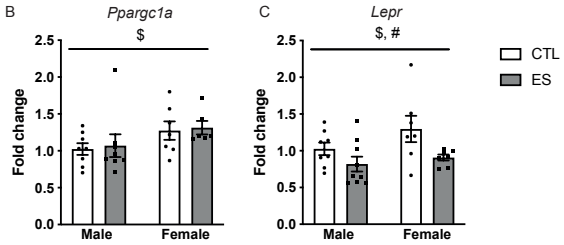

Figure 1. A: Effects of ES and sex on hypothalamic gene expression at P9. B: Hypothalamic *Ppargc1a* expression was higher in females. C: *Lepr* expression was higher in females and lower in ES-exposed animals. Indicated is mean +/- SEM, p<0.05. # main effect of condition; $ main effect of sex.

3.2 Effects of ES on hypothalamic gene expression in male and female mice at P180

Fig. 2A shows an overview of the effects of ES and sex on hypothalamic gene expression in adulthood (P180). *Acacb* expression was higher in females (Fig. 2B). Moreover *Agrp* (Fig. 2C) and *Npy* (Fig. 2D) expression were higher in females compared to males. There were no effects of ES on the hypothalamic expression of hormone receptors, genes in fatty acid metabolism, or neuropeptides in adulthood.

A

Function	Gene	Condition	Sex	Interaction
Fatty acid metabolism	*Acacb*	F(1,24)=0.557, p=0.463	F(1,24)=5.37, p=0.029*	F(1,24)=2.906, p=0.101
	Cpt1a	F(1,24)=0.001, p=0.981	F(1,24)=1.574, p=0.222	F(1,24)=0.816, p=0.375
	Cpt1c	F(1,20)=1.134, p=0.300	F(1,20)=0.291, p=0.595	F(1,20)=0.234, p=0.634
	Fasn	F(1,25)=0.417, p=0.525	F(1,25)=1.183, p=0.155	F(1,25)=2.153, p=0.155
	Ppara	F(1,22)=0.065 p=0.801	F(1,22)=0.792, p=0.383	F(1,22)=1.92, p=0.180
	Ppard	F(1,23)=2.056, p=0.165	F(1,23)=0.020, p=0.889	F(1,23)=0.003, p=0.954
	Pparg	F(1,20)=0.002, p=0.966	F(1,20)=2.567, p=0.125	F(1,20)=0.001, p=0.97
	Ppargc1a	F(1,22)=0.109, p=0.744	F(1,22)=0.506,p=0.484	F(1,22)=0.065, p=0.801
Receptors	*Ghsr*	F(1,26)=2.512, p=0.125	F(1,26)=3.269, p=0.082	F(1,26)=0.387, p=0.539
	Insr	F(1,26)=2.682, p=0.1114	F(1,26)=0.459, p=0.504	F(1,26)=0.481, p=0.494
	Lepr	F(1,26)=1.626, p=0.214	F(1,26)=3.753, p=0.064	F(1,26)=0.271, p=0.607
Neuropeptides	*Npy*	F(1,24)=0.145, p=0.707	F(1,24)=25.181, p<0.001*	F(1,24)=0.119, p=0.734
	Agrp	F(1,23)=1.203, p=0.284	F(1,23)=34.232, p<0.001*	F(1,23)=3.416, p=0.077
	Pomc	F(1,25)=0.159, p=0.693	F(1,25)=0.058, p=0.811	F(1,25)=0.074, p=0.788

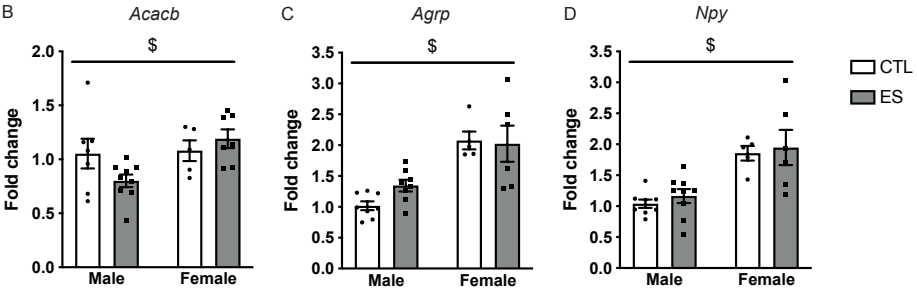

Figure 2. A: Effects of ES and sex on hypothalamic gene expression in adulthood. B: *Acacb* **expression was higher in females. C: Expression of** *Agrp* **was higher in females. D:** *Npy* **expression was higher in females. Indicated is mean +/- SEM, p<0.05. # main effect of condition; $ main effect of sex.**

3.3 Hypothalamic gene expression correlates with metabolic readouts in adulthood

In the same cohort of mice as used in the current study, we previously reported that ES decreased white adipose tissue (WAT) levels at P9 and in adulthood, as well as reduced leptin expression and circulating leptin levels in both males and females (see Yam et al., 2017a). To explore whether hypothalamic gene expression is related to the metabolic profile, we correlated the genes that were significantly affected by either ES or sex with white adipose tissue (WAT) levels and circulating leptin levels (Fig. 3A). At P9, *Ppargc1a* and *LepR* expression did not correlate with either WAT or leptin levels. In adulthood, *Acacb* expression correlated with leptin levels (Fig. 3B), *Agrp* expression correlated with both WAT and leptin levels (Fig. 3C,D), and *Npy* expression correlated with WAT levels (Fig. 3E).

A

Age	Correlation	r value	p value
P9	*Ppargc1a* : WAT	-0.178	0.439
	Ppargc1a : leptin	-0.155	0.527
	LepR : WAT	0.126	0.577
	LepR : leptin	0.156	0.511
Adult	*Acacb* : WAT	-0.265	0.173
	Acacb : leptin	-0.411	0.046
	Agrp : WAT	-0.706	<0.001
	Agrp : leptin	-0.719	<0.001
	Npy : WAT	-0.377	0.044
	Npy : leptin	-0.308	0.134

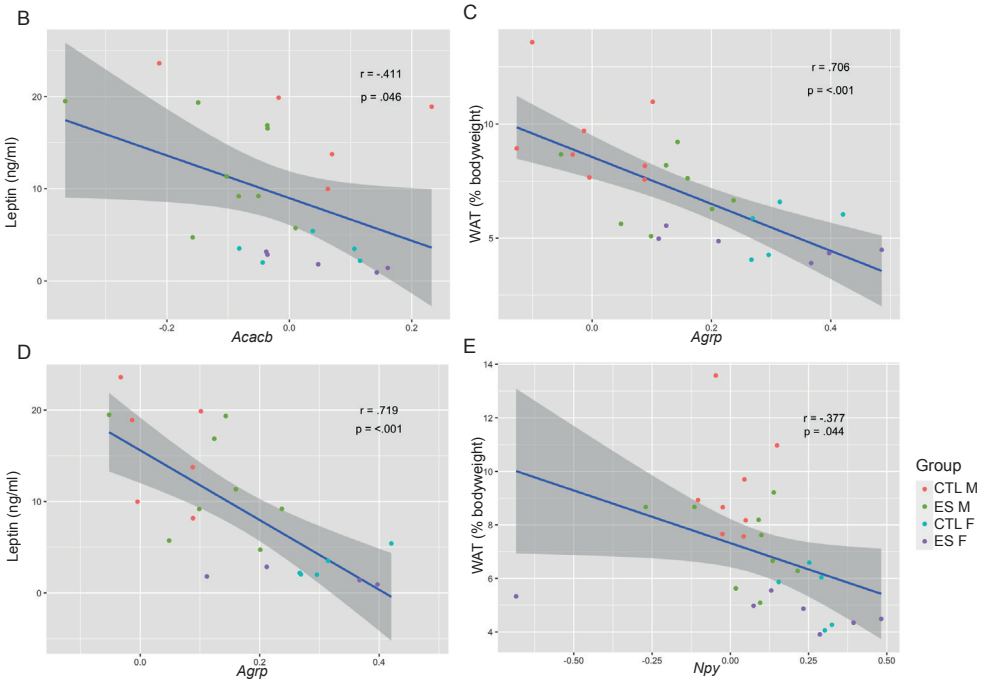

Figure 3. A: Correlations between significantly affected genes and metabolic readouts at P9 and in adulthood. B: ***Acacb*** **expression correlated with leptin levels. C:** ***Agrp*** **expression correlated with white adipose tissue (WAT) levels. D:** ***Agrp*** **expression correlated with leptin levels. E:** ***Npy*** **expression correlated with WAT levels.**

4. Discussion

We presented here for the first time the effect of sex and ES on hypothalamic fatty acid metabolism, hormone receptors and neuropeptides at P9 and in adulthood. Pervasive sex differences emerge at both ages with a targeted impact of ES at P9, which was independent of sex. In particular, at P9, females had higher *Ppargc1a* expression compared to males. Moreover, while *Lepr* expression was higher in females compared to males, ES decreased *Lepr* expression independent of sex. In adulthood, females, compared to males, had higher *Acacb*, *Npy* and *Agrp* expression. We will first discuss the sex differences in hypothalamic

gene expression at P9 and adulthood, followed by the effects of ES, and will relate this to the previously described metabolic phenotype (Yam et al., 2017b, 2017a).

4.1 Sex differences in hypothalamic nutrient sensing

At P9, females had higher *Ppargc1a* and *Lepr* expression compared to males. At this age, females also have a lower bodyweight compared to males, whereas their adiposity (as percentage of bodyweight) is similar (Yam et al., 2017a). PGC-1α (*Ppargc1a)* is known as transcription factor that responds to environmental and energy signals and targets the PPAR family (Sugden et al., 2010). As such, it is involved in the regulation of mitochondrial oxidative metabolism, glucose and lipid homeostasis, and was implicated in metabolic diseases (Lin et al., 2005). Whole body PGC-1α null mice are lean, resistant to diet-induced obesity and show hyperactivity (Lin et al., 2004), and also a selective inactivation of hypothalamic PGC-1α protected mice against diet-induced obesity (Ma et al., 2010). A high-fat diet furthermore downregulated hypothalamic PGC-1α in male but not female mice (Morselli et al., 2014). Since we did not find significant correlations between *Ppargc1a* expression and adiposity or leptin levels, it remains to be determined what is the functional implication of the observed sex differences in *Ppargc1a* expression early in life.

The increased *Lepr* expression in females at P9, despite similar leptin levels in male and female pups (Yam et al., 2017a), suggests there are sex-differences in leptin signaling in the hypothalamus. Sex differences in *Lepr* expression seem to be brain region specific, as hippocampal *LepR* expression was similar in males and females (Yam et al., 2017a). Leptin signaling early in life, rather than modulating food intake, is crucial for the development of the hypothalamic circuitry (Ahima et al., 1998; Bouret et al., 2004; Granado et al., 2012; Mistry et al., 1999). Although in the current study we did not observe sex differences in whole hypothalamic neuropeptide expression levels at this age, with a more anatomically precise approach we previously described higher ARH NPY fiber density in females compared to males at P14 (Yam et al., 2017b).

In adulthood, females had higher *Acacb*, *Agrp* and *Npy* levels in the hypothalamus compared to males. Female mice at this age weigh less, and have lower adiposity independent of bodyweight, as well as lower circulating leptin levels when fed a standard chow diet (Yam et al., 2017a). Acetyl-CoA carboxylases (ACCs) occur in two isozymes (ACC1 (*Acaca*) and ACC2 (*Acacb*)) that are activated in the fed state. While ACC1 plays a key role in fatty acid synthases, ACC2 inhibits β-oxidation (Tong et al., 2006), and higher *Acacb* expression thus indicates more inhibition of β-oxidation. The higher hypothalamic *Acacb* expression in female hypothalamus is in line with a previous report showing higher hepatic *Acacb* levels in female mice (Tuazon et al., 2015). To get a better understanding of what could be the functional implication of such sex-specific change in expression, is notable that polymorphisms in the *Acacb* gene influence the risk on developing metabolic syndrome in humans (Phillips et al., 2010). Whether ACCs are involved in sex differences in metabolism remains to be determined.

Increased *Acacb* expression in females suggests lower *Npy* and *Agrp* gene expression (Rijnsburger et al., 2016). In contrast, we observed higher hypothalamic expression of *Npy* and *Agrp* mRNA in females compared to males. A previous study found lower numbers of NPY expressing cells in the ARH of females (in proestrus) compared to males (Urban et al., 1993), and for *Agrp*, both lower (Viveros et al., 2010) as well as higher (Lensing et al., 2016) expression levels have been described in females. In fact, *Npy* and *Agrp* expression are affected by sex steroids. Testosterone increases *Npy* expression and release (Sahu et al., 1989; Urban et al., 1993), and in females, *Npy* and *Agrp* expression fluctuates throughout the estrous cycle, parallel to the cyclic changes in food intake and bodyweight (Fontana et al., 2014; Olofsson et al., 2009). Although estrous cycle was not a contributing factor in our statistical models, our study was not designed to specifically investigate the effect of estrous cycle in females. Another difference between our study and others is whether the animals were fasted before sacrificing. We fasted the mice for 4 hours, while other studies describe no fasting period (Lensing et al., 2016; Urban et al., 1993; Viveros et al., 2010). Indeed, female rats show an increase in food intake after fasting, while male rats do not show such rebound feeding (Funabashi et al., 2009). This was accompanied by an increased number of activated hypothalamic orexin neurons in females (independent of estrous cycle) but not males. Sex differences in *Agrp* and *Npy* expression dynamics thus require further investigation.

In our study, despite increased *Npy* and *Agrp* expression in females, which stimulate food intake and reduce energy expenditure (Timper and Brüning, 2017), females were leaner and did not eat more of the standard chow than males (food intake after fasting was not investigated) (Yam et al., 2017a). In fact, male rodents are more vulnerable to develop adiposity and have more greatly impaired glucose tolerance upon long-term high-fat diet feeding (Estrany et al., 2013, 2011; Grove et al., 2010). Sex differences in the quantity and functioning of fat depots (Fuente-Martín et al., 2013) likely contribute to this increased metabolic vulnerability in males, and the same applies to the sympathetic innervation and projections from the hypothalamus to the adipose tissue (Adler et al., 2012). We here show that *Acacb*, *Agrp* and *Npy,* genes that were differentially expressed between males and females, correlated with metabolic readouts (adiposity and/or leptin levels). Although these correlation analyses cannot imply causality nor directionality, our data suggest that sex-differences in nutrient sensing pathways could also contribute to this sex-bias in metabolic vulnerability, an avenue worth investigating in the future.

4.2 Effects of ES on nutrient sensing pathways

ES exposure affected hypothalamic nutrient sensing pathways early in life, in a targeted manner. More specifically, at P9, *Lepr* expression was lower in ES-exposed mice. At this age, ES also led to a reduction in circulating leptin levels, that was notably similar in males and females, and did not affect hippocampal *Lepr* expression (Yam et al., 2017a), indicative of brain-region specific effects of ES on *Lepr* expression. Moreover, the reduction in leptin levels

together with the lower hypothalamic *Lepr* expression suggests reductions in leptin signaling during hypothalamic development. In line with the altered hypothalamic leptin signaling at P9, we had previously found that at P14, ES also affected NPY and AgRP fiber density in the ARH and paraventricular nucleus, respectively (Yam et al., 2017b). In our current study, however, ES did not affect the gene expression *Npy* and *Agrp* in whole hypothalamic lysates. This could be due to the different method of assessment (i.e. gene expression of the whole hypothalamus versus fiber density in specific regions), and/or depend on the specific developmental stage due to the rapid development of the hypothalamus during the first two postnatal weeks (Bouret, 2012; Bouret and Simerly, 2006). Despite ES-induced changes in ghrelin levels in females at P9 (Yam et al., 2017b), hypothalamic *Ghsr* levels were not affected by ES, nor did ES affect the expression of genes involved in fatty acid metabolism. Thus, ES alters the leptin system at P9, whereas hypothalamic fatty acid metabolism seems unaffected. Interestingly, the leptin signaling pathway has been suggested to be a top upstream regulator of the effects of chronic stress in the prefrontal cortex, indicating leptin could be one of the key mediators by which stress exerts its effect on the brain (Musaelyan et al., 2020). Whether this might be the case also in the context of ES remains to be addressed.

In adulthood, ES did no longer impact the expression of genes involved in hypothalamic nutrient sensing. While we and others have previously shown lasting effects of ES on adipose tissue levels under basal circumstances and/or in the response to unhealthy diets (Murphy et al., 2018, 2017; Yam et al., 2017a), it remains unclear what leads to these ES-induced metabolic alterations. Investigating the expression of genes involved in nutrient sensing in (whole) hypothalamic samples provides a first indication of potential effects of ES on these pathways. However, it would be important in future studies to investigate these pathways with better spatial resolution and on the protein level, and next to under basal circumstances, also in response to an unhealthy diet. For example, we have previously shown that ES alters hypothalamic microglia in response to a western-style diet, but not under standard dietary circumstances (Ruigrok et al., 2021). Next to the hypothalamus, also other brain circuits and/or peripheral organs could be involved in the ES-induced metabolic risk. For example, we have previously shown ES-induced changes in the adipose tissue gene expression (Ruigrok et al., 2021; Yam et al., 2017a). Adipose tissue has a crucial role in regulating energy homeostasis, and its dysfunction has been related to the negative health consequences associated with obesity (Longo et al., 2019). In addition, the microbiome is implicated in obesity development (Davis, 2016), and has been shown to be affected by ES exposure (O'Mahony et al., 2009). ES has furthermore been shown to affect the reward circuitry, which is involved in the hedonic aspects of food intake, an important aspect of human obesity development, and there is initial evidence that food preference indeed is affected by ES exposure (Jackson and Vaughn, 2019; Lussana et al., 2008). In addition to the above described sex differences in the hypothalamus and adipose tissue, sex differences in the microbiome and reward circuitry have also been reported (Becker and Chartoff, 2019; Fuente-Martín et al., 2013; Kim et al., 2020), but it remains to be determined whether ES

affects these systems in a sex-dependent manner. More research is thus needed to further elucidate how ES increases the risk for developing obesity, potentially in a sex-specific manner.

Conclusion

To the best of our knowledge, this is the first description of effects of ES and sex on hypothalamic nutrient sensing pathways. Clear sex differences were present in these pathways, while ES induced a minor modulation early in life that was similar in both sexes. Our data contribute to a further understanding of sex differences in the circuits that regulate metabolism and the impact of ES on these pathways.

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Supplementary table 1. Primers for RT-PCR

Function	Gene	Forward primer	Reverse primer
Fatty acid metabolism	*Acacb*	AGATGGCCGATCAGTACGTC	GGGGACCTAGGAAAGCAATC
	CPT1a	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
	CPT1c	CGTGTCTGGAATGACTTTCTTGCT	TCTCCATCAGTCCTAGGGAAGG
	Fasn	GCGCTCCTCGCTTGTCGTCT	TAGAGCCCAGCCTTCCATCTCCTG
	Ppara	ATGCCAGTACTGCCGTTTTC	GGCCTTGACCTTGTTCATGT
	Ppard	AACCCACGGTAAAGGCAGTC	CTTCCTCTTTCTCCTCTTCCCG
	Pparg	GTCTCACAATGCCATCAGGTT	CAAATGCTTTGCCAGGGCTC
	Ppargc1a	TGCTAGCGGTTCTCACAGAG	AGTGCTAAGACCGCTGCATT
Receptors	*Ghsr*	GATGCTTGCTGTGGTGGTGTT	GAAGAGGACAAAGGACACCAGG
	Insr	GCCACTAATCCTTCTGTCCCC	AGGTAGTGTGTGATGTTGCCA
Neuropeptides	*Npy*	TCACCAGACAGAGATATGGCAA	AAGTCGGGAGAACAAGTTTCA
	Agrp	AAGTCTGAATGGCCTCAAGAAGA	GACTCGTGCAGCCTTACACAG
	Pomc	CGAGGCCTTTCCCCTAGAGT	CCAGGACTTGCTCCAAGCC
Reference genes	*Rpl13a*	CCCTCCACCCTATGACAAGA	CTGCCTGTTTCCGTAACCTC
	Rplp0	GCTTCATTGTGGGAGCAGACA	CATGGTGTTCTTGCCCATCAG
	Tbp	GTCATTTTCTCCGCAGTGCC	GCTGTTGTTCTGGTCCATGAT
	Tuba1a	CCCTCGCCTTCTAACGCGTTGC	TGGTCTTGTCACTTGGCATCTGGC

CHAPTER 6

6

Effects of early-life stress on peripheral and central mitochondria in male mice across ages

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Abstract

Exposure to early-life stress (ES) increases the vulnerability to develop metabolic diseases as well as cognitive dysfunction, but the specific biological underpinning of the ES-induced programming is unknown. Metabolic and cognitive disorders are often comorbid, suggesting possible converging underlying pathways. Mitochondrial dysfunction is implicated in both metabolic diseases and cognitive dysfunction and chronic stress impairs mitochondrial functioning. However, if and how mitochondria are impacted by ES and whether they are implicated in the ES-induced programming remains to be determined. ES was applied by providing mice with limited nesting and bedding material from postnatal (P) day 2-P9, and studied metabolic parameters, cognitive functions and multiple aspects of mitochondria biology (i.e. mitochondrial electron transport chain (ETC) complex activity, mitochondrial DNA copy number, expression of genes relevant for mitochondrial function, and the antioxidant capacity) in muscle, hypothalamus and hippocampus at P9 and late adulthood (10-12 months of age). We show that ES altered bodyweight (gain), adiposity and glucose levels at P9, but not in late adulthood. At this age however, ES exposure led to cognitive impairments. ES affected peripheral and central mitochondria in an age-dependent manner. At P9, both muscle and hypothalamic ETC activity were affected by ES, while in hippocampus, ES altered the expression of genes involved in fission and antioxidant defence. In adulthood, alterations in ETC complex activity were observed in the hypothalamus specifically, whereas in muscle and hippocampus ES affected the expression of genes involved in mitophagy and fission, respectively. Our study demonstrates that ES affects peripheral and central mitochondria biology throughout life, thereby uncovering a converging mechanism that might contribute to the ES-induced vulnerability for both metabolic diseases and cognitive dysfunction, which could serve as a novel target for intervention.

1. Introduction

Exposure to early-life stress (ES) programs individuals for life, leading to an increased vulnerability to develop both metabolic diseases, such as obesity, as well as cognitive dysfunction (Chugani et al., 2001; Danese and Tan, 2014). Such programming effects of ES have also been shown in preclinical studies (Maniam et al., 2014; Naninck et al., 2015; Walker et al., 2017; Yam et al., 2017a, 2017b). There is evidence that ES affects metabolic functions by altering the adipose tissue, glucose metabolism and hypothalamic neuropeptides (Maniam et al., 2014; Yam et al., 2017a, 2017b), and leads to a differential response to an unhealthy diet later in life with more fat accumulation compared to controls (Yam et al., 2017a). Furthermore, exposure to ES in rodents has been shown to lead to cognitive impairments and altered hippocampal structure and function (Naninck et al., 2015; Walker et al., 2017). Interestingly, metabolic and cognitive disorders are often comorbid (Dye et al., 2017), suggesting that converging pathways might be involved in how ES increases the vulnerability to both metabolic and cognitive dysfunction. We hypothesize that mitochondria could be such a converging entity because they are i) the essential cellular energy power houses across most cells in our body (Nolfi-Donegan et al., 2020), ii) critical in both responding and adapting to stress (Hoffmann and Spengler, 2018; Picard et al., 2014), and iii) have been implicated in both metabolic disease and cognitive dysfunction (Guo et al., 2017; Khacho et al., 2017; Sivitz and Yorek, 2010).

Mitochondria generate energy derived from glucose, fatty acids or protein in the form of adenosine triphosphate (ATP), via a process called oxidative phosphorylation (OXPHOS). OXPHOS is regulated by protein complexes (complex (C)I-V) encoded in both the mitochondrial DNA (mtDNA) and nuclear DNA (Nolfi-Donegan et al., 2020). Electrons travel from complex I to IV (together called the electron transport chain (ETC)) located in the inner mitochondrial membrane thereby creating a proton gradient which is used as a driving force for ATP production by complex V. In this process, reactive oxygen species (ROS) are produced as a natural side product, which can serve as signalling molecules but also damage mitochondria and other components of the cell (Nolfi-Donegan et al., 2020). Mitochondria are highly dynamic organelles that readily sense and respond to internal and environmental changes (Manoli et al., 2007). During acute stress, energy demand increases to facilitate the flight or fight response. In response to stress mitochondria increase their energy production via mitochondrial biogenesis and increasing the activity of ETC complexes, and generate signals to promote adaptation (Manoli et al., 2007; Picard et al., 2014). While these adaptations are beneficial on the short-term, chronic stress exposure seems detrimental for mitochondria, resulting in accumulation of mtDNA damage, increased ROS production, decreased energy producing capacity and increased mitochondrial fragmentation (Manoli et al., 2007; Picard et al., 2014; Picard and McEwen, 2018). Importantly, mitochondria do not only respond to stress, but they may also drive the stress response. Glucocorticoid synthesis depends on mitochondria (Midzak and Papadopoulos, 2016), and mitochondrial dysfunction

has been shown to alter the neuroendocrine, metabolic and behavioural response to stress (Emmerzaal et al., 2020; Picard et al., 2015). Thus, mitochondria both mediate the stress response and are affected by stress.

Mitochondrial dysfunction has been implicated in both metabolic diseases and cognitive disorders. For example, changes in muscle mitochondrial dynamics and function have been linked to (the development of) insulin insensitivity (Sivitz and Yorek, 2010). Moreover, in the hypothalamus, a brain region critical for the regulation of energy homeostasis, mitochondria are involved in the regulation of food intake and fat accumulation upon high-fat diet feeding (Kim et al., 2019), whereas in the hippocampus, mitochondrial dysfunction has been related to disrupted synaptic transmission and a loss of adult neurogenesis, both critical for proper cognitive functioning (Guo et al., 2017; Khacho et al., 2017).

Although the effects of chronic (adult) stress on mitochondria have been described more extensively (Picard and McEwen, 2018), whether ES programs mitochondria and if this is involved in disease risk, has only recently gained interest (Hoffmann and Spengler, 2018; Zitkovsky et al., 2021). Studies in humans reported that ES exposure increases leukocyte mtDNA copy number (mtDNAcn) (Ridout et al., 2020), as well as mitochondrial respiration and ROS production in peripheral blood mononuclear cells (Boeck et al., 2016). In mice, ES induced by either limiting the bedding and nesting material (LBN) or maternal separation (MS) reduced ATP production, mitochondrial respiration and complex I activity and increased ROS production in the hippocampus at 2-4 months of age (Amini-Khoei et al., 2017; Eagleson et al., 2020), and MS in rats led to alterations in mitochondrial protein levels in the prefrontal cortex (PFC) at 3 months of age (van Zyl et al., 2016). If such ES-induced mitochondrial alterations last into late adulthood is unknown, and similarly, if ES effects on mitochondria are brain region and/or tissue specific is under-investigated.

Due to their central role in mediating and adapting to stress, metabolic disease and cognitive function, we hypothesized that ES alters mitochondrial functions both peripherally and centrally up into late adulthood. We used an established ES mouse model, in which LBN is provided from postnatal day (P)2-P9, and studied the activity of the ETC complexes, antioxidant activity, mtDNAcn, as well as the expression of genes relevant for mitochondrial function in hippocampus, hypothalamus and muscle at two ages (immediately after the ES exposure as well as in late adulthood). To increase our understanding in whether there could be a role for mitochondria in ES-induced programming, we relate the mitochondrial measures to cognitive function and metabolic readouts (bodyweight, adiposity and glucose levels). We show that ES affects metabolic parameters at P9 and cognitive functions in adulthood, that peripheral and central mitochondria are affected by ES in an age- and tissue-dependent manner, and that muscle and hypothalamic mitochondrial functions correlate to metabolic parameters at P9, but not in adulthood. We provide evidence for direct and long-term effects of ES on mitochondria and identify mitochondria as a potential target for intervention strategies.

2. Materials and methods

2.1 Mice and breeding

Animals were kept under standard housing conditions (temperature 20–22C, 40–60% humidity, 12/12h light/dark schedule, standard chow and water *ad libitum*). For the long-term studies, animals were weaned at postnatal day 21 and group housed with same sex littermates in conventional type III cages (2-4 per cage). Only male mice were used for these studies. All experimental procedures were conducted under national law and European Union directives on animal experiments and were approved by the animal welfare committee of the University of Amsterdam.

Experimental animals were bred in house to standardize the perinatal environment. 8-10 week old C57BL/6J female and male mice were purchased from Envigo Laboratories B.V. (Venray, The Netherlands) and allowed to habituate for 1-2 weeks. Then, to allow for mating, two primiparous females were housed together with one male for one week. Females were housed together for another week in clean cages with nesting material (square piece of cotton) to practice. Afterwards, females were individually housed in a conventional type II cage with filtertop and nesting material, and placed in a ventilated cabinet providing a standardized and quiet environment. From day 18 onwards, females were monitored daily before 09:00 A.M. When a litter was born, the previous day was designated as P0.

The following cohorts were used for P9 experiments: complex activity (muscle, hypothalamus, hippocampus), gene expression (muscle and hippocampus), mtDNAcn (muscle and hippocampus), adipose tissue levels, bodyweight gain, muscle strength, glucose and CORT (sacrificed in the light phase): CTL: n=7 (2 litters), ES: n=10 (3 litters); gene expression (hypothalamus) (sacrificed in the light phase): CTL: n=8 (3 litters), ES: n=9 (4 litters); mtDNAcn (hypothalamus) (sacrificed in the light phase): CTL: n=8 (5 litters), ES: n=8 (4 litters); antioxidant capacity (muscle, hypothalamus, hippocampus) (sacrificed in the light phase): CTL: n=9 (5 litters), ES: n=9 (5 litters). For adult experiments, the following cohorts were used: complex activity (muscle, hypothalamus, hippocampus), gene expression (muscle, hippocampus), mtDNAcn (muscle, hippocampus), bodyweight, adipose tissue levels, behaviour, glucose and CORT (10-12 months of age; sacrificed in the dark phase): CTL: n=10 (7 litters), ES: n=16 (8 litters). Gene expression (hypothalamus) (6 months of age, sacrificed in the dark phase): CTL: n=8 (3 litters), ES: n=9 (3 litters); mtDNAcn (hypothalamus) (10-12 months of age, sacrificed in the dark phase) CTL: n=7 (5 litters), ES: n=11 (7 litters); antioxidant capacity (muscle, hypothalamus, hippocampus) (10-12 months of age, sacrificed in the light phase): CTL: n=12 (3 litters), ES: n=10 (3 litters).

2.2 Early-life stress paradigm

The early-life stress (ES) paradigm consisted of the LBN model as described previously (Walker et al., 2017). ES was applied from postnatal day (P)2-P9. To standardize litter size and composition, large litters were culled to 6 pups and no litters of less than 5 pups or litters

with only one sex were included. ELS cages consisted of a little amount of sawdust on the bottom, covered with a fine-gauge stainless steel mesh, and half a square piece of cotton nesting material (2.5 x 5 cm, Tecnilab-BMI, Someren, The Netherlands), while control (CTL) cages had a standard amount of sawdust and one square piece of cotton nesting material (5 x 5 cm). All cages were covered with a filtertop and placed in ventilated cabinets until P9. At P2 and P9 the pups, dams, and food were weighted. At P9, all litters were moved to new cages containing standard amounts of sawdust and were left undisturbed until weaning at P21. See figure 2 for the experimental overview.

2.3 Muscle strength test

At P9, CTL and ES animals were subjected to a muscle strength task. Pups were placed on a fine mesh, which was slowly inverted from a 90° (horizontal) angle to a 180° (vertical) angle. The angle at which the pup would lose grip was reported. This procedure was repeated 3 times per pup and an average was calculated as an indication of muscle strength.

2.4 Behaviour tests

At 10 months of age, a subset of mice (n=10 for CTL, n=16 for ES) underwent behaviour test to assess cognitive functions, in the following order: object location task (OLT), object recognition task (ORT), Morris water maze (MWM). Mice were housed on a reversed day/light cycle starting three weeks before the testing, and tested in the dark (active) phase between 1 and 5 P.M. The experimenter performing the tasks was blinded for the condition of the animals. All behaviors were recorded by a video camera connected to a computer with Ethovision software (Noldus, The Netherlands). Prior to testing, mice were handled to habituate to the experimenter for 3 consecutive days.

The ORT is a non-spatial, emotionally neutral memory test in which mice have to discriminate a novel object from an object they have explored before. First, mice were habituated to the test arena for 5 min for 3 consecutive days. The testing arena consisted of a rectangular plastic box (dimensions: 23.5*31 cm, height wall: 27 cm) with a little amount of clean sawdust covering the floor. Between trials the sawdust was refreshed and the arena cleaned with 25% ethanol. During the training trial mice were exposed to two identical objects (plastic egg-shaped object) that were placed at the midline of the arena with an equal distance between the objects and the wall and allowed to explore them for 5 min. In the testing trial (5 min) one of the objects was replaced for a novel object (tall LEGO tower). We modified the intertrial time (time between training and testing trial) to the age of the animals. As these are rather old animals, we chose a short (40 min) intertrial time. Exploration of the objects in the training and testing trials was scored manually by an experimenter blinded for condition. The ratio of novel/familiar object exploration time (in the testing trial) was used as a memory index. Mice that spent <10 seconds exploring were excluded from the analysis.

The OLT is a spatial memory test in which the location of an object should be remembered. The same arena and setup was used as for the ORT. First, mice were habituated to the empty arena for 5 min. The next day, the training session took place in which the mice were exposed to two novel identical objects (white coffee cups) placed on the midline with an equal distance from each other and the walls. Mice were allowed to explore the objects for 5 min. In the testing trial (40 min later), one object was moved to a novel position in the arena and mice were reintroduced into the arena for 5 min. Time spent exploring the objects was scored manually and a novel/familiar ratio was calculated similar as for the ORT.

The MWM is a spatial memory test in which mice are supposed to find a platform that is hidden under the water surface by using visual cues on the walls. The water was made opaque with latex, and the water temperature was kept at 23±1 °C. After each trial, mice were placed under a heating lamp to prevent hypothermia. At day 1 (2 trials, 1 minute each), mice were placed in the pool while the platform was visible in the middle of the pool, so they could become aware of the presence of the platform. No spatial cues were available yet in this phase. If by the end of the 1 minute trial the mouse had not climbed on the platform by itself, the mouse would be guided to the platform and allowed to sit on it for 5-10 seconds. In the following days (training phase), spatial cues were placed on the walls and the platform was hidden in one of the quadrants of the pool (always the same place) under the water surface. During the training phase, mice underwent 3 trials per day (max 1 minute each). When the platform was found, the time was noted, mice were left on the platform for 5-10 seconds before taken out of the pool, and placed under a heating lamp. If the platform was not found after 1 minute, mice were guided to the platform and allowed to sit on it for 5-10 seconds. Due to technical problems, mice underwent 1 training day, followed by a 4 week break, and then another 3 training days. 24 hours after the training phase, a probe trial was conducted in which the platform was removed (but the spatial cues remained) and mice were allowed to swim for 1 minute. The time spent in the quadrant where the platform used to be, was measured. Learning and memory deficits can be shown by a slower learning curve, or by showing less memory (spending less time in the target quadrant) in the probe trail. Animals that at the final training day failed to perform the task (50-60 seconds to find the platform), were excluded from the analysis of the acquisition phase and probe trial. This was the case for 2 out of 10 CTL animals (20%), and 4 out of 16 ES animals (25%).

2.5 Tissue collection

At P9, 6 months (hypothalamic gene expression only) and 10-12 months of age, animals were sacrificed (Fig. 1). Mice were sacrificed by rapid decapitation. Trunk blood was collected in EDTA-coated tubes (Sarstedt, Germany, 20.1288), centrifuged, and plasma was stored at -40°C. Hypothalamus, hippocampus and muscle (quadriceps femoris) were rapidly dissected, snap frozen and stored at -80°C. Inguinal (P9) or gonadal (adulthood) adipose tissue was carefully dissected and weighted.

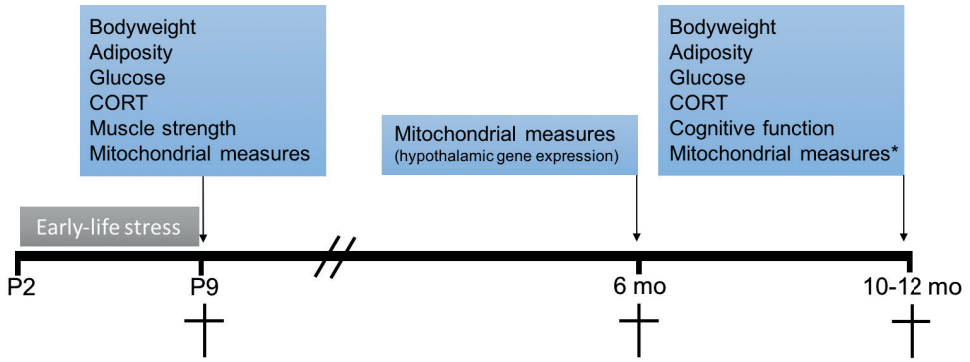


Figure 1. Experimental overview. Multiple cohorts of animals were exposed to control (CTL) or early-life stress (ES) conditions from postnatal day (P)2 to P9, and sacrificed at either P9 or in adulthood (10-12 months of age). Metabolic (P9 and adulthood) and cognitive measures (adulthood only) were taken, together with multiple measures involving mitochondrial function (ETC complex activity, gene expression, mtDNAcn, antioxidant capacity assay). *all mitochondrial measures in adulthood were taken at 10-12 months of age, except hypothalamic gene expression which was measured at 6 months of age.

2.6 Glucocorticoid and glucose measurements

Glucose was measured with a FreeStyle Optium Neo glucose meter (Abbott Diabetes Care Ltd., United Kingdom). Corticosterone levels were measured with either a radioimmunoassay kit (adult measures; MP Biomedicals, The Netherlands) or ELISA (P9 measures; Tecan, Germany, RE52211).

2.7 ETC complex activity measurements

To measure the activity of the mitochondrial complexes in hippocampus, hypothalamus and muscle, tissue was homogenized with a glass-glass potter tube in ice-cold SEF buffer (0.25 M sucrose, 2 mM EDTA in 10 mM kPi, pH 7.4) to obtain a homogenate of approximately 5%. Next, the samples were centrifuged at 600 *g* for 10 min at 2°C. The supernatant was snap frozen in liquid nitrogen and stored at –80°C until measurements were performed. Activity of complex I-IV, as well as the activity of succinate:cytochrome c oxidoreductase (SCC), and citrate synthase (CS) activity were measured. Measurements were performed with spectrophotometric assays on a KoneLab 20XT analyzer (Thermo Scientific) following standard procedures and as previously described (Emmerzaal et al., 2020). Complex I activity was measured in two ways: via the reduction of decylubiquinone (CID) and via reduction of coenzyme Q1 (CIQ). SCC activity gives an indication of the complex II > coenzyme Q > complex III route, while CS activity is a proxy for the number of mitochondria. Complex I-IV and SCC measurements were normalized for CS as a proxy for mitochondrial content.

2.8 RNA and DNA extraction

RNA and DNA were obtained from the same tissue sample using TRIzol (Invitrogen), according to manufacturer's instructions. In short, brain (hippocampus and hypothalamus)

and muscle tissue was homogenized in TRIzol. Muscle samples were centrifuged for 10 min at 12000g and the supernatant was used for further processing. Samples were incubated at room temperature (RT) for 5 min, and chloroform was added. After 15 min of centrifuging at 12000g, the aqueous phase containing RNA was transferred to a new tube, while the remaining organic and interphase were kept for DNA extraction. RNA was cleaned and purified with one 100% isopropanol wash and two 75% ethanol washes, before being diluted in miliQ water. RNA was stored at -80 ˚C until cDNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen). cDNA was stored at -20 ˚C until further use.
To obtain DNA from the remainder, samples (containing the organic and interphase) were treated with RNase and incubated at 37˚C for two hours. Next, 100% ethanol was added to precipitate the DNA, samples were centrifuged, washed three times with sodium citrate in 10% ethanol (pH 8.5), and two times with 75% ethanol. The DNA pellet was diluted in miliQ water and kept on 55˚C for 30 min to solubilize. DNA was stored at -20˚C until further use.

2.9 Real-time PCR

Relative gene expression and mtDNAcn were assessed by RT-PCR performed on a 7500 Real-time PCR system (Applied Biosystems). Hot FirePol EvaGreen Mastermix (Solis Biodyne), 150 nM of gene specific forward and reverse primers and 0.135 ng/µl cDNA template were added to the reaction mix. Primers (Eurogentec, supplementary table 1) all had an efficiency between 90-110%. Cycling conditions were as follows: 15 min polymerase activation at 95°C and 40 cycles of replication (15 s at 95°C, 20 s at 65°C, and 35 s at 72°C). The ΔΔCt method was used to calculate relative gene expression, and was performed in Qbase+ software (Biogazelle). For gene expression analysis, expression was normalized for two reference genes (for P9 and adult muscle and hippocampus: *Rpl0* and *Rpl19*; for P9 hypothalamus: *Rpl0* and *Rpl13a*; for adult hypothalamus: *Tbp* and *Tuba1a*), which were not affected by experimental conditions and tested for stability in Qbase+. For mtDNAcn analysis, the ratio of mitochondrial DNA to nuclear DNA was calculated.

2.10 Antioxidant capacity assay

Snap-frozen tissue was homogenized in 500 µl ice cold PBS, incubated on ice for 10 minutes, and centrifuged for 5 minutes at 4°C on top speed. The supernatant was used for antioxidant capacity measurements. Total antioxidant capacity was measured with a colorimetric kit (Abcam, ab65329), in which Cu^{2+} ion is reduced to Cu^{+} by both small molecules and proteins. Cu^{+} is chelated with a colorimetric probe, and absorbance at OD 570nm is proportional to the antioxidant capacity of the sample. To correct for variation in tissue input, antioxidant capacity was normalized for protein concentration in the sample. Protein concentration was measured using a BCA Protein Assay (Pierce Thermo Fischer, 23225).

2.11 Statistical approach

Data were analysed with SPSS 25.0 (IBM software), and Graphpad Prism 6 (Graphpad software). All data are presented as mean ± standard error of the mean (SEM), and when $p<0.05$, data was considered statistically significant. For the statistical analysis of gene expression, mtDNAcn, and total antioxidant capacity data, log transformed values were used. For animals that performed in all 3 behaviour tasks, a composite learning z-score was computed by calculating a z-score for each task ($z = (x-\mu)/\sigma$), with x being the animal's score, μ being the group mean, and σ being the group standard deviation. The z-scores for each task were then averaged to calculate a composite learning score. In addition, we calculated a composite ETC activity score by computing mean-centered scores for each complex (for complex I, measurements IQ and ID were averaged into a single complex I score). Mean-centered scores of all 4 complexes were then added together to obtain the ETC activity score. The formula was as follows: ETC activity score = $((x-\mu)_{CID +} (x-\mu)_{CIQ})/2 + (x-\mu)_{CII} + (x-\mu)_{CIII} + (x-\mu)_{CIV,}$ with x being the activity of a specific complex for an animal, and μ being the group average for the respective complex.

Outliers were identified in SPSS and removed from the dataset, before testing the data for the assumptions for parametric testing. For CTL/ES comparisons, data was analysed with either an independent Students' *t*-test or Mann-Whitney U. For behavioural analysis, to test if animals performed above chance level in the OLT, ORT and MWM probe trial, one sample t-tests were used, and a repeated measures ANOVA was performed to test for learning deficits in the acquisition phase of the MWM. Multiple animals from one litter were included in these studies, resulting in nested data. We therefore always tested for potential contributing effects of litter to the outcome variable and corrected when necessary by performing mixed model analysis with litter as random factor.

For correlations between the mitochondrial (ETC activity score) and metabolic readouts, CORT and cognitive function, Pearson correlation was performed. For the correlations between CORT and ETC activity scores in adulthood, the PM CORT levels were used (on reversed day/night cycle). Pearson correlations were calculated based on complete pairwise cases, and correlation coefficients were tested against critical values on a two-tailed distribution (alpha=0.05).

3. Results

3.1 ES leads to lower bodyweight gain, adiposity, glucose levels and muscle strength at P9

ES exposure led to a lower bodyweight gain from P2 to P9 in the offspring ($t(15)=4.166$, $p=0.001$) (Fig. 2A). This was accompanied by a reduction in fat mass ($t(6.298)=6.596$, $p<0.001$) and lower blood glucose levels ($t(13.64)=2.964$, $p=0.01$) at P9 (Fig. 2B,C). CORT

levels were not affected by ES at P9 (fig 2D), but ES-exposed offspring had reduced muscle strength (t(8.941)=4.869, p=0.001) (Fig. 2E).

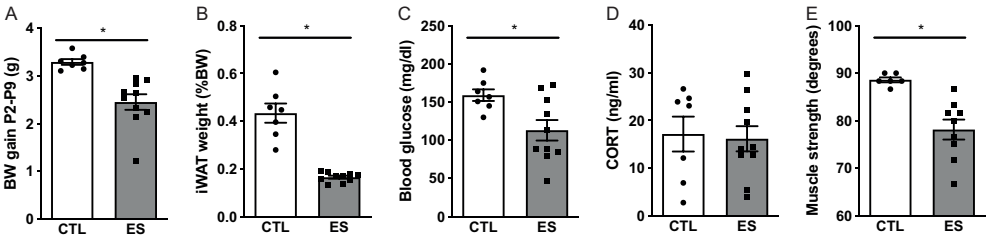


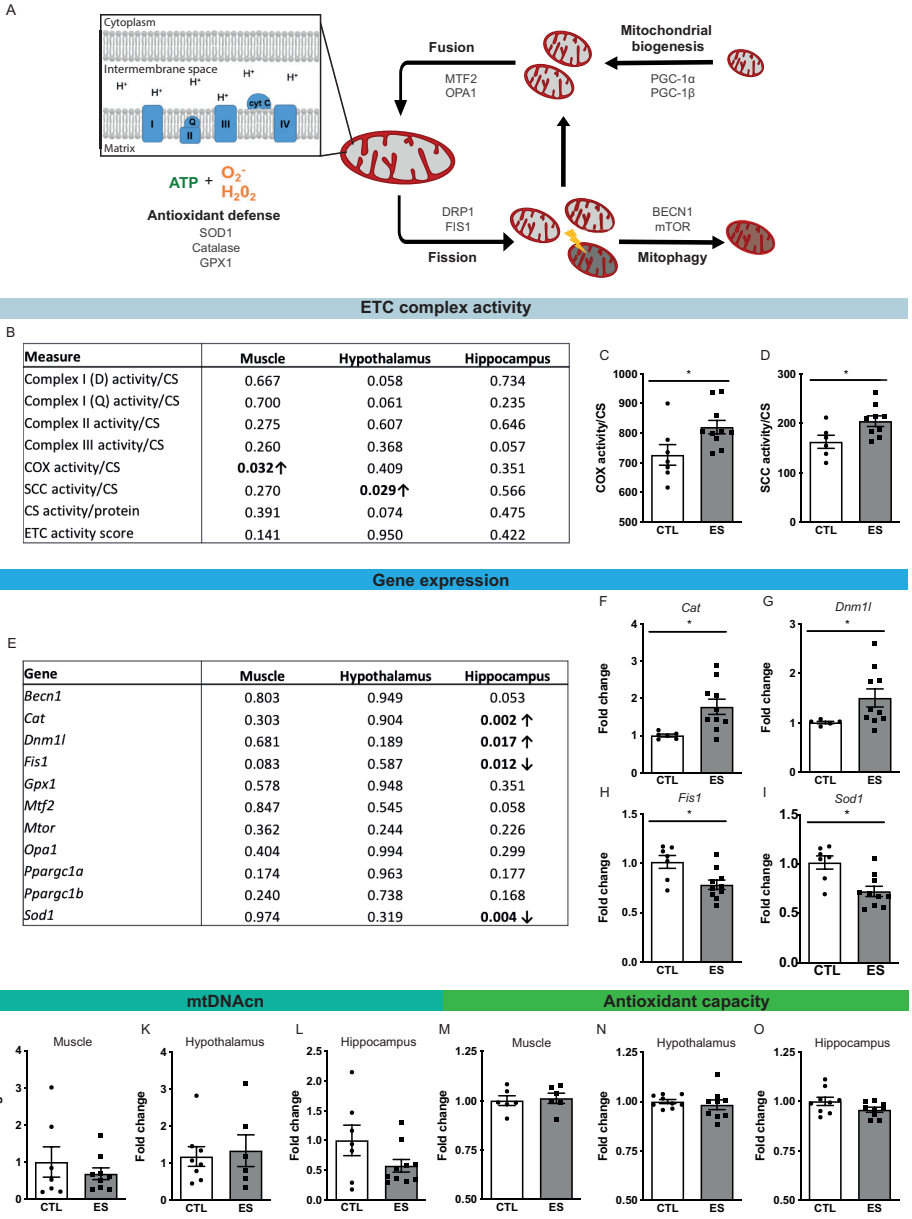
Figure 2. ES-induced phenotype at P9. A: ES mice have a lower bodyweight (BW) gain from P2-P9 compared to CTL mice. B: Adiposity (inguinal depot as percentage of BW) is reduced after ES exposure. C: ES exposed mice have lower blood glucose levels. D: Glucocorticoid (CORT) levels are not affected by ES at P9. E: muscle strength is reduced in ES mice. Indicated is mean ± SEM, p<0.05.

3.2 ES affects peripheral and central mitochondria at P9

We investigated the effects of ES on multiple aspects of mitochondria biology in muscle, hypothalamus and hippocampus (Fig. 3A). In muscle, ES increased cytochrome C oxidase (COX, CIV) activity (t(15)=-2367, p=0.032), whereas in the hypothalamus, ES increased cytochrome C oxidoreductase (SCC) activity indicating increased activity in the CII > CoQ > CIII route (t(13)=-2.448, p=0.029) at P9 (fig 3B-D). No effects of ES were observed in the activity of the individual complexes in the hippocampus, nor in the citrate synthase (CS) activity (indication of the number of mitochondria) or the composite ETC activity score in any of the measured tissues. Furthermore, ES affected the expression of several key (nuclear encoded) genes important for mitochondrial function and dynamics in the hippocampus, but not muscle or hypothalamus at P9 (Fig. 3E). Hippocampal expression of *Cat* t(10.699)=-4.182, p=0.002) and *Dnm1l* (t(9.792)=-2.857, p=0.017) were increased by ES (Fig. 3F,G), while expression of *Fis1* (t(15)=2.876, p=0.012) and *Sod1* (t(15)=3.397, p=0.004) in the hippocampus were decreased by ES (Fig. 3H,I). We did not observe effects of ES on mtDNAcn in muscle, hypothalamus or hippocampus (Fig. 3J-L), nor in antioxidant capacity in any of these tissues at P9 (Fig. 3M-O).

3.3 ES affects cognitive functions, but not bodyweight, abdominal adiposity and glucose levels in late adulthood

In late adulthood, ES did not affect bodyweight, abdominal adiposity or plasma glucose levels (Fig. 4A-C). CORT levels measured at 08:00 AM and 8:00 PM (reversed day-night cycle) were not affected by ES (Fig. 4D,E). However, ES exposure led to memory deficits specifically in tasks related to spatial memory. In both the OLT and ORT, exploration time of the left and right object during the training phase was not different between CTL and ES animals (Fig. 4F,H). In the OLT, in contrast to the CTL group (t(7)=4.366, p=0.003), ES mice did not perform above chance level (t(11)=2.041, p=0.066) (Fig. 4G).



Measure	Muscle	Hypothalamus	Hippocampus
Complex I (D) activity/CS	0.667	0.058	0.734
Complex I (Q) activity/CS	0.700	0.061	0.235
Complex II activity/CS	0.275	0.607	0.646
Complex III activity/CS	0.260	0.368	0.057
COX activity/CS	**0.032↑**	0.409	0.351
SCC activity/CS	0.270	**0.029↑**	0.566
CS activity/protein	0.391	0.074	0.475
ETC activity score	0.141	0.950	0.422

Gene	Muscle	Hypothalamus	Hippocampus
Becn1	0.803	0.949	0.053
Cat	0.303	0.904	**0.002 ↑**
Dnm1l	0.681	0.189	**0.017 ↑**
Fis1	0.083	0.587	**0.012 ↓**
Gpx1	0.578	0.948	0.351
Mtf2	0.847	0.545	0.058
Mtor	0.362	0.244	0.226
Opa1	0.404	0.994	0.299
Ppargc1a	0.174	0.963	0.177
Ppargc1b	0.240	0.738	0.168
Sod1	0.974	0.319	**0.004 ↓**

Figure 3. Effects of ES on mitochondria at P9. A: Overview of mitochondrial functions and dynamics. B-D: ES affects ETC complex activity at P9. B: p values for the comparison between CTL and ES for each complex, arrows indicate the direction of a significant ES effect. C: ES increases COX activity in muscle. D: SCC activity in the hypothalamus is higher in ES-exposed offspring. E-I: Effects of ES on the expression of genes important for mitochondrial function. E: p values for the comparison between CTL and ES for each gene, arrows indicate the direction of a significant ES effect. F: ES increased the hippocampal expression of *Cat*. G: ES increased hippocampal *Drp1* expression. H: *Fis1* expression in the hippocampus was decreased by ES. I: *Sod1* expression was lower in ES-exposed mice. J-L: ES does not affect mtDNAcn in muscle (J), hypothalamus (L) and hippocampus (M) at P9. M-O: ES does not affect the total antioxidant capacity at P9 in muscle (M), hypothalamus (N) and hippocampus (O). Indicated is mean ± SEM, p<0.05.

Performance in the ORT was not affected by previous ES exposure as both CTL (t(5)=5.051, p=0.004) and ES (t(10)=2.978, p=0.014) mice performed above chance level (Fig. 4I). In addition, CTL and ES mice both learned to find the platform in the acquisition phase of the MWM (F_{time}(2)=13.625, p<0.001) (Fig. 4J). However, during the probe trial, while CTL and ES mice swam similar distances (Fig. 4K), only CTL animals spent significantly more time than chance (25%) in the target quadrant during the probe trial (CTL: t(6)=3.682, p=0.01; ES: t(11)=2.055, p=0.064) (Fig. 4L). Finally, when taking the performance in all cognitive tasks together in a composite z-score, it was shown that ES animals overall performed worse compared to CTL animals (t(10)=2.555, p=0.029) (Fig. 4M).

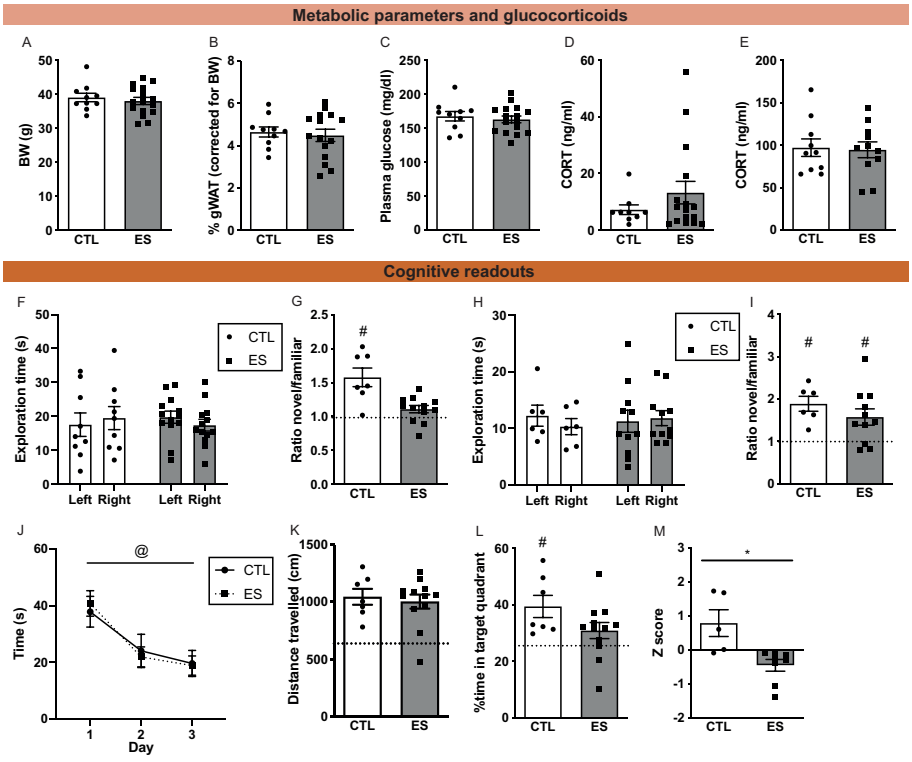


Figure 4. ES effects on metabolic and cognitive parameters in adulthood. A-E: ES does not affect bodyweight (A), abdominal adiposity (B), CORT levels at 08:00 PM (D) or 08:00 AM (E). F: In the training phase of the OLT, exploration time was similar for both objects, and similar for CTL and ES animals. G: only CTL animals preferred the novel location in the OLT testing phase. H: Exploration in the ORT training phase was similar for both objects. I: both CTL and ES animals preferred the novel object over the familiar one. J: CTL and ES animals both learned to find the platform in the MWM. M: ES did not affect the travelled distance in the MWM probe trial. L: only CTL animals spent more time in the target quadrant during the MWM probe trial. M: ES animals perform worse in the cognitive tasks indicated by the composite z-score. Indicated is mean ± SEM, p<0.05. * different between CTL and ES; # performed above chance level; @ main effect of time.

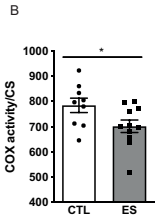
3.3 ES affects mitochondria in adulthood

In adulthood ES decreased COX activity in the hypothalamus (t(18)=2.21, p=0.04) without affecting any of the other complexes or the composite ETC activity score (Fig. 5A,B). Complex activity in hippocampus and muscle were not affected by ES. Moreover, ES exposure increased the expression of *Mtor* in muscle, a protein involved in mitophagy (U=37.5, p=0.023), as well as the expression of *Fis1* in the hippocampus (t(19)=-2.304, p=0.033) in adulthood (Fig. 5C-E). The expression of the other genes, mtDNAcn and the antioxidant capacity were not affected by ES in any of the studied tissues (Fig. 5F-K).

ETC complex activity

A

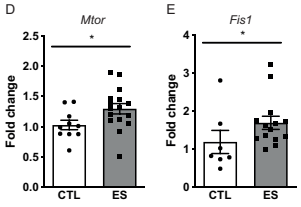
Measure	Muscle	Hypothalamus	Hippocampus
Complex I (D) activity/CS	0.549	0.999	0.855
Complex I (Q) activity/CS	0.211	0.959	0.928
Complex II activity/CS	0.520	0.180	0.856
Complex III activity/CS	0.715	0.074	0.108
COX activity/CS	0.578	**0.040↓**	0.737
SCC activity/CS	0.676	0.210	0.172
CS activity/protein	0.908	0.594	0.563
ETC activity score	0.496	0.133	0.221



Gene expression

C

Gene	Muscle	Hypothalamus	Hippocampus
Becn1	0.563	0.707	0.575
Cat	0.828	0.927	0.846
Dnm1l	0.897	0.716	0.803
Fis1	0.942	0.824	**0.033 ↑**
Gpx1	0.830	0.835	0.890
Mtf2	0.810	0.962	0.171
Mtor	**0.023 ↑**	0.963	0.243
Opa1	0.698	0.549	0.487
Ppargc1a	0.938	0.598	0.760
Ppargc1b	0.092	0.828	0.637
Sod1	0.412	0.741	0.522



mtDNAcn | **Antioxidant capacity**

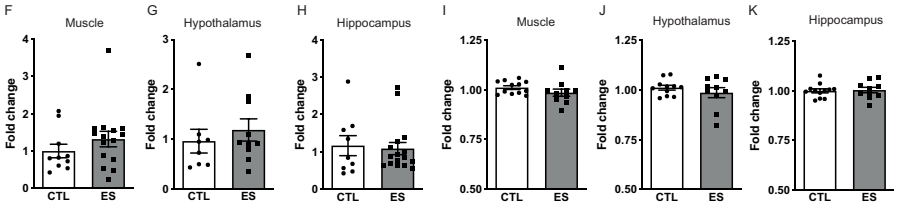


Figure 5. Effects of ES on mitochondria in adulthood. A-B: ES effects on ETC complex activity. A: p values for the comparison between CTL and ES for each complex, arrows indicate the direction of a significant ES effect. B: COX activity in the hypothalamus is lower in ES-exposed animals. C-E: ES effects on the expression of genes important for mitochondrial functions in adulthood. C: p values for the comparison between CTL and ES for each gene, arrows indicate the direction of a significant ES effect. D: ES increases the expression of *Mtor* in muscle, and E: ES increases *Fis1* in hippocampus. F-H: ES does not alter mtDNAcn in adulthood in muscle (F), hypothalamus (G) and hippocampus (H). I-K: ES does not alter antioxidant capacity in adulthood in muscle (I), hypothalamus (J) and hippocampus (K). Indicated is mean ± SEM, p<0.05.

3.4 Mitochondrial function correlates to metabolic readouts at P9

A composite score was calculated that integrated the activity of all four ETC complexes (CI-CIV) and correlated to metabolic and CORT readouts at both ages and learning in adulthood (Fig. 6A). Even though there were no significant effects of ES on the muscle, hypothalamic and hippocampal ETC activity score at P9 (Fig. 3B) or in adulthood (Fig. 5A), at P9, muscle ETC activity negatively correlated to adiposity (r=-0.541, p=0.025) (Fig. 6B), and hypothalamic ETC activity negatively correlated to glucose levels (r=-0.577, p=0.039) (Fig. 6C). In adulthood, CORT (PM levels) correlated positively with the hippocampal ETC score (r=0.509, p=0.031) (Fig. 6D).

A

Correlation	P9	Adult
ETC activity score muscle : bodyweight	-0.301	-0.006
ETC activity score muscle : adiposity	**-0.541•**	-0.195
ETC activity score muscle : glucose	0.031	-0.249
ETC activity score muscle : muscle strength	-0.072	x
ETC activity score muscle : CORT	0.308	-0.222
ETC activity score hypothalamus : bodyweight	-0.343	0.019
ETC activity score hypothalamus : adiposity	-0.021	0.084
ETC activity score hypothalamus : glucose	**-0.577•**	-0.160
ETC activity score hypothalamus : CORT	-0.400	-0.063
ETC activity score hippocampus : learning score	x	-0.230
ETC activity score hippocampus : CORT	-0.093	**0.509•**

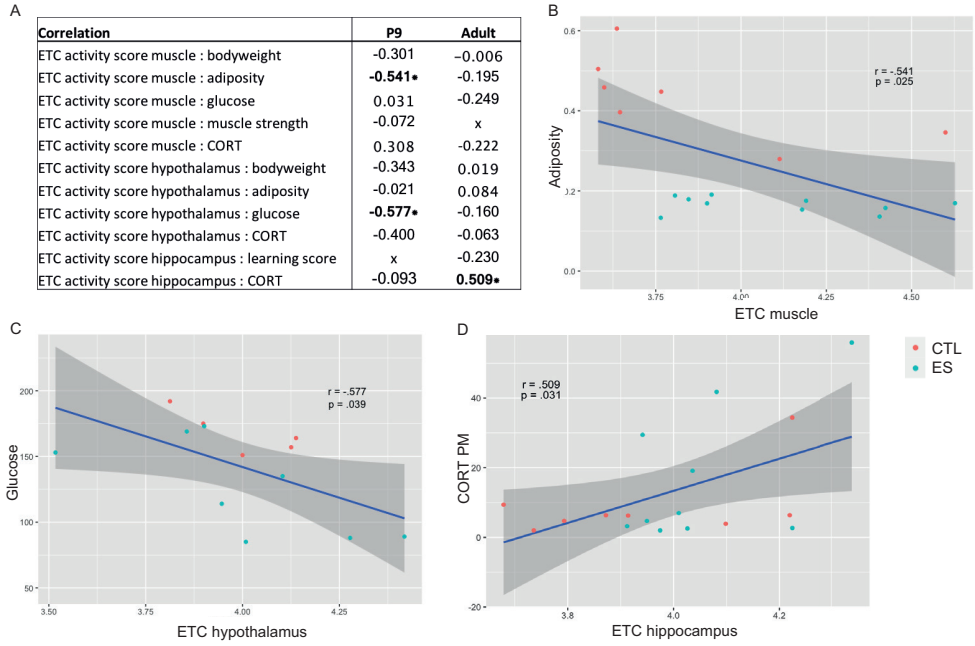


Figure 6. Correlations between ETC activity and the ES-induced phenotype. A: overview of the r values of correlations between ETC activity, metabolic readouts and learning. B: Correlation between adiposity and muscle ETC activity at P9. C: Correlation between glucose and hypothalamic ETC activity at P9. D: Correlation between CORT and hippocampal ETC activity in adulthood. * = p<0.05.

4. Discussion

We investigated the short- and long-term effects of ES on mitochondria, and if and how this relates to the ES-induced metabolic and cognitive phenotype. Early in life we observed increased COX activity in muscle, increased SCC activity in the hypothalamus, and alterations in hippocampal gene expression related to antioxidant defence and fission. In adulthood, ES reduced COX activity in the hypothalamus, and increased hippocampal *Fis1* and muscle

Mtor expression. Thus, ES affected mitochondria-related measures in both muscle and the brain directly after stress exposure and in adulthood, although differential effects were found depending on age and tissue. We observed a strong metabolic phenotype with reduced BW gain, adiposity and circulating glucose levels directly after ES exposure at P9, confirming previous studies (Walker et al., 2017; Yam et al., 2017a). Moreover, to the best of our knowledge we are the first to describe that ES reduces muscle strength at this age. In late adulthood, no apparent metabolic alterations under these standard dietary circumstances were found, but ES-exposed mice did show cognitive deficits. Indeed, such ES-induced cognitive impairments have repeatedly been shown before (Naninck et al., 2015; Walker et al., 2017). While muscle and hypothalamic ETC activity correlated to metabolic readouts at P9, hippocampal ETC activity correlated with CORT levels in adulthood, whereas no association between hippocampal ETC activity and cognitive function was observed. Below, we will discuss the ES-induced effects on mitochondria across the different tissues, and relate our findings on mitochondria biology to the ES-induced metabolic and cognitive alterations.

4.1 Effects of ES on muscle mitochondria

Directly after ES exposure at P9, ES mice showed increased COX (CIV) activity in muscle. COX is thought to be the pacesetter for mitochondrial respiration and ATP synthesis (Srinivasan and Avadhani, 2012). Long-term dietary restriction resulting in a leaner phenotype has previously been reported to increase mitochondrial respiration and COX content in muscle (Hempenstall et al., 2012). Increased COX activity is thus in line with the reduced bodyweight, adiposity and glucose that we observed in ES-exposed offspring at P9. Indeed, higher total ETC activity was associated with lower adiposity. However, no correlations with body weight or glucose levels were observed.

In adulthood, we no longer found such ES effects on ETC complex activity in muscle. Instead, ES increased *Mtor* expression in muscle. mTOR is a serine/threonine protein kinase which has many functions including regulating cell growth and proliferation, stimulating mitochondrial biogenesis and activity, as well as suppressing mitophagy (de la Cruz López et al., 2019). Mitophagy is key to maintain mitochondrial health due to the elimination of damaged mitochondria, and defects in mitophagy have been implicated in ageing and age-related disorders (Fivenson et al., 2017). Indeed, higher mTOR has been related to increased mitochondrial protein ageing (Bartolomé et al., 2017), and increased *Mtor* expression levels could thus be detrimental for mitochondria functioning. However, we did not observe higher mitochondrial numbers indicated by CS activity or mtDNAcn, nor defects in ETC complex activity at this age. Due to an absence of ES effects in these measures, it is unclear if the increased *Mtor* expression has functional implications for mitochondria at this age when not being further challenged by e.g. exercise or an unhealthy diet. In muscle specifically, mTOR is important for maintaining muscle mass (Yoon, 2017). We observed decreased muscle strength at P9, but did not perform such tasks in adulthood. Of note, reduced muscle

coordination in the rotarod has been reported before in male mice exposed to MS at 2-2.5 months of age (Kokubo et al., 2018). To better understand the functional implications of increased *Mtor* expression upon ES exposure, it is important to further study muscle mass and strength in (late) adulthood.

A few studies investigated effects of ES on muscle mitochondria, but only in adulthood. ES induced by MS in rats increased ROS formation, decreased glutathione (antioxidant) levels, and reduced ATP production in cardiac muscle at P60 (Sahafi et al., 2018). In addition, MS in rats lowered mtDNAcn and the expression of mitochondrial biogenesis regulator *Pgc-1a* and the antioxidant *Cat* at 4-8 months of age in skeletal muscle (Ghosh et al., 2016). However, we did not find similar effects of ES on mtDNAcn, antioxidant capacity or gene expression, either directly after ES at P9 or at an older age (10-12 months). This could suggest that the effects of ES on muscle mitochondria are ES model- or age-specific.

4.2 ES has long-term effects on hypothalamic ETC complex activity

In the hypothalamus, we report increased SCC activity in ES-exposed mice early in life, and a reduction in complex IV (COX) activity in adulthood. To the best of our knowledge we are the first to describe such lasting effects of ES on mitochondrial function in the hypothalamus. An increase in SCC activity could indicate an increase in complex II, coenzyme Q or complex III activity. As we do not observe effects on complex II or III activity, it is likely that the increased SCC activity in ES is due to increased coenzyme Q (CoQ) activity. CoQ is a lipid that functions as an ETC electron carrier, but also regulates other aspects of mitochondrial function including the activation of uncoupling proteins and the permeability of mitochondrial transition pores (Turunen et al., 2004). Changes in CoQ activity can thus have multiple functional implications. Notably, plasma CoQ10 (most common form of CoQ in humans) is increased in patients with metabolic syndrome (Miles et al., 2004).

In adulthood, ES reduced hypothalamic COX activity. COX is thought to determine the pace of ATP production (Srinivasan and Avadhani, 2012), and metabolic diseases have been associated with both decreased expression and increased activity of COX in peripheral cells (Čapková et al., 2002; Van Der Schueren et al., 2015). However, so far it is unknown if hypothalamic COX activity is related to metabolic disease. Interestingly, in one study it was shown that chronic prenatal stress (gestational day 1-7) in mice decreased hypothalamic COX activity in offspring at P2 (Howerton and Bale, 2014). Although the timing of both stress and measurement are different from our study, this could suggest that specifically hypothalamic COX is sensitive for programming by early-life stress exposure.

Hypothalamic mitochondrial respiration has been shown to be key in regulating energy homeostasis (Kim et al., 2019). At P9 we observed a strong ES effect on bodyweight gain, adiposity and glucose levels, and at this age, hypothalamic ETC activity correlated to blood glucose levels. In adulthood we did not observe effects of ES on bodyweight, abdominal fat or glucose levels, and total ETC activity also did not correlate to any of these measures. Hypothalamic mitochondria have been shown to specifically be involved in high-fat diet

(HFD)-induced weight gain (Kim et al., 2019). Therefore, to better understand whether the ES-induced alteration in ETC complex activity is involved in increased metabolic vulnerability, animals should be challenged with a HFD.

4.3 ES alters the expression of genes implicated in mitochondrial function in the hippocampus

In the hippocampus, ES reduced the expression of mitochondrial antioxidant enzyme *Sod1*, while increasing the expression of *Cat* at P9. Expression of the antioxidant *Gpx1* was not affected by ES. During normal cellular metabolism, reactive species are produced which can chemically react with and cause damage to nucleic acids, proteins and lipids. In fact, mitochondria itself are a main target for ROS-induced damage (Zorov et al., 2006). Especially free radicals (i.e. having unpaired electrons) such as superoxide anion are chemically reactive and can cause cellular damage. SOD1, localized in the outer mitochondrial membrane, catalyses the conversion from superoxide anion to hydrogen peroxide (Sea et al., 2015), whereas (peroxisomal) catalase is responsible for the decomposition of hydrogen peroxide to water and oxygen (Nandi et al., 2019). Deficiency in SOD1 and catalase has been related to the pathogenesis of (age-related) diseases including diabetes and Alzheimer's disease (Góth and Eaton, 2000; Murakami and Shimizu, 2012; Nandi et al., 2019). Alterations in the expression of these important antioxidant enzymes could affect antioxidant capacity. However, in our study the total antioxidant capacity (consisting of antioxidant activities of both enzymes and small molecules) was unaltered in the hippocampus at P9, suggesting that the increase in *Cat* expression could compensate for the decrease in *Sod1* expression. Due to the highly reactive nature of superoxide anion, it would however be important to understand if ES leads to oxidative damage in the hippocampus at P9. Indeed, hippocampal oxidative damage has been reported after perinatal stress exposure: prenatal stress increased ROS-dependent mitochondrial DNA damage in the hippocampus at 1 month of age (Song et al., 2009), and also ES-induced lipid peroxidation has been reported at P60 (Réus et al., 2018).

Moreover, we observed an increase in hippocampal *Dnm1l* expression (protein name DRP1) and a decrease in hippocampal *Fis1* expression directly after ES exposure. Both proteins are involved in mitochondrial fission: FIS1 is a receptor protein that recruits cytosolic DRP1 to the site of fission (Su et al., 2010). Whereas ES reduced hippocampal *Fis1* expression at P9, in adulthood ES exposure increased hippocampal *Fis1* expression, without affecting *Dnm1l* expression any longer. It has been suggested that acute cellular stress promotes mitochondrial fusion, thereby increasing energy production and survival, while mitochondrial fission occurs upon severe and/or long-term stress (Picard et al., 2014). It is possible that such effects of chronic stress on fission persist even after the stress period ended, as is the case in ES exposure. It would thus be interesting to further investigate whether *Fis1* expression could serve as a hallmark of previous chronic stress exposure. However, as we did not observe differences in CS activity or mtDNAcn (indicators

of mitochondrial content), it remains speculative whether ES affects mitochondrial fission, and it is possible that, especially at P9, compensatory mechanisms might be at play due to the increased *Dnm1l* expression.

Besides these ES-induced changes in gene expression, in the hippocampus we did not observe effects of ES on hippocampal ETC activity, CS activity and mtDNAcn or on antioxidant capacity, at P9 nor in adulthood. Other studies investigating effects of ES induced by MS on hippocampal mitochondria report reduced ATP production (Amini-Khoei et al., 2017), increased oxidative stress and ROS production (Amini-Khoei et al., 2017; Réus et al., 2018; Zugno et al., 2015), as well as altered ETC complex activity (Zugno et al., 2015) at 2 months of age. These studies investigated offspring in young adulthood, which is different from our study, in which we investigated mitochondria either directly after ES at P9 or in late adulthood. Nonetheless, as fission is related to lower ATP and higher ROS production, these findings could match with the increased *Fis1* expression we observed in our study. Hippocampal mitochondria have a pivotal role in (synaptic) plasticity and cognitive functioning (Guo et al., 2017; Khacho et al., 2017). However, hippocampal ETC complex activity was not affected by ES in our study, and also did not correlate to cognitive performance. It would therefore be important to investigate the relationship between other functional mitochondrial measures and cognitive performance in future studies. Of note, the hippocampal ETC activity score positively correlated with CORT levels, while neither of these measures were affected by ES. This positive association between mitochondrial ETC activity in the hippocampus and CORT levels is in line with our previous study in which impairing mitochondrial functions reduced CORT levels under basal circumstances (Emmerzaal et al., 2020). In addition, stress (inducing increased CORT levels) in turn also impacts mitochondrial functions (Picard et al., 2014; Picard and McEwen, 2018). However, thus far it is unknown what the exact effects of variations in basal, physiological CORT levels, as measured in our study, are on mitochondrial physiology. Thus, the positive relationship between CORT levels and the hippocampal ETC activity (both the directionality and its consequences) should be explored further in future studies

In the current study, we extensively characterized the effects of ES on mitochondria by investigating them both peripherally and centrally, directly after stress exposure as well as in late adulthood. Interestingly, despite the described bidirectional relationship between CORT and mitochondrial function (Emmerzaal et al., 2020; Picard et al., 2015, 2014; Picard and McEwen, 2018), in our study ES did not affect CORT levels, nor did CORT correlate to ETC activity in most tissues/ages (with the exception of adult hippocampal ETC activity). Previous studies show conflicting results, as ES has been shown to increase, decrease or have no effect on plasma CORT levels (Walker et al., 2017). Thus far, the source of this variation is unclear. We have previously hypothesized that likely multiple elements in the early-life environment (e.g. nutrition, tactile stimulation and stress system) interact to program offspring by ES exposure (Lucassen et al., 2013). It thus remains to be understood

how exposure to ES affects mitochondrial function, as well as what is the exact relationship between mitochondrial functions and the ES-induced phenotype.

A limitation of our study is that we investigated mitochondria under basal circumstances without further challenging the system e.g. with a high caloric diet or acute stress exposure which could aid to possibly unmask additional effects of ES on mitochondria. Indeed, Ferreira *et al*. showed that ES (MD) did not affect antioxidant enzyme activities, mitochondrial membrane potential or ETC activity. However, when also exposed to an omega 3 deficient diet for 15 weeks, ES exposed animals had higher hippocampal glutathione peroxidase, free radicals production and thiol content (Ferreira et al., 2015). Moreover, the effect of ES on mitochondria might be sexually dimorphic, with females being more affected compared to males (Eagleson et al., 2020), and thus in future studies it would be important to also include females. Considering the differential effects of ES at P9 compared to adulthood, a factor other than age that could potentially contribute to the observed differences is the day/light cycle, as P9 samples were collected in the light phase while the adult samples were mostly collected in the dark phase. In fact, there is emerging evidence for rhythmic changes in the morphology, proteome and lipidome of mitochondria (Manella and Asher, 2016). While such rhythmicity was not taken into account in the current study, this might be important to control for in future research. Nonetheless, our study provides important insights in the direct and later life effects of ES on peripheral and central mitochondria, and contributes to a further understanding of how ES exposure programs individuals for life.

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Supplementary table 1. Primers used for real time-PCR

Gene symbol	Forward primer	Reverse primer
18SrRNA (nuclear DNA)	CTTAGAGGGACAAGTGGCGTTC	CGCTGAGCCAGTCAGTGTAG
16SrRNA (mtDNA)	CCGCAAGGGAAAGATGAAAGAC	TCGTTTGGTTTCGGGGTTTC
Becn1	CTTGGAGGAGGAGAGGCTGAT	TATACTCCCGCTGGTACTGAGCTT
Cat	CTCGCAGAGACCTGATGTCC	TGTGCCATCTCGTCAGTGAA
Dnm1l (DRP1)	TGACCAAAGTACCTGTAGGCG	GCATCAGTACCCGCATCCAT
Fis1	GGCAACTACCGGCTCAAGGA	GCCTACCAGTCCATCTTTCTTCA
Gpx1	ATCAGTTCGGACACCAGAATGG	GGAAGGTAAAGAGCGGGTGAG
Mtf2	CTCTCGATGCAACTCTATCGTC	TCCTGTACGTGTCTTCAAGGAA
Mtor	ATGGAAATGGCGTCCAAGGC	GAGAACGAGGACAGCGGCAT
Opa1	CCTGTGCATCCAAGACGGAT	TGGGAAGAGCTTGCCTTCAA
Ppargc1a (PGC-1α)	TGCTAGCGGTTCTCACAGAG	AGTGCTAAGACCGCTGCATT
Ppargc1b (PGC-1β)	CTGACGTGGACGAGCTTTCA	TGCCATCCACCTTGACACAA
Sod1	GCGGATGAAGAGAGGCATGTT	GTACGGCCAATGATGGAATGC
Tuba1a	CCCTCGCCTTCTAACGCGTTGC	TGGTCTTGTCACTTGGCATCTGGC
Tbp	GTCATTTTCTCCGCAGTGCC	GCTGTTGTTCTGGTCCATGAT
Rpl0	GCTTCATTGTGGGAGCAGACA	CATGGTGTTCTTGCCCATCAG
Rpl13a	CCCTCCACCCTATGACAAGA	CTGCCTGTTTCCGTAACCTC
Rpl19	TTGCCTCTAGTGTCCTCCGC	CTTCCTGATCTGCTGACGGG

CHAPTER 7

7

Impact of early-life stress on hypothalamic microglial mitochondria in male and female mice

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Abstract

Early-life stress (ES) exposure has been associated with an increased risk to develop obesity, and this is suggested to be sex-specific. Hypothalamic inflammation has been implicated in diet-induced obesity development, and there is initial evidence that microglial mitochondria drive the high-fat diet-induced neuroinflammation. We have previously shown in mice that ES increases fat accumulation upon western-style diet exposure, and that such a diet elicited a different hypothalamic microglia response in ES-exposed animals. We here hypothesized that ES impacts on microglial mitochondrial biology to affect hypothalamic inflammation and metabolic vulnerability. To test this, we exposed mice to limited nesting and bedding material from postnatal day (P)2-P9, and investigated at P9 as well as at 8-9 weeks of age after exposure to a high-fat high-sugar (HFHS) diet i) hypothalamic microglia density and morphology, ii) hypothalamic microglial mitochondrial structure and density at the ultrastructural level, as well as iii) microglial mitochondrial function in response to a basal or HFHS environment *in vitro* at P9, and iv) if the studied effects of ES were sex-specific. In this preliminary report, we show that ES does not affect microglial mitochondrial density and structure in the hypothalamus at P9. At 8-9 weeks of age, HFHS diet exposure increased microglia cell density as well as affected mitochondrial circularity and coverage, the latter depending on previous ES exposure. Functionally, under basal circumstances female microglial mitochondria showed higher respiration, and ES affected aspects of mitochondrial functions mostly similarly in both sexes. However, when exposed to the HFHS environment, ES affected mitochondrial respiration more so in females. Thus, we provide preliminary evidence for effects of ES on microglial mitochondria structure and function that were partly sexually dimorphic. More research is needed to confirm these findings, as well as to uncover if microglial mitochondria are implicated in the (sex-specific) effects of ES on metabolism.

1. Introduction

Metabolic health is heavily influenced by the perinatal environment one is exposed to (Bouret, 2009; Bouret and Simerly, 2006; Dearden and Ozanne, 2015; Levin, 2006). Exposure to stressful experiences during this period programs offspring for life, and has been associated with increased risk to develop obesity and diabetes (Alciati et al., 2013; Basu et al., 2017; Danese and Tan, 2014), potentially in a sex-specific manner (Boynton-Jarrett et al., 2010; Dearden et al., 2018; Murphy et al., 2018, 2017).

The arcuate nucleus of the hypothalamus (ARH) regulates energy homeostasis by sensing circulating hormones and nutrients (Barsh and Schwartz, 2002; Lam et al., 2005; Timper and Brüning, 2017), and inflammation in the ARH has been proposed to be involved in diet induced obesity (DIO) development (Dorfman and Thaler, 2015; Kim et al., 2019; Thaler et al., 2012). In fact, hypothalamic microglia are activated by high-fat diet (HFD) feeding within 3 days of diet exposure, before noticeable bodyweight gain (Thaler et al., 2012), and blocking inflammation in the hypothalamus attenuates HFD-induced weight gain (Benzler et al., 2015; Kim et al., 2019), suggesting microglia activation is pivotal in DIO development.

HFD-induced activation of hypothalamic microglia depends on mitochondria (Kim et al., 2019). Impeding the mitochondrial response to a HFD in microglia protected against HFD-induced inflammation and obesity, by reducing feeding and enhancing energy expenditure. Mitochondria are the cellular powerhouses of the cell which produce ATP from glucose and fatty acids (Nolfi-Donegan et al., 2020). Both acute and chronic stress impact on mitochondrial functions, and mitochondria have been proposed to be (one of the) subcellular substrates of early-life stress (ES)-induced programming (Hoffmann and Spengler, 2018; Picard et al., 2014; Picard and McEwen, 2018; Zitkovsky et al., 2021).

We have previously shown in mice that ES exposure results in an exacerbated response to an unhealthy western-style diet (WSD). In fact, while ES exposure led to lower adiposity in both males and females when fed a standard chow diet, long-term western-style diet (WSD) increased fat accumulation in ES-exposed males whereas this was not investigated in females (Yam et al., 2017). Importantly, WSD also led to a higher microglia density with more amoeboid (immature) morphology directly after stress exposure at postnatal day (P)9, and increased microglia coverage in adulthood upon long-term WSD exposure (Ruigrok et al., 2021). Due to the effects of ES on i) the vulnerability to an unhealthy diet, ii) hypothalamic microglia, and iii) the proposed role for mitochondria in ES-induced programming, we hypothesized that ES alters microglial mitochondria structure and function in the hypothalamus at basal state and in response to high-fat high-sugar diet (HFHS).

In this preliminary report, we exposed mice to ES from P2-P9, and used an *in vivo* and *in vitro* approach to test the effects of ES i) on hypothalamic microglial mitochondrial density and structure at P9, as well as at 8-9 weeks of age in response to a HFHS, and ii) on microglial mitochondrial function in response to a high-fat high-sugar environment *in vitro*. Due to the evidence for sex differences in programming by ES, we investigated these questions

in both males and females. It should be noted that the data presented in this chapter are preliminary. Due to COVID-19 related delays, the structural analysis of mitochondria remains to be completed and the functional (*in vitro*) assay should be repeated.

2. Materials and methods

2.1 Animals and breeding

In total 148 mice were used for these studies (for P9 electron microscopy (EM) studies: n=20 (n=5 per group); for experiments at 8-9 weeks of age: n=83 were exposed to STD or HFHS of which food intake and bodyweight were analyzed, of which n=40 were used for EM (n=5 per group); for seahorse studies performed at P9: n=45 (n=10-12 per group)). The cohorts for EM experiments were bred at the University of Amsterdam, the cohort for the seahorse experiment were bred at Yale University School of Medicine. All mice were kept under standard housing conditions (12h/12h light dark cycle, temperature 20-23°C, humidity 40-60%, standard rodent chow unless noted otherwise (see below) and water *ad libitum)*. For the Amsterdam cohorts, experimental procedures were conducted under the national Dutch law and European Union directives on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam. For the Yale cohorts, all experimental procedures were approved by the Yale University Institutional Animal Care and Use Committee. Mice were housed in facilities operated by the Yale Animal Resources Center. (YARC). YARC is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

To standardize the perinatal environment, breeding was performed in house. For the Amsterdam cohorts, 8-week-old male and female mice were ordered from Envigo Laboratories (Venray, The Netherlands). For the Yale cohort, 8-week-old male and female mice were ordered from YARC. Animals were allowed to acclimatize for 1-2 weeks prior to breeding. For breeding, one male was housed with two primiparous females for one week. After another week of paired-housing, pregnant females were housed individually. When a litter was born before 10:00 A.M., the previous day was designated as P0. For adolescent experiments, mice were weaned at P21, housed with same sex littermates (2-4 per cage), and left undisturbed until the start of the experiment (P57-P62).

2.2 ES paradigm

ES was induced by limiting the nesting and bedding material from P2-P9, as described previously (Naninck et al., 2015; Rice et al., 2008). At P2, litters were culled to 6 pups (litters with <5 pups were excluded) including both males and females, weighted, and assigned to the control (CTL) or ES condition. For the Amsterdam cohorts, for the CTL condition, type 2 conventional cages contained standard amount of sawdust and one square piece of cotton nesting material (5x5 cm, Techninlab-BMI, Someren, The Netherlands). ES cages (also

type 2 conventional cage) had a thin layer of sawdust on the bottom, covered with a fine-gauge stainless steel mesh, and half a square piece of cotton nesting material (2.5x5 cm). All cages were covered with a filtertop. For the Yale cohort, litters were housed in standard individually ventilated cages. CTL cages contained standard amount of bedding and one square piece of cotton material (5x5 cm, Techninlab-BMI, Someren, The Netherlands). For ES cages, a similar fine-gauge stainless steel mesh was used adapted for IVC cages, the floor was covered with a minimal amount of bedding, and half a square piece of cotton nesting material was provided. Litters were left undisturbed until the morning of P9, when they were weighted, and either directly used for experiments at P9 or transferred to a clean cage.

2.3 Diets

For P9 experiments, animals were fed standard rodent chow diet throughout the experiment (Amsterdam cohorts: CRM (P), 801722, Standard Diets Services, Essex, United Kingdom, 3.585 kcal/g, where: 22% energy from protein, 9% energy from fat, and 69% energy from carbohydrates; Yale cohort: Harlan Teklad #2018, Envigo, Indianapolis, IN, USA, 3.1 kcal/g, where: 18.6% energy from protein, 6.2% energy from fat). For the experiments in adolescence, mice (aged 8-9 weeks) were switched to either standard diet (STD), or high-fat high-sugar diet (HFHS) for 3 days (Research Diets, New Brunswick, NJ, USA). The HFHS contained 45% kcal from fat, 35% kcal from carbohydrate (21.3% sucrose), and 20% kcal from protein (cat# D12451). The STD was specifically designed to resemble the CRM (P) standard chow, and contained 10% kcal from fat, 70% kcal from carbohydrate (3.9% sucrose), and 20% kcal from protein.

2.4 Tissue preparation

For EM experiments, animals were anesthetized via an intraperitoneal injection of pentobarbital (120 mg/kg Euthasol®), and transcardially perfused with 0.9% saline, followed by fixative (4% paraformaldehyde, 15% picric acid, 0.1% glutaraldehyde in 0.1 M PB). For pups and adolescent experiments, perfusion speeds of 1 mL/min and 2.5 mL/min were used, respectively. Brains were post-fixed for 2 more days and stored in PB with 0.01% sodium azide at 4°C. Brains were sliced with a vibratome in 50 µm thick sections, and kept in PB with 0.01% sodium azide until further processing. For seahorse experiments, mice were rapidly decapitated at P9. Brains were quickly removed and immediately used for further processing (see below).

2.5 Immunofluorescence and confocal microscopy

To quantify microglia density and coverage in the ARH, brain sections were washed (0.05M PBS, pH 7.6) and incubated in 1% BSA, 0.3% triton in PBS for 30 min (blocking solution). Next, sections were incubated in anti-Iba1 antibody (1:5000 in blocking solution, Wako Pure Chemical, Richmond, VA, USA, Cat#019-19741) at room temperature (RT) for 1h, followed by an overnight incubation at 4°C. The next day, sections were washed in PBS

containing 0.3% triton, and incubated in biotinylated goat anti-rabbit IgG for 2h (1:200 in blocking solution; Vector Laboratories). After several washes in PBS, sections were incubated with Streptavidin 594 in PBS (1:2000, Life technologies, Carlsbad, CA, USA, Cat# S32357) for 2h at RT. Sections were then washed and mounted with DAPI containing mounting medium (Invitrogen, Carlsbad, CA, USA).

Z-stacks (15 stacks per photo, 1 µm distance between stacks) of Iba1 stained tissue were taken using a 40x objective on a Nikon A1 confocal microscope. For each animal, both sides of the ARH in 3 different sections were imaged between bregma -1.055 and -1.955, based on the Allen Brain Atlas (2011).

2.6 Electron microscopy

Sections containing intact ARH were kept in 10% sucrose for 30 minutes and 20% sucrose for 1 hour. The sections were rapidly frozen/thawed for 3 times and washed 3 times with 0.1% PB. Sections were stained with anti-Iba1 antibody (1:5000 Wako Pure Chemical, Richmond, VA, USA, Cat#019-19741 in goat blocking serum) for 2 days on a shaker in the cold room. After 3 PB washes, slices were incubated for 1 hour 30 minutes in biotinylated goat anti-rabbit IgG (1:200 in PB; Vector Laboratories, Burlingame, CA, USA) at RT. Sections were again washed 3 times and incubated in avidin–biotin–peroxidase (ABC; 1:100 in PB; ABC Elite kit, Vector Laboratories, Burlingame, CA, USA) for 1 hours 30 minutes at RT. After washing 3 times, sections were placed in a nickel DAB solution for 1 minute and 30 seconds, and washed 3 times. The sections were then osmicated (1% osmiumtetroxide) for 30 minutes. Next, the sections were washed 3x in PB, followed by a ddh_20 and a 50% ethanol wash. Sections were kept in 1% uranyl acetate in the 70% ethanol for 1 hour. Slices were washed 3 times each in 95% ethanol and absolute ethanol. Slices were then washed 2 times in propylene oxide and left in a solution of 50% propylene oxide, 50% durcupan for 3 hours. Sections were left overnight in pure durcupan and flat-embedded on liquid release-coated slides, and coverslipped with Aclar (Electron Microscopy Sciences, Fort Washington, PA, USA), glued to a blank and trimmed. Sections were collected on Formvar-coated single slot copper grids, and imaged using a Philips Tecnai T-10 electron microscope.

2.7 Image analysis

ImageJ software (version 2.0.0-rc-69/1.52n) was used to analyze both the EM and confocal images. For the analysis of microglia numbers, microglia were counted manually in a defined region of interest (ROI; square-shaped) throughout the Z stack. The ROI was placed, depending on the bregma, with one or two corners touching the third ventricle. The number of microglia per μm^3 was calculated.

For EM analysis, the cell membrane and nucleus were outlined to measure the area of the cytosol. Next, all mitochondria of the specific cell were drawn. Average mitochondrial size, mitochondrial density (number/μm^2 cytosol), mitochondrial coverage (area covered

by mitochondria/μm^2 cytosol*100), and the average circularity (width by height ratio) were determined.

2.8 Microglia cell culture and seahorse

Mixed glia cultures were obtained at P9 by pulling the brains of 10-12 mice per experimental group. Following decapitation, brains were rapidly dissected and kept in culture medium (Dulbecco's modified eagle medium 4.5 g/L glucose (Thermofisher Scientific, Waltham, MA, USA, Cat# 11965092), containing 1% penicillin-streptomycin and 10% heat-inactivated fetal bovine serum). Brains were minced into a fine slurry with corneal scissors, and further homogenized by gentle pipetting. Next, suspended cells were passed through a 40mm filter, and centrifuged for 5 min at 1000 rpm. The pellet was resuspended in culture medium and plated in poly-D-lysine (Gibco Thermofisher Scientific, Waltham, MA, USA, Cat# a38904-01) coated T75 flasks (Cat# 0030.711.122, Eppendorf). 10-11 days later, flasks were shaken (200 rpm) for 2 hours (37°C) to obtain microglia enriched cultures. Cells were then seeded in Poly-D-lysine-coated XF96 cell culture microplates (Cat# 101085-004, Agilent Technologies) at 1x105 cells/well density, and kept for 3 days before the Seahorse experiment was performed. Oxygen consumption rate (OCR; reflects mitochondrial oxidation) and the extracellular acidification rate (ECAR; reflects glycolytic activity) were measured using a microfluorimetric Seahorse XF96 Analyzer (Agilent Technologies, Santa Clara, CA, USA). In short, 1 hour prior to the start of the assay, cells were incubated in Seahorse XF Base medium (assay medium, Cat# 102353-100, Agilent Technologies) containing either 2 mM L-glutamine (Sigma, Saint Louis, MO, USA, Cat# G8540) (basal environment), or 2 mM L-glutamine, 25 mM glucose (Sigma, Saint Louis, MO, USA, Cat# G7021) and 200 mM palmitate (Sigma, Saint Louis, MO, USA, Cat# P9767) (high energy environment) at 37°C without CO_2. Next, OCR and ECAR were measured in response to the following compounds in this order: 1) 5 mM of oligomycin (Cat# 495455, EMD Millipore), 2) 10 mmM of FCCP [carbonyl cyanide-p-(trifluoromethoxy) phenylhydrazone] (Sigma, Saint Louis, MO, USA, Cat# C2920), and 3) 10 mM of antimycin A (Sigma, Saint Louis, MO, USA, Cat# A8674) and 5 mM of rotenone (Sigma, Saint Louis, MO, USA, Cat# R8875). OCR was determined as the change after injection of these compounds compared to the last OCR value before injection and analyzed with Wave 2.6.0 (Agilent Technologies software, Santa Clara, CA, USA) software. The average ECAR before oligomycin injection was taken as indication for cellular acidification.

2.9 Statistical approach

Data are expressed as mean ± standard error of the mean (SEM), and considered significant when $p < 0.05$. Data were analyzed using SPSS 25.0 (IBM software, Chicago, IL, USA), Graphpad Prism 6 (Graphpad software, La Jolla, CA, USA) and R Studio 1.2.1335 (R Core Team, 2018). The general statistical approach was as follows. Significant outliers were removed from the dataset. For the analysis of data with two predictor variables (e.g. condition and sex, or condition and diet), 2-way ANOVA was used. When a 2-way

interaction was found, post hoc tests were performed with Bonferroni correction. For data with 3 predictor variables (condition, sex and diet), 3-way ANOVA was used. Only in case of a 3-way interaction in the 3-way ANOVA, post hoc tests were performed with Bonferroni correction. As a 2-way interaction indicates there is no contribution of the third predictor to the interaction, we did not explore these 2-way interaction further with pairwise comparisons between each individual experimental group. Multiple mice from the same litter were included in this study, resulting in nested data. We therefore always tested if litter contributed to the outcome with mixed model analysis with litter as random factor, and corrected for this when needed. Moreover, for adolescent analysis, mixed model with estrous cycle as random factor was performed to test if estrous cycle contributed to the outcome variable across the female groups. When needed, estrous cycle was corrected for.

3. Results

3.1 Effect of ES, diet and sex on bodyweight, food intake and CORT

ES decreased bodyweight (BW) gain from P2-P9 independent of sex ($F_{condition}(1, 39.844)=49.009$, $p<0.001$) (Fig. 1A). At the start of diet exposure (P57-P62) female mice were lighter than male mice ($F_{sex}(1, 76)=397.571$, $p<0.001$), independent of early-life condition (Fig. 1B). BW gain over the 3-day dietary exposure (as a percentage of the BW at the start of the diet) was higher in males compared to females ($F_{sex}(1, 76)=25.479$, $p<0.001$), and higher in mice fed a HFHS diet compared to those fed a STD ($F_{diet}(1, 76)=25.99$, $p<0.001$) (Fig. 1C). Similarly, caloric intake was higher in males ($F_{sex}(1, 73)=81.728$, $p<0.001$), and higher in animals on HFHS diet ($F_{diet}(1, 73)=279.722$, $p<0.001$) (Fig. 1D). Finally, CORT levels were higher in females ($F_{sex}(1, 65)=14.858$, $p<0.001$), but not affected by early-life condition or diet (Fig. 1E).

3.2 Effects of ES, diet and sex on microglia and microglial mitochondrial structure in the ARH

Figure 2A shows the different aspects of mitochondrial structure that were analyzed in hypothalamic microglia. At P9, ES did not affect the density, size, coverage or circularity of mitochondria in ARH microglia, and there were also no sex differences on any of these measures (Fig. 2B-E). At 8-9 weeks of age, HFHS exposure increased $Iba1^+$ cell numbers in the ARH (Fig. 2F). Since these analyses are not complete yet, we will also report on trends ($p=0.05$-0.06). While microglial mitochondrial density and size were not affected by ES, diet, nor sex (Fig. 2G,H), the area of cytoplasm covered by mitochondria was higher in animals fed HFHS but this could be observed in CTL mice in particular ($F_{condition*diet}(1, 211)=4.616$, $p=0.033$). Furthermore, mitochondrial circularity was affected by HFHS feeding so that mitochondria had a higher width by height ratio in HFHS-fed mice compared to STD fed mice ($F_{diet}(1, 210)=5.976$, $p=0.015$), but this effect of the diet tended to depend on previous ES exposure suggesting this was only the case for CTL mice ($F_{condition*diet}(1, 210)=3.705$, $p=0.056$) (Fig. 2I,J).

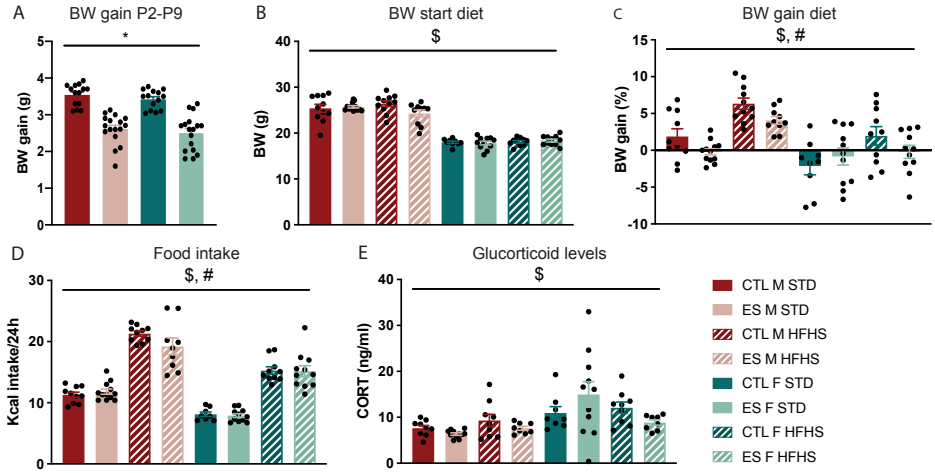

Figure 1. Effects of ES on BW, food intake and CORT levels. A: ES decreased BW gain from P2-P9. B: Female mice weighted less at the start of the diet. C: BW gain during dietary exposure was affected by early-life condition, sex and diet. D: Caloric intake was higher when fed HFHS, and higher in males. E: CORT levels after dietary exposure were higher in females but not affected by the type of diet or ES. Shown is mean ± SEM; p<0.05; * condition effect; $ sex effect; # diet effect; $# sex by diet interaction effect.

3.3 ES and sex impact microglial mitochondrial functions

Mitochondrial functions were assessed by measuring mitochondrial respiration in microglia enriched cultures from CTL and ES-exposed mice in the presence of different nutrients (Fig. 3A,B). When only glutamine was present in the medium (basal), females had higher non-mitochondrial respiration (F_{sex}(1, 19)=15.84, p=0.001), basal respiration (F_{sex}(1, 19)=17.773, p<0.001), maximal respiration (F_{sex}(1, 20.236)=20.236, p<0.001), proton leak (F_{sex}(1, 19)=7.216, p=0.015) an ATP production (F_{sex}(1, 19)=17.008, p=0.001) compared to males (Fig. 3C). ES furthermore decreased proton leak ($F_{condition}$(1, 19)=7.686, p=0.015), and increased coupling efficiency ($F_{condition}$(1, 19)=5.201, p=0.034) similarly in males and females. In the presence of glutamine, glucose and palmitate (high-fat high-sugar environment), basal respiration was affected by sex depending on early-life condition ($F_{condition*sex}$(1, 21)=6.372, p=0.02), so that CTL females had higher basal respiration compared to CTL males (p<0.001), while ES females had lower levels than CTL females (p=0.006) and were comparable to males (Fig. 3D). Similarly, maximal respiration was affected by sex depending on early-life condition ($F_{condition*sex}$(1, 21)=4.485, p=0.046). When comparing individual experimental groups, CTL females had higher maximal respiration compared to CTL males (p=0.002). Proton leak was higher in females (F_{sex}(1, 21)=5.966, p=0.024), and lower in ES exposed independent of sex ($F_{condition}$(1, 21)=6.226, p=0.021). Finally, ATP production was affected by sex depending on ES ($F_{condition*sex}$(1, 21)=6.672, p=0.017). Post hoc analysis revealed that CTL females had higher ATP production compared to CTL males (p<0.001), whereas ES females had lower levels than CTL females (p=0.015) and were comparable to males.

Cellular acidification (glycolytic + non-glycolytic acidification) was measured with ECAR (Fig. 3E,F). Under glutamine conditions, cellular acidification was affected by sex depending on early life condition ($F_{condition*sex}(1, 19)=5.784$, $p=0.027$). Post hoc analysis revealed that CTL males had lower cellular acidification levels compared to CTL females ($p<0.001$). Under glutamine, glucose and palmitate conditions, cellular acidification was affected by sex depending on previous ES exposure ($F_{condition*sex}(1, 21)=7.469$, $p=0.012$), so that CTL had lower levels compared to CTL females ($p=0.018$) and ES males had higher levels compared to CTL males ($p=0.019$).

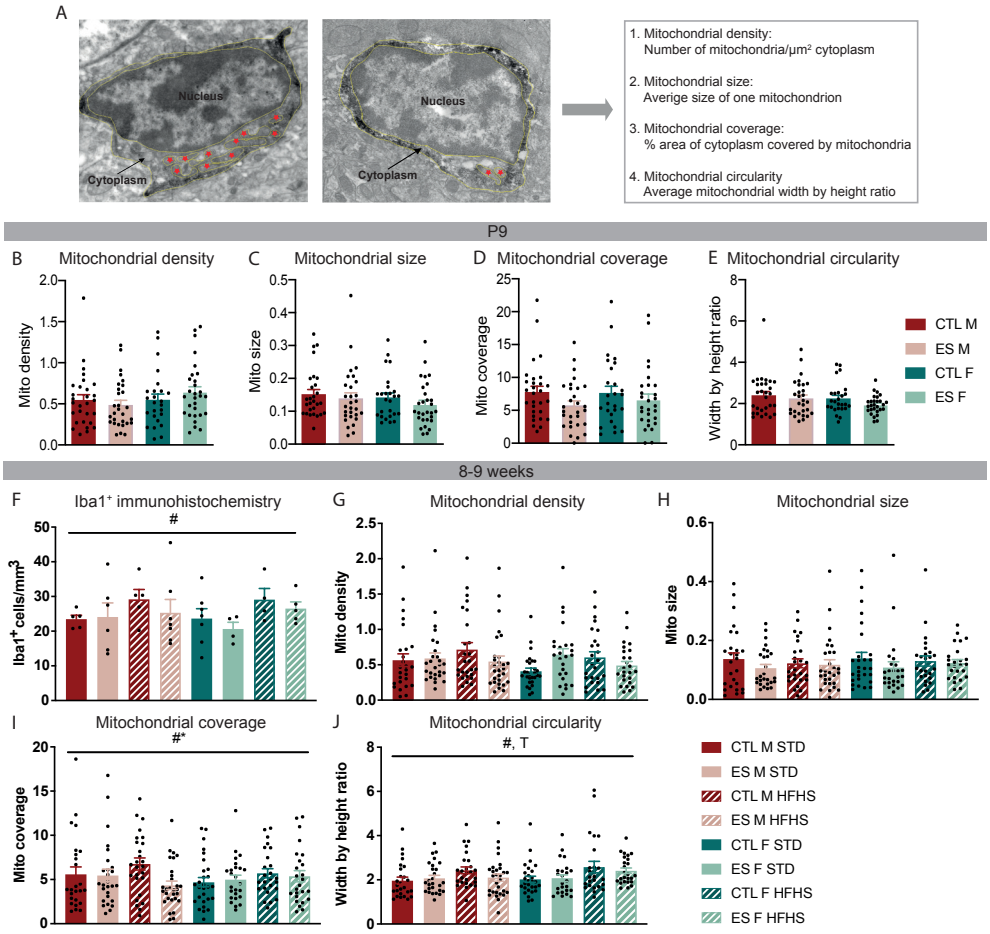

Figure 2. Effects of ES and diet on mitochondrial morphology of microglia in the ARH. A: Examples of ARH microglia and their mitochondria (indicated by red stars). B-E: ES does not affect mitochondrial density, size, coverage or circularity in male and female pups at P9. F: HFHS exposure increases microglia cell density in the ARH. G-H: ES, diet and sex do not affect mitochondrial density or size. I: Mitochondrial coverage is affected by diet depending on previous ES exposure. J: HFHS increased the mitochondrial width by height ratio (indicator of circularity). Shown is mean ± SEM; $p<0.05$; # diet effect; #* diet by condition interaction effect; T trend towards diet by condition effect.

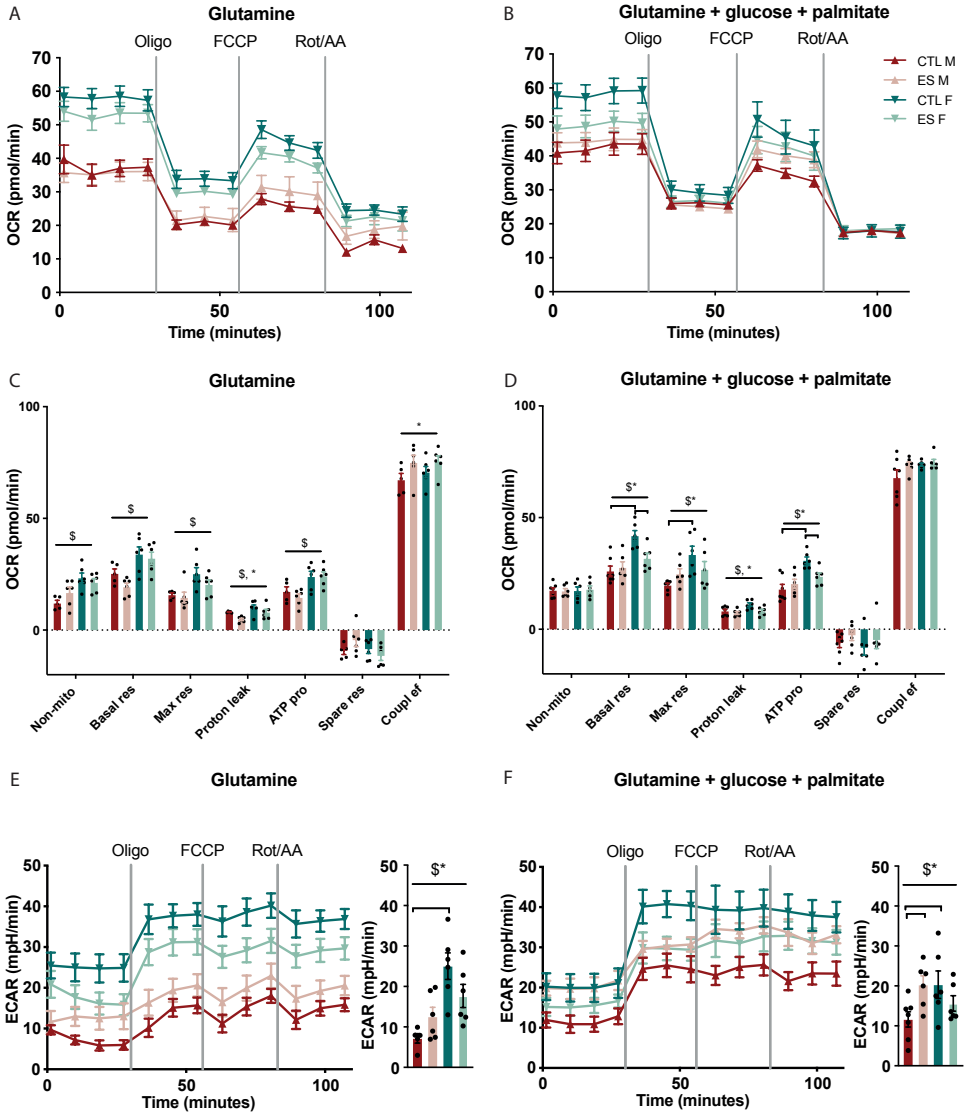

Figure 3. Effects of ES on mitochondrial respiration of microglia enriched primary cultures under different nutritional environments. A: OCR under glutamine incubation. B: OCR under glutamine, glucose and palmitate incubation. C: Under glutamine incubation, non-mitochondrial respiration, basal respiration, maximal respiration proton leak and ATP production are higher in females. ES lowers proton leak and coupling efficiency. D: Under glutamine, glucose and palmitate incubation, basal respiration, maximal respiration and ATP production are affected by early-life condition depending on sex. Proton leak is higher in females and lower in ES exposed males and females. E: ECAR under glutamine incubation, insert: cellular acidification was affected by sex depending on previous ES exposure. F: ECAR under glutamine, glucose and palmitate incubation, insert: cellular acidification was affected by sex depending on early-life condition. Shown is mean ± SEM; p<0.05; $ sex effect; $* sex by condition interaction effect.

4. Discussion

In this preliminary report, we investigated if and how ES exposure affects microglial mitochondrial structure and function under basal circumstances and in response to a high-fat high-sugar challenge, and if this is similar for both sexes. We show sex differences in BW, caloric intake and CORT, and that males and females have a different BW gain response to the HFHS diet. Moreover, ES reduced BW gain, both early in life and during the 3-day dietary exposure. At P9, neither ES or sex affected the density and structure of mitochondria of microglia in the ARH. At 8-9 weeks of age, HFHS exposure increased Iba1 cell density in the ARH, as well as led to a higher mitochondrial width by height ratio, indicating mitochondria were less circular. Moreover, mitochondrial coverage was higher upon HFHS exposure but in particular in CTL animals. Mitochondrial respiration in microglia-enriched cultures was, when exposed to a basal environment, higher in females compared to males and affected by ES exposure similarly in both sexes. Under HFHS conditions, (basal) mitochondrial respiration and ATP production were lower in ES-exposed females compared to CTL females, while proton leak was decreased after ES exposure similarly in both sexes. Below, we will first discuss the sex differences and ES effects on metabolic readouts, followed by their effect on mitochondrial structure and function.

4.1 Effects of ES and sex on physiological readouts under STD and HFHS conditions

In line with previous studies, ES lowered BW gain from P2 to P9 similarly in both sexes (Naninck et al., 2015; Rice et al., 2008; Yam et al., 2017). To study the effects of ES on the microglial mitochondrial response to a HFHS diet, mice were exposed to either STD or HFHS for 3 days at 8-9 weeks of age. The 3-day HFHS exposure was selected as an optimal time window to study the microglial mitochondrial response to the HFHS (Kim et al., 2019), and was not expected to greatly impact metabolic readouts. This is different from studies that report increased fat accumulation in ES-exposed animals when fed unhealthy diets, which use notoriously longer diet exposures (Murphy et al., 2018, 2017; Yam et al., 2017). At the start of the 3-day dietary exposure, BW was higher in males but no longer affected by ES. Moreover, although the HFHS did not lead to a higher BW at the end of the 3-day exposure, BW gain *during* the 3-day dietary exposure was higher in mice fed a HFHS. ES did not impact BW at this age or in response to the diet. Indeed, previous studies did not show effects of ES on adolescent and adult BW (Fuentes et al., 2018; Moussaoui et al., 2016; Naninck et al., 2015; Zimmerberg and Kajunski, 2004). Moreover, caloric intake was higher in males compared to females and higher in mice fed a HFHS compared to those fed STD, but not affected by early-life condition. This matches previous studies that fed ES-exposed animals either chow or high-caloric diets *ad libitum* and without a choice for different diets (Abbink et al., 2020; Murphy et al., 2018, 2017; Yam et al., 2017). At 8-9 weeks of age, CORT levels were higher in females, but not affected by either ES or diet. These effects of sex and ES on

CORT levels are in line with our observations in adulthood (Naninck et al., 2015). Of note, Murphy et al. reported a higher CORT response to a prolonged (12-week) HFD in ES-exposed females (Murphy et al., 2017), indicating the length of dietary exposure matters for the effects of ES on the CORT response.

4.2. Effects of ES, sex and diet on microglia and their mitochondrial structure

The HFHS exposure increased Iba1^{+} cell density in the ARH. In line with this, a 3-day HFD (60% kcal from fat) has previously been shown to increase ARH microglia cell numbers (Thaler et al., 2012). Interestingly, in our previous study we observed changes in microglia after 3 days, however this concerned rather the size of the microglia cell bodies, while the density only increased after 7 days of diet. In the current study we focused on the effects of a 3-day HFHS diet exposure based on our previously reported results on mitochondria (Kim et al., 2019), so we cannot further compare such temporal dynamics. Possible reasons for the subtle differences in the microglia response to the HFHS diet could lie in subtle design differences (e.g. synthetic control diet (current study) versus grain-based control diet (previous study)). ES did not impact the microglia response to the 3-day HFHS diet. However, we have previously shown that a prolonged (27 week) WSD increased microglia coverage in the ARH, suggesting that microglia are in fact programmed by ES which becomes apparent only upon longer exposures (Ruigrok et al., 2021).

At P9, there were no effects of ES or sex on microglial mitochondrial density and structure in the ARH. At 8-9 weeks of age, the HFHS diet resulted in a less mitochondrial circularity (indicated by a higher width to height ratio), and there was a trend for an interaction effect between diet and ES. In addition, mitochondrial coverage was higher in mice fed a HFHS, and this in particular affected CTL animals. We previously showed that a similar 3-day HFHS diet decreased mitochondrial size while increasing mitochondrial numbers in hypothalamic microglia (Kim et al., 2019). Thus, we confirm changes in mitochondria in response to the diet, however with some subtle differences between studies that awaits further clarification. As the morphology of mitochondria has functional implications (Picard et al., 2014; Shutt and McBride, 2013; Westermann, 2010), it is important to understand where these effects of diet and ES on coverage and circularity originate from. We are currently increasing the power of these experiments in order to strengthen our conclusions on these aspects.

To the best of our knowledge, we are the first to characterize microglial mitochondrial density and structure upon ES exposure in the hypothalamus. Previous studies have found effects of ES on various measures of mitochondrial function in the hippocampus (Amini-Khoei et al., 2017; Réus et al., 2018; Zugno et al., 2015) and PFC (van Zyl et al., 2016). One study investigated effects of prenatal stress on mitochondria in the hypothalamus at P2, and found higher mitochondrial complex IV activity, but animals were not followed into adulthood (Howerton and Bale, 2014). Different from our ultrastructural analysis, these studies focused on functional readouts such as complex activity, ATP and ROS production, and none investigated cell type specificity. To assess functional implications of ES exposure

on mitochondria specifically in microglia, we assessed mitochondrial respiration *in vitro*, which will be discussed below.

4.3 Effects of ES and sex on mitochondrial functions

In primary microglia enriched cultures, we observed strong sex differences in mitochondrial respiration, under both the basal (glutamine) and HFHS (glutamine + glucose + palmitate) environment, with females showing higher basal respiration, maximal respiration and ATP production. Sex differences in mitochondrial respiration and complex activity have been described before in the brain (full brain and cortex, no cell type specificity) (Gaignard et al., 2018; Guevara et al., 2011; Khalifa et al., 2017) with females showing higher metabolic rates compared to males. Interestingly, cell type-specific sex differences have been described before for astrocytes, where males had higher mitochondrial respiration compared to females when exposed to low oxygen concentrations (Jaber et al., 2018). In this study no sex differences were observed in microglia, indicating that the nature of exposure (varying oxygen or nutrient levels) can be determinant for cell type specific effects.

ES also impacted microglial mitochondrial respiration. Under basal conditions ES reduced proton leak while increasing coupling efficiency similarly in males and females. In contrast, under HFHS conditions, interesting sex-dependent effects of ES emerged. While ES-exposed females had lower basal respiration and ATP production compared to CTL females, no such effects were observed for males. Indeed, there is evidence that females are more vulnerable for effects of ES on mitochondrial function in hippocampus, nucleus accumbens shell and prefrontal cortex (Eagleson et al., 2020; González-Pardo et al., 2020). To the best of our knowledge, we are the first to provide evidence that sex-specific effects of ES on mitochondrial function are also present in microglia specifically, and in the hypothalamus. Thus, our results are in line with previous studies showing increased vulnerability for brain mitochondrial dysfunction in ES females, however, it needs to be emphasized that the data are preliminary and the experiment should be repeated to confirm these findings.

Similarly, cellular acidification (indicator of glycolytic ATP production) also showed a nutrient- and sex-dependent effect of ES. Under basal circumstances, CTL but not ES females had higher acidification levels compared to their male counterparts, whereas under HFHS conditions, next to higher levels in CTL females, also ES males had higher levels compared to CTL males. To the best of our knowledge such sex-specific ES effects on cellular acidification are not described before.

In this preliminary report, we provide initial evidence that ES impacts on microglial mitochondria biology, partly in a sex-specific manner. Additional experiments are needed to confirm these findings and to increase the power of the experiments, as well as to understand whether mitochondria in hypothalamic microglia are involved in the ES-induced metabolic vulnerability.

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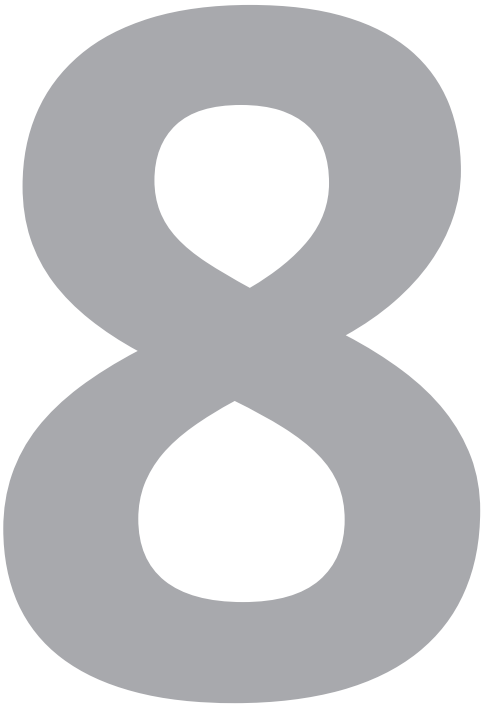
CHAPTER 8



General discussion

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1. Introduction

Exposure to stress during the early life period (early-life stress; ES) programs individuals for life. Exposure to adversity (e.g. neglect or physical, sexual or emotional abuse) during this critical developmental period increases the risk for metabolic diseases and cognitive disorders (Alciati et al., 2013; Chugani et al., 2001; Danese and Tan, 2014; Saleh et al., 2016), and these effects of ES might be sex specific (Boynton-Jarrett et al., 2010; Hay et al., 2008; Kozyrskyj et al., 2011; Park et al., 2018).

Unfortunately, ES is very common (Merrick et al., 2018) and difficult to prevent. In order to develop intervention strategies, it is important to increase our understanding of how ES programs offspring for life. While a substantial body of literature has investigated the association between mental and cognitive dysfunction and ES exposure, little is known about the metabolic programming by ES. Similarly, the subcellular substrates of programming by ES remain largely elusive.

Metabolic and cognitive disorder are often comorbid (Dye et al., 2017; Gunstad et al., 2020), suggesting similar subcellular substrates or entities could be involved ES-induced programming of metabolic and cognitive functions. If such converging substrates can be identified, these could eventually serve as targets for intervention.

The main aims of this thesis were:
i) to better understand which elements in the early-life environment mediate the programming by ES;
ii) to increase the knowledge on metabolic programming by ES-exposure, focusing on food choice and the underlying brain circuits that regulate metabolism;
iii) to identify subcellular substrates of ES-induced programming, for which we concentrated on mitochondria;
iv) to assess if there are sex differences in ES-induced programming.

We will start by discussing the elements in the early environment that may mediate programming by ES, highlighting the interaction between different elements (e.g. nutrition, hormones, tactile stimulation). We will continue by deliberating on the ES-induced metabolic programming, and attempt to integrate the different aspects that could be involved in the increased risk for metabolic disease (e.g. altered food choice, altered metabolic response), as well as discuss the underlying brain circuits and substrates. We will next discuss (the origin of) sex-specific vulnerability, focusing on sex differences in development and metabolism, and touch upon the comorbidity between metabolic and brain disorders. We will finish by discussing the clinical relevance of the work described in this thesis.

2. Early-life programming: which elements in the early environment are involved?

The early life environment encompasses many essential elements, comprising exogenous factors (e.g. tactile stimulation, temperature, nutritional provision) and endogenous modulators (e.g. stress hormones) (Hoeijmakers et al., 2015; Lucassen et al., 2013). It is likely that different aspects (maternal tactile stimulation, stress hormones, availability of nutrients) in the early environment interact and contribute to the ES-induced programming and later-life disease risk (Hoeijmakers et al., 2015; Lauby et al., 2021; Lucassen et al., 2013). In fact, stress often co-occurs with e.g. a suboptimal nutritional environment, and vice versa. For example, low food availability can cause stress (Knowles et al., 2015; McCurdy et al., 2010) and the stress response, in turn, can affect food intake and food preference (Dallman et al., 2005; Pecoraro et al., 2004). Thus, it is likely that these different elements interact to exert the effects of ES on later life health.

2.1 Beyond HPA-axis activation and maternal care

Stress activates the hypothalamic-pituitary-adrenal (HPA)-axis, which stimulates the release of corticotrophin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus. CRH then stimulates adrenocorticotrophic hormone (ACTH) release from the pituitary, and ACTH in turn stimulates the release of glucocorticoids (CORT) from the adrenals. Effects of ES on the offspring have often been attributed to the quantity and/or quality of maternal care and its effect on CORT. For example, fragmentation of maternal care induced by limiting the bedding and nesting material, activates the HPA-axis and results in increased CORT levels in mice (Naninck et al., 2015; Rice et al., 2008). In addition, rats receiving high licking and grooming (LG) had a lower glucocorticoid (CORT) response to acute stress, compared to those that received low levels of LG (Liu et al., 1997), and stroking has been shown to reduce stress reactivity human neonates (Feldman et al., 2010).

Several studies investigated the effects of such an early exposure to CORT and show similar consequences later in life as ES exposure. For example, neonatal treatment with a synthetic CORT (dexamethasone, DEX) reduced spatial learning and hippocampal plasticity (Kamphuis et al., 2003). In addition, DEX treatment during pregnancy led to insulin resistance and adiposity in mouse offspring (Chen et al., 2020). However, there is some controversy as to whether CORT is involved in programming by ES, since different studies and different animal models show different effects on CORT levels (Brunson et al., 2005; Lajud et al., 2012; Mirescu et al., 2004). Indeed, in **chapter 2**, we did not find an increase in CORT, but rather a decrease in ES-exposed offspring at P9. Moreover, preventing the rise in CORT induced by ES (maternal deprivation, MD) did not prevent alterations in HPA-axis activation at P20 (Van Oers et al., 2001). In addition, while preventing the ES-induced rise in CORT levels at P9 via supplementation of the diet with essential micronutrients restored object recognition memory in adulthood, Morris water maze performance and object location performance

were still impaired (Naninck et al., 2017). These results highlight that although alterations in CORT early in life play an important role and can affect adult metabolic and cognitive function, they are likely not the sole source of ES-induced programming.

2.2 Role for nutrition

Interestingly, while preventing the rise in CORT after MD did not affect HPA-axis responsivity, stroking combined with feeding, but not stroking alone, did (Van Oers et al., 2001). Indeed, ES and nutrition likely interact and affect one another (Lucassen et al., 2013), and stress can affect food intake and metabolism (Dallman et al., 2005; Pecoraro et al., 2004). In line with this, **in chapter 2**, we show that stress increases food intake in the dams from postnatal day (P)2-P9.

Interestingly, early-life malnutrition as well as maternal obesity increase the risk to develop various metabolic and brain disorders in the offspring (O'Reilly and Reynolds, 2013; Roseboom et al., 2006, 2011; Santangeli et al., 2015) that are similar to ES. Maternal undernutrition in turn also affects maternal behaviour (Wiener et al., 1976) and increases CORT levels in offspring (Núñez et al., 2008), suggesting that ES and nutrition interact with one another. In particular during the first 1000 days of human life, nutrition quality has a substantial impact on later life health, and breast milk provides an optimal form of nutrition during this important developmental period (Cusick and Georgieff, 2016; Horta et al., 2015; Lee et al., 2016; Mameli et al., 2016; Mayer-Davis et al., 2006).

The early life fatty acid quality has in particular been shown to be important for later life metabolic and brain function (Innis, 2008; Oosting et al., 2010; Schipper et al., 2020). Fatty acids (FAs) are incorporated in cellular membranes, function as signaling molecules, and are important for myelin formation and neuronal differentiation (Martínez and Mougan, 1998; Tabernero et al., 2001). In **chapter 2**, we show that stress alters the milk and offspring FA status in mice. Effects of ES on brain FA status have been shown before (Clarke et al., 2009; Yam et al., 2019) and we had previously shown that manipulating the dietary FA composition in mice can prevent ES-induced cognitive impairments in adulthood (Yam et al., 2019). Together, these studies suggest a pivotal role for FAs in programming by ES. However, it remains to be determined if postnatal stress affects the milk and brain fatty acid composition in humans.

There is initial evidence that depressive symptoms during pregnancy affect milk FA status in humans, although also no association between antenatal and postnatal depression and milk FA status has been reported (Keim et al., 2012; Urech et al., 2020). Notably, milk FA composition has been linked to child temperament (Hahn-Holbrook et al., 2019). It thus is possible that milk and offspring FA status could be involved in the programming by ES, but this requires further investigation. Since milk FA status reflects dietary intake (Koletzko et al., 1992; Yuhas et al., 2006), and is therefore relatively easy to modify, this is an exciting avenue to further explore for intervention strategies. Next to the milk FA composition, psychological state, stress and disorders have also been related to alterations in the milk microbiome

(Browne et al., 2019), immune factors (Kawano and Emori, 2015; Moirasgenti et al., 2019; Shariat et al., 2017) and glucocorticoid levels (Aparicio et al., 2020). Moreover, in another study, the total caloric content of milk was associated with child BMI and adiposity (Prentice et al., 2016). It is thus important to further characterize the effects of stress on the milk composition, and if such alterations are involved in ES-induced programming.

3. Metabolic programming by early-life stress exposure

A main focus in this thesis was the ES-induced metabolic programming, and whether ES differently affected males and females (sex differences are discussed in section 4). Clinical studies show that ES exposure increases the risk to develop obesity and diabetes type 2 (Alciati et al., 2013; Balsevich et al., 2019; Bellis et al., 2015; Danese and Tan, 2014), but it is not yet clear how ES increases these risks. The development of obesity is often multifactorial and depends on energy intake (food quantity and quality), energy expenditure (e.g. basal metabolic rate, exercise), as well as several less well known factors including the microbiome (Davis, 2016). Studies trying to understand the ES-induced metabolic risk in humans are confounded by factors such as poverty and food availability and food cues (Conroy et al., 2010; Smoyer-Tomic et al., 2008), making it challenging to disentangle the separate effects of biological vs environmental (e.g. the type of diet) elements. Preclinical studies have therefore opted to understand what factors contribute to these effects of ES on adiposity by exposing rodents to different dietary circumstances, that were provided *ad libitum* and without the option for a choice, allowing to study the physiological response (e.g. adiposity) to such diets. However, another important factor that could be implicated in the ES-induced metabolic risk is food choice (i.e. choosing more unhealthy foods). Evidence for effects of ES on both these aspects will be discussed below, followed by a discussion of the underlying neurobiological circuits (hypothalamic feeding circuitry and reward system) and subcellular substrates (mitochondria), that might be involved in ES-induced metabolic programming.

3.1 Effects of ES on energy intake: is there a role for food choice?

Preclinical studies suggest that the effect of ES on bodyweight and/or adiposity depend on, among others, the later life diet and sex, with ES-exposed females being more affected when they are fed an unhealthy diet (**chapter 1**). We have previously shown in mice that ES induced by limiting the bedding and nesting material (LBN) resulted in a lasting reduction of adipose tissue under standard dietary (STD) circumstances in both sexes, while upon exposure to an 8-week western-style diet (WSD) ES-exposed males accumulated more fat (females not investigated) (Yam et al., 2017a). The caloric intake of these diets was not affected by ES. Another study found that while ES (induced by repeated maternal separation (MS)) did not affect bodyweight on STD, when exposed to a 12-week high-fat diet (HFD), females, but not males, revealed higher bodyweights and adiposity compared to their

respective controls (CTLs)(Murphy et al., 2017). Also here, ES did not alter food intake on either STD or HFD. ES thus seems to alter the response to unhealthy diets without affecting the total intake of these diets when they are provided without a choice option. This points towards an altered metabolic regulation in ES-exposed animals, which is discussed below.

Next to an altered metabolic response, ES may also increase the preference for unhealthy foods, thereby further contributing to the observed obesity risk in humans. In **chapter 4**, we indeed report that ES increases the intake of the fat component (when provided the choice between fat, sugar water, chow and normal water) after an acute stressor, specifically in females. This is in line with a clinical study in which exposure to famine during gestation increased the intake of fatty foods (Lussana et al., 2008). The question of whether ES affects food choice is understudied, and studies in rodents using food choice paradigms reported mixed results. For example, while two studies found an increased intake of a palatable component (combining fat and sugar) in females (males not investigated) (Machado et al., 2013) and both sexes (de Lima et al., 2020), when exposed to LBN and MS, respectively, another study found a reduced chocolate intake in male rats (females not tested) (Bolton et al., 2018a). These differences likely depend on the exact composition of the diet (e.g. combined palatable chow versus a multiple component choice diet) and form (solid versus liquid), all aspects that affect food intake (la Fleur et al., 2010; La Fleur et al., 2014).

In line with our result on an increased fat intake in ES-exposed females upon acute stress exposure, human studies showed that women demonstrate more comfort feeding upon exposure to a stressful task, whereas men actually decrease their snack intake (Zellner et al., 2007, 2006). Such increased comfort eating might contribute to the sex-dependent metabolic vulnerability upon ES exposure observed in clinical studies.

Does ES alter energy expenditure?

Next to effects of ES on energy intake, alterations in energy expenditure could also contribute to fat accumulation. Energy expenditure can be investigated by measuring activity levels, thermogenesis (heat production) and assessing O_2/CO_2 levels with indirect calorimetry (Speakman, 2013). Only a few papers investigated the effects of ES on (indirect and direct) measures of energy expenditure. For example, we have previously shown that ES (LBN) increases UCP-1 expression in the brown adipose tissue of male and female offspring at P9, indicating a higher heat production, which however was normalized in adulthood (Yam et al., 2017a). Another study found that ES (MS) led to hyperactivity in the open field test at P50 in males but not females (Aya-Ramos et al., 2017). However, ES (LBN) did not affect activity levels in the elevated plus maze in both sexes (Naninck et al., 2015). These tasks may not be optimal to study activity levels, as they only provide a snapshot in time and also contain an anxiety component. Of note, when ES (MS) rats were exposed to an activity-based anorexia paradigm (22h of wheel running combined with 1h of food restriction for 5 days), ES-exposed rats ran more, ate less and lost weight more rapidly (Hancock and Grant, 2009). Finally, one study showed that MS affected multiple parameters of the hypothalamic-

pituitary-thyroid axis in both sexes (Jaimes-Hoy et al., 2019), which is key in regulating metabolic rate as well (Mullur et al., 2014). Thus, while these studies suggest that ES may alter energy expenditure, in the future it would be important to use indirect calorimetry and home cage activity tracking to study this in more detail.

3.2 Does ES program the adipose tissue?

We previously reported that the ES-induced reduction of adipose tissue levels under a standard diet (STD) was accompanied by a reduction in leptin expression, indicating that next to adipose tissue *quantity*, adipose tissue *function* was also affected by ES (Yam et al., 2017a). Adipose tissue dysfunction seems to be involved in obesity-associated metabolic complications (e.g. insulin resistance) (Longo et al., 2019), and we therefore further explored the effects of ES on adipose tissue under different dietary circumstances. In **chapter 3**, we investigated whether ES altered adipose tissue inflammatory gene expression in male mice at multiple ages, and under both STD and WSD. Adipose tissue inflammation is thought to be involved in the negative health consequences following obesity (Mraz and Haluzik, 2014; Ouchi et al., 2011). Gene expression analysis indicated lower macrophage numbers in the adipose tissue at P9, while at P42, ES increased expression of a major cytokine tumor necrosis factor alpha (**chapter 3;** Ruigrok et al., 2021). These effects of ES however did not last into adulthood (P230) either under STD or WSD. In addition, in **chapter 4**, we show that a 5-week free choice high-fat high-sugar diet (fcHFHS) increased adipose tissue fatty acid synthase expression in females, which was further increased by ES. Thus, taken together, we observed (partially sex-dependent) effects of ES on adipose tissue quantity and gene expression. Such programming of the adipose tissue is in line with another study investigating the effects of MS on metabolic gene expression in the adipose tissue at 10 weeks of age in females (Miki et al., 2013). Thus far, it is unknown whether ES directly programs the adipose tissue, or if it is rather an indirect effect due to an altered central innervation of the adipose tissue. Nonetheless, such changes in the adipose tissue may have important functional implications, for example for the development of insulin resistance (Longo et al., 2019).

3.3 What is the underlying neurobiological substrates for metabolic programming by early-life stress?

In this thesis, we investigated the effects of ES on two brain circuits involved in the regulation of energy metabolism and/or food intake: the hypothalamus, involved in energy expenditure and the homeostatic regulation of food intake, and the reward circuitry, that is implicated in the more hedonic-driven aspects of food intake (De Macedo et al., 2016; Timper and Brüning, 2017). These circuits also communicate with one another (Dietrich et al., 2012), and it is thus possible that ES can alter both these circuits, and/or the communication between them, to affect both peripheral metabolic functions and food choice.

With regard to the hypothalamus, we focused on two aspects: i) hypothalamic nutrient sensing pathways, in which fatty acid metabolism, metabolic hormone receptors and

hypothalamic neuropeptides are key, as well as ii) hypothalamic inflammation. The nutrient sensing pathway in the hypothalamus integrates multiple sources (nutrients, hormones) of information about the energy state of the body and thereby regulates energy homeostasis (Lam et al., 2005). In **chapter 5**, we show that ES modulated this pathway in a targeted manner early, but not later, in life, and similarly in both sexes. Next to this, in **chapter 3** we further show that ES affects the hypothalamic microglia response to a WSD in male mice (females not investigated). In fact, hypothalamic inflammation occurs within a few days after the initiation of high-fat diet (HFD) feeding and may mediate diet-induced obesity (DIO) development, since blocking hypothalamic inflammation prevents HFD-induced weight gain (Benzler et al., 2015; Kim et al., 2019). Importantly, hypothalamic inflammation also affects the neuronal populations involved in nutrient sensing (Kim et al., 2019). Because of the proposed important and integral role of hypothalamic microglia in DIO development, and the finding that microglia are pivotal for the induction of microglia activation upon HFD feeding (Kim et al., 2019), we next investigated whether ES affects microglial mitochondria (**chapter 7**). While this study is still preliminary, we indeed provide initial evidence for effects of ES on mitochondrial morphology and function, partly in a sex-dependent manner.

Next to these effects of ES on the hypothalamic circuitry, in **chapter 4**, we show that ES also altered aspects of the reward circuitry, similarly in both sexes (i.e. on dopamine cell numbers and on the inhibitory input on these cells in the ventral tegmental area (VTA)). It remains difficult to pinpoint the exact consequences of each separate change in these circuits, and likely a combination of factors is involved in ES-induced metabolic programming. In fact, while the homeostatic and hedonic aspects are often studied independently, there is communication between these circuits at multiple fronts. This connection is likely involved in a hunger/satiety regulation of food motivation and rewards (Castro et al., 2015). In a recent study it was shown that while both nutrients and drugs of abuse inhibit hypothalamic neurons expressing agouti-related protein (AgRP), which are known for their role in homeostatic metabolic regulation (Timper and Brüning, 2017), these, at the same time, also increase dopamine signaling in the reward circuitry (Alhadeff et al., 2019). The effects in the two brain circuits depended on each other, since modulation of either AgRP neurons or midbrain dopamine signaling influenced reward-evoked activity in the other area (Alhadeff et al., 2019). In fact, it had been shown before that AgRP neurons directly innervate the VTA (Dietrich et al., 2012). Interestingly, we showed in **chapter 4** that AgRP fiber density in the VTA was lower in ES-exposed males but not females compared their CTLs (on STD), suggesting the connection between homeostatic and reward areas may also be affected by ES exposure. Impairing the AgRP circuitry increases the response to rewards (Dietrich et al., 2012), however, it is unclear whether these sex-specific changes are involved in the sex-specific effect of ES on food choice.

Next to the ARH (homeostatic regulation) and VTA (hedonic regulation) and the connection between these areas, other central pathways could be at play in the ES-induced metabolic vulnerability. For example, the lateral hypothalamus links feeding and reward and

is important for the motivational aspect of food intake (Castro et al., 2015; Stuber and Wise, 2016). It receives input from the ARH and nucleus accumbens among others, and projects to the VTA (Betley et al., 2013; Stuber and Wise, 2016). In addition, the reward circuitry also expresses receptors for the metabolic hormones leptin and ghrelin (Hommel et al., 2006; Zigman et al., 2006). Direct infusion of leptin in the VTA decreases food intake (Hommel et al., 2006), while VTA ghrelin infusion stimulates palatable food intake (Skibicka et al., 2011; Wei et al., 2015). This indicates that these metabolic hormones, which are well known for their role in the hypothalamus, may also affect the reward circuits and reward-related food intake. Since ES lowers circulating leptin levels in both sexes and affects ghrelin levels in ES-exposed females (Yam et al., 2017b, 2017a), these hormone signaling pathways might be another mechanism by which ES affects the reward circuitry. While these pathways were not studied in this thesis, they would be interesting targets for future research.

Taken together, ES impacts on multiple metabolic systems, including the adipose tissue and the brain circuits that regulate food intake and energy expenditure, and these systems closely interact with one another. Coping with stress requires an individual to adequately respond as a whole. For example, when exposed to acute stress we breath more rapidly, our heartrate goes up, muscles become tense, and blood glucose levels rise. All these physiological changes are interrelated and needed to cope with stress, but become detrimental upon prolonged exposure. Receptors for stress hormones are widely expressed to accomplish such responses, and as mentioned in section 1, it is likely that multiple elements in the early-life environment (e.g. maternal care, nutrition, hormones) are affected by ES exposure. It can therefore merely be expected that multiple systems (adipose tissue, homeostatic and hedonic brain circuits) are programmed by ES exposure and together contribute to the increased disease risk.

4. Sex-specific metabolic programming by ES

One of the main scopes of this thesis was to increase our understanding of the sex-specific, metabolic programing by ES exposure. We identified a plethora of sex differences in metabolic functions and regulation, including sex differences in adiposity, food choice and the underlying brain circuits, as well as sex-specific modulations by ES. For example, in **chapter 1**, we review clinical and preclinical literature and show that males/boys and females/girls are affected by ES on different domains (cognitive, behavioural and metabolic domains), and that females/girls seem more vulnerable for the effects of ES on adiposity. In **chapter 4**, we show strong sex differences in food choice and the physiological response to a high-fat high-sugar choice diet, as well as sex-specific modulations by ES, and identify brain circuits that are affected by ES partly in a sex-specific manner. In **chapter 5** we show a sexually dimorphic expression pattern of genes critical in hypothalamic nutrient sensing,

and a targeted modulation of this pathway by ES, and in **chapter 7** we provide preliminary evidence that female microglial mitochondria have higher mitochondrial respiration, and that more aspects of mitochondrial respiration were affected by ES in females compared to males. In the following section we will discuss what could underlie this sex-specific metabolic programming and how ES could impact on this, with a focus on sex steroids.

4.1 A role for sex steroids in sex-specific metabolic programming by ES?

Sex hormones such as progesterone, testosterone and estradiol are steroid hormones that belong to the same synthetic pathway and start from a cholesterol backbone. Progesterone and testosterone, besides having their direct actions by binding to the progesterone and androgen receptor (PR, AR), also provide a precursor for estrogen synthesis (Miller, 1988). Glucocorticoids also belong to this synthetic pathway. In fact, it has been hypothesized that crosstalk between glucocorticoids and sex steroids is involved in ES-induced sex-specific programming of metabolism (Ruiz et al., 2020). Sex steroids both regulate the sex depended early-life development as well as influence the functioning of organ systems later in life, including the brain. Below, we will first discuss sex differences in development under the influence of sex steroids, followed by the role of sex steroids in regulating adiposity and insulin sensitivity, the hypothalamus, reward-related behaviors, and mitochondria. We finish with elaborating on whether there could be a role for these hormones in mediating sex-specific programming by ES.

4.2 Sex differences in development and sexual differentiation of the brain

Sex differentiation is a sequential process. Early in development, the genitalia of both sexes are similar and have the capacity to develop in either male or female genitalia. However, (in humans) after 6-7 weeks of gestation, the expression of the Y-chromosome based SRY gene, among others, leads to development of the testes. Testosterone synthesis from the testes occurs from around the 9th week of gestation onwards, and peaks around 16 weeks (Scott et al., 2009). In females, the ovaries are able to produce steroids, but do so marginally: both male and female fetuses mostly receive estrogen from the placenta (Costa, 2016). Upon birth, males have a luteinizing hormone (LH) surge, followed by an increase in circulating testosterone, which is absent in females. During childhood, both sexes have very low levels of circulating testosterone, and girls have higher levels of estradiol compared to boys (Klein et al., 1994). Secondary sexual characteristics appear during puberty, mediated by the hypothalamic-pituitary-gonadotropin-gonadal (HPGG) axis. The HPGG system sexually differentiates during prenatal and early postnatal development but is suppressed during childhood, to become active again in puberty. In adulthood, women have more estrogen and progestin and less androgen (including testosterone) as compared to men.

In humans, adipose tissue appears around mid-gestation, and its mass will increase through late gestation (Poissonnet et al., 1984; Symonds et al., 2012). At this age, the adipose tissue is a mix of brown adipocytes (involved in heat production) and white adipocytes

(involved in fat storage and adipokine production), while during postnatal life, most depots are replaced by white fat (Symonds et al., 2012). The early development of adipose tissue during gestation does not seem to be much different for males and females (Poissonnet et al., 1984). However, the percentage of body fat postnatally is higher in girls compared to boys (Hawkes et al., 2011), and sex differences in adipose tissue become more pronounced after puberty, when women have more subcutaneous fat, while men have more visceral fat (Fuente-Martín et al., 2013).

The brain continues to develop far into postnatal life. In humans, the brains reaches maturity around approximately 25 years of age, while in rodents this happens arounds postnatal day 60 (Arain et al., 2013; Semple et al., 2013). The brain is therefore extremely vulnerable for programming by environmental disturbances (e.g. stress) during pregnancy and postnatal life. The sex-dependent organization of different aspects of the brain occurs under the influence of sex steroids (Kudwa et al., 2006; Wu et al., 2009), as well as sex chromosomes and environmental influences (McCarthy and Arnold, 2011). While in males, male-typical neural and behavioral differentiation largely seems to occur *prenatally* by testosterone, that is conversed to estradiol, there is evidence that in females, *postnatal* estradiol is important for female-typical neural and behavioural differentiation (Bakker and Brock, 2010). Sexually dimorphic organization of the brain by sex steroids, sex chromosomes and environmental factors persists throughout life and affects the way the brain responds to circulating sex steroids later in life (McCarthy and Arnold, 2011).

4.3 Effects of estrogen on adiposity and insulin sensitivity

Premenopausal women, compared to postmenopausal women as well as similar-aged men, have been shown to be less likely to develop the metabolic syndrome (central obesity, insulin insensitivity, dyslipidemia) (Carr, 2003; Cignarella et al., 2010; Janssen et al., 2008; Kim et al., 2004). In line with this, in **chapter 4**, we showed that males were more vulnerable to the effects of the fcHFHS on circulating glucose levels. After menopause, when circulating estrogens decline, alterations in energy homeostasis occur that cause intra-abdominal body fat to increase (Carr, 2003). Indeed, estrogen deficiency promotes metabolic dysfunction, obesity and type 2 diabetes development (Frank et al., 2014; Mauvais-Jarvis et al., 2013). Estrogen has strong metabolic actions, and works on the adipose tissue, liver, pancreas and hypothalamus, among others (Mauvais-Jarvis et al., 2013). 17β-estradiol (E2) is the main circulating estrogen and is produced by the ovaries by aromatization of androstenedione into estrone, with a subsequent conversion of estrone (E1) into E2. E2 can also be produced locally from circulating androgens in tissues such as the brain, breast, muscle and adipose tissue (Simpson, 2003). Estrogen and its receptor have been shown to affect body composition by acting on food intake, energy expenditure and physical activity (Blaustein and Wade, 1976; Geary et al., 2001; Heine et al., 2000; Jones et al., 2000; Rogers et al., 2009). Adipocytes produce estrogens (via aromatization of androgenic precursors) while expressing the estrogen receptor alpha (ERα) themselves as well (Mizutani et al., 1994). Adipocyte

ERα knockdown increases adiposity and adipose tissue inflammation, characterized by macrophage infiltration and increased cytokine expression in the adipose tissue (Davis et al., 2013). E2 decreases lipogenesis and fatty acid uptake (D'Eon et al., 2005). In addition to these effects on the adipose tissue, estrogen also acts on insulin sensitivity. Ovariectomy leads e.g. to glucose intolerance, while the restoration of E2 levels maintains proper insulin action (Stubbins et al., 2012). Taken together, these data indicate that estrogens protect against metabolic perturbations.

4.4 Effects of testosterone on adiposity and insulin sensitivity

Next to estrogen, also, testosterone (T) is of interest in this respect. Although less extensively described, T has also been associated with metabolic disease risk and development (Jones, 2010). For example, men with low T levels have a higher body mass index compared to those with T levels in the normal range (Simon et al., 1997), and low T levels predict diabetes type 2 development during a 5 year follow-up in adult men (Haffner et al., 1996). Indeed, T modulates insulin sensitivity in male rats. Both the absence of T (via castration) and the presence of high T levels markedly reduced insulin sensitivity, while restoring T levels to a physiological range in castrated rats abolished insulin sensitivity perturbations (Holmang and Bjorntorp, 1992). The effects of T on adiposity and insulin sensitivity could potentially be explained by the fact that testosterone serves as a precursor for E2 production in adipose tissue (Cohen, 1999). However, it is possible that T also regulates metabolism in a more direct manner e.g. via acting on the hypothalamus. How sex steroids modulate hypothalamic functions will be discussed next.

4.5 Sex steroids and hypothalamic functions

In the ARH, ERα receptors are predominantly expressed in proopiomelanocortin (POMC) neurons (Xu et al., 2011). These neurons are known to inhibit food intake, while increasing energy expenditure. Excitatory synaptic inputs onto ARH POMC neurons fluctuate throughout the estrous cycle, and E2 treatment increases the number of these synapses onto POMC neurons (Gao et al., 2007). Next to the ARH, the ventromedial nucleus of the hypothalamus (VMH) also regulates food intake and is affected by estrogen. In fact, while a selective deletion of ERα from steroidogenic factor-1 neurons in the VMH resulted in hypometabolism and abdominal obesity, but did not affect food intake per se, a deletion of the ERα from ARH POMC neurons led to hyperphagia, without altering energy expenditure or fat distribution in females (Xu et al., 2011). In addition, leptin and estrogen seem to interact in the hypothalamus to regulate metabolism (Clegg et al., 2006; Diano et al., 1998). We have shown before that ES lastingly lowered circulating leptin levels, and lowered leptin receptor expression in the hypothalamus at P9 as well, suggesting that ES could also impact this interaction (Yam et al., 2017a) (**chapter 5**).

The protective metabolic effects of estrogens could, at least in part, be mediated by their effects on hypothalamic inflammation (Butler et al., 2020; Morselli et al., 2014; Yang et

al., 2020). Under standard dietary circumstances, ovariectomy increased microglia numbers and cytokine mRNA levels (Yang et al., 2020), and triggered microglia activation in response to a high-fat diet (Butler et al., 2020), which could again be attenuated by E2 replacement (Butler et al., 2020). In addition to being sensitive to effects of estrogen, the hypothalamus also expresses ARs (Fernández-Guasti et al., 2000), and T treatment, next to counteracting the effects on adiposity and glucose metabolism, was recently also shown to be able to prevent HFD-induced hypothalamic inflammation (Sarchielli et al., 2021).

4.6 Sex steroids and reward-related behaviour

Interestingly, next to the regulation of metabolism by sex steroids, these hormones also affect the reward circuitry (de Souza Silva et al., 2009; Vandegrift et al., 2017). In **chapter 4**, we show that specifically ES-exposed females, but not males, ate more fat after a mild acute stress exposure. Palatable foods, such as fat and sugar, activate the mesocorticolimbic dopamine (DA) circuitry. This circuitry is implicated in reward and encompasses the VTA and nucleus accumbens (N.Acc), among others (De Macedo et al., 2016). In **chapter 4**, we show that ES increased TH^+ cell density (as readout for DA cells) in the VTA, while decreasing the inhibitory input onto TH^+ fibers, and to a similar extent in both sexes. In addition, AgRP fiber density in the VTA, which also provides inhibitory control (Dietrich et al., 2012), was affected only in ES-exposed males. While the latter effect could be involved in the ES-induced sexual dimorphic food intake response to acute stress, there could also be a role for sex-steroids in modulating such sex differences. In fact, it has been hypothesized that sex steroids may modulate the effects of ES on the dopamine system (Eck and Bangasser, 2020).

Notably, sex differences exist in the neural mechanisms that regulate reward (Becker and Chartoff, 2019). For instance, compared to males, female rodents acquire drug self-administration tasks faster (Hu et al., 2004) and have greater motivation to seek drugs (Cummings et al., 2011). Both human and rodent studies furthermore indicate that women/females have a higher propensity to drug relapse (Hudson and Stamp, 2011). These sex differences might be explained by differences in E2, as ovariectomized females have a faster acquisition of drug self-administration upon E2 administration (Hu et al., 2004). Indeed, the VTA contains ERs and VTA DA neuron survival seems to be regulated by ER (Creutz and Kritzer, 2004, 2002; Johnson et al., 2010). For example, DA firing in the VTA fluctuates over the estrous cycle (Zhang et al., 2008), and rats with high E2 levels have an increased sensitivity of their DA neurons in the VTA (Vandegrift et al., 2017).

Next to E2, T might also influence reward-related behaviour: T may do so indirectly by conversion to E2, but also in a more direct manner due to the presence of ARs in the VTA (Creutz and Kritzer, 2004, 2002). It has for instance been shown that prenatal T exposure increases TH^+ cell number in female sheep (Brown et al., 2015). Also, intranasal T treatment increases dopamine release in the N.Acc (de Souza Silva et al., 2009). Altogether, these data indicate that sex steroids modulate the reward circuitry and reward-based behaviour.

Thus far, it is unknown whether sex steroids are involved in the sex-dependent effects of ES on the (acute stress-induced) intake of palatable food. The effects of ES on reward are usually studied in only one sex and different studies investigate different aspects of the reward circuitry. This makes it difficult to understand if ES differently affects the reward circuitry in males or females (Bolton et al., 2018b; Brake et al., 2004; Peña et al., 2017; Spyrka et al., 2020; Ventura et al., 2013). However, given our current findings that such sex differences indeed exist, and the initial evidence that ES may alter sex steroid levels (see section 4.8), it would be interesting to investigate in future studies also the role of these steroids in programming the reward circuitry after exposure to ES (Eck and Bangasser, 2020).

4.7 Sex steroids and mitochondrial functions

Mitochondrial dysfunction is a common feature in several brain and metabolic disorders (Guo et al., 2017; Khacho et al., 2017; Sivitz and Yorek, 2010), and sex differences in mitochondrial function have been described in multiple tissues (Demarest and McCarthy, 2015; Ventura-Clapier et al., 2017). In **chapter 7**, we show preliminary evidence for sex differences in mitochondrial functions in microglia-enriched cultures, with females showing higher basal respiration rates, maximal respiration rates and ATP production compared to males. Indeed, a higher mitochondrial respiration rate has also been shown in the female liver and brain (Gaignard et al., 2015; Valle et al., 2007), whereas oxidative stress was reported to be higher in males (Gaignard et al., 2015). Male and female mitochondria further respond differently to metabolic stressors, like starvation: while neurons from males decrease their mitochondrial respiration more strongly, undergo autophagy and die more rapidly, neurons from females survive longer, and mobilize more fatty acids and lipid droplets (Du et al., 2009). Such differences in mitochondrial functions and vulnerability could also be implicated in the sex differences in disease risk (Demarest and McCarthy, 2015; Ventura-Clapier et al., 2017). In **chapter 7**, we provide preliminary evidence that while CTL females maintain higher respiration rates upon exposure to an environment with an excess of nutrients ('high-fat high-sugar'), ES exposure lowered the mitochondrial respiration of females to male-like levels of respiration. Whether ES-induced differences in mitochondrial function are indeed implicated in the vulnerability to develop later metabolic and/or brain dysfunction, remains, however, to be determined.

Sex steroids can directly regulate mitochondrial function (Vasconsuelo et al., 2013). Mitochondrial genomes contain e.g. sequences similar to those for nuclear hormone response elements, for type I and type II receptors, including ERs and ARs (Demonacos et al., 1996; Sekeris, 1990). In line with this, E2 upregulates the expression of several mtDNA-encoded proteins involved in the electron transport chain (ETC), and also affects the activity of ETC complexes (Bettini and Maggi, 1992; Chen, 2003, 1998). Next to directly affecting mtDNA transcription, estrogens' effect on mitochondria may, at least in part, be mediated by its effect on the expression of the nuclear encoded PGC-1a, which is a master regulator of energy metabolism and mitochondrial biogenesis (Tcherepanova et al., 2000).

Indeed, we observed higher PGC-1a expression in the hypothalamus of females early in life (**chapter 5**). Yet, less is known about the effect of T on mitochondria, although T deficiency causes mitochondrial damage and increased mitochondrial ROS accumulation in gastric mucosa (Kang et al., 2018). T administration after traumatic brain injury further preserved mitochondrial functions (Carteri et al., 2019). However, more research is needed to understand the actions of T on mitochondria.

4.8 Does ES impact on sex steroids and sexual maturation?

E2 strongly impacts on metabolism and brain functions, and, although less well studied, T might also exert such effects, either in a direct or indirect manner (via estrogenic conversion). Notably, it has been hypothesized that neuroactive steroids could be involved in the ES-induced programming of the brain (Brunton, 2015), but this area of research has remained rather unexplored so far. One study showed that exposure to LBN from P2-P9 reduced circulating estradiol (E2) levels in adult rat offspring, which was only significant for males and not females (Eck et al., 2020). Furthermore, both lower T levels (Tsuda et al., 2011) and no effect of ES on T levels (Eck et al., 2020) have been reported in adult males (females not investigated). A study on prenatal stress has further shown that T dynamics were altered in male, but not female rat fetuses (Ward and Weisz, 1984). In the brain, hippocampal T and E2 levels were not affected by MS in adult male rat offspring (Wang et al., 2013). In a study on natural variation in maternal care (low LG versus high LG), it was further shown that female offspring from high LG mothers had higher ERα mRNA levels in the medial preoptic area (MPOA) of the hypothalamus, and the cFOS response in the MPOA to E2 was different between low and high LG offspring (Champagne et al., 2003). Finally, a human study showed that exposure to maternal stress during pregnancy (caused by the Chernobyl disaster) increased T levels in girls at 14 years of age, while boys were unaffected (Huizink et al., 2008).

Taken together, different studies have looked at a variety of aspects of the sex steroid signaling pathways (e.g. circulating hormone levels, brain hormone levels and receptor expression) at multiple ages, and in different species and ES models. While there is initial evidence for effects of ES on sex steroid levels and receptors, it remains to be understood how ES exactly modulates these pathways. Interestingly, in a mouse study on *adult* chronic social defeat stress, ERα was identified as the top regulator of pro-resilient transcriptional changes in the N.Acc, and overexpression of ERα in the N.Acc promoted stress resilience in both sexes. These data provide further evidence for an interaction between stress and sex steroids, and underscore the importance of studying these pathways with regard to ES.

Sexual maturation is mediated by the HPGG axis, and the HPGG axis is regulated by leptin, among others (Elias, 2012). Notably, we previously found circulating leptin levels to be lower in ES-exposed offspring (Yam et al., 2017a), and ES induced by MS has been shown to alter puberty onset in a sex-specific manner (Cowan and Richardson, 2019): while ES-exposed females showed an earlier onset of puberty, males showed a later puberty onset.

In line with this, female offspring that received less maternal care (low LG) showed an earlier puberty based on vaginal opening, compared to those that received high LG (Borrow et al., 2013). In addition, low LG female offspring showed more lordosis behaviour (body posture for sexual receptivity), received more ejaculations and were more likely to get pregnant (Cameron et al., 2008). In **chapter 1,** we discuss evidence from human studies for a more rapid maturation in ES-exposed girls, who seem to show better adaptation in class and have less externalizing problems, as well as faster sexual maturation (Belsky et al., 2015; Kersten-Alvarez et al., 2012; Sinclair and Murray, 1998). Thus, although there is only limited evidence so far, ES seems to impact sex hormone levels as well as sexual maturation itself. While other mechanisms could also be involved in the sex-dependent effects of programming by ES (e.g. sex chromosomes), it is important to better understand if and how sex steroids are involved in ES-induced sex-specific programming.

5. Comorbidity between metabolic and brain disorders

ES increases the vulnerability to develop both metabolic and cognitive disorders (Chugani et al., 2001; Danese and Tan, 2014; Gilbert et al., 2009; Saleh et al., 2016), diseases that are often comorbid. Also, obesity and diabetes type 2 have often been associated with an increased risk to develop cognitive and mental (e.g. depression) disorders (Nguyen et al., 2014; Nooyen, 2010)(Dye et al., 2017). Indeed, metabolic hormones as well as obesity-associated inflammation and vascular changes are related to brain (dys)function (McGregor et al., 2015; Nguyen et al., 2014). However, cognitive dysfunction itself is also a risk factor for overfeeding (Gunstad et al., 2020), and a reciprocal association between depression and obesity is described (Luppino et al., 2010).

In **chapter 1**, we reviewed the effects of early-life adversity (ELA; encompassing pre- and postnatal stress) on both metabolic and brain readouts in both sexes. One of the aims of this literature review was to understand whether metabolic and brain alterations following ES exposure show a comorbid (sex-dependent) occurrence. When we aligned the brain and metabolic outcomes, we found that girls were more likely to develop adiposity upon ELA, and were also more often affected on the internalizing behavioral domain (including depressive and anxiety symptoms), following prenatal stress exposure. The comorbidity between metabolic and brain disorders suggest that converging pathways might be involved in ES-induced programming of metabolic and brain functions.

In **chapter 6**, we explored whether mitochondria could be such a converging substrate because they are; i) the essential 'power houses' of our cells, ii) involved in both responding and adapting to stress (Hoffmann and Spengler, 2018; Picard et al., 2014; Zitkovsky et al., 2021), and iii) implicated in both metabolic disease and cognitive dysfunction (Guo et al., 2017; Khacho et al., 2017; Sivitz and Yorek, 2010). Indeed, in **chapter 6** we found peripheral and central mitochondria to be affected by ES in an age- and tissue-dependent

manner. Thus, although ES had widespread effects on mitochondria (i.e. in hippocampus, hypothalamus and muscle), these effects were different for each tissue type and brain region. Indeed, mitochondria in different organs may respond differently to early stress (Eagleson et al., 2020). Nonetheless, this indicates that mitochondria could be potential targets for intervention. Mitochondrial function and oxidative stress levels are modulated by nutrition, and also these elements could be an interesting avenue for future treatment strategies. This is supported by a study showing that ES-induced oxidative stress and depressive-like behaviour could be prevented by a treatment with omega-3 FAs, folic acid and n-acetylcysteine (Réus et al., 2018).

6. Clinical relevance

All experiments in this thesis were performed in mice. This allows for a more controlled study of the effects of ES on food choice and metabolism, as well as for the possibility to examine the underlying brain circuits. An important question that arises is whether the results obtained from such rodent experiments are indeed translatable to humans, for example with regard to interspecies differences in development, that indeed exist between rodents and humans. Firstly, rodents are born in large litters, whereas humans usually deliver one baby per pregnancy. Moreover, humans have a greater phase of intra-uterine development compared to rodents (Xue et al., 2013). Whereas the mouse embryo is born directly after the initial development of its organs, the human embryo stays in the uterus for several more months after its initial organ development, during which they continue to grow. In addition, differences in brain development between rodents and humans are well described: just after birth (postnatal day (P)1-3), the rodent brain is comparable to a human brain at gestational week 23-32, and at P7-P10, the rodent brain can be compared with that of an *a term* infant (Semple et al., 2013). Of note, while the timescale of brain maturation is different between humans and rodents, the sequence of events is largely comparable (Semple et al., 2013). In humans, adipose tissue depots largely develop in the second trimester (Poissonnet et al., 1984), but in rodents, most depots develop postnatally, except from the inguinal fat store which already starts to develop prenatally (Slavin, 1979). Altogether, the early postnatal period in rodents could thus best be compared with the mid to late prenatal period in humans. Except for this early phase, and as concluded in **chapter 1,** preclinical and clinical studies are in general terms well comparable and from a translational perspective, preclinical studies thus provide a useful tool to investigate programming by ES exposure.

7. Conclusion

In this thesis, we have provided evidence that i) early-life nutrition could play a role in ES-induced programming, ii) ES has sex-specific effects on food choice, iii) effects of ES on peripheral metabolic readouts depend on diet and sex, iv) the brain circuits involved in both the homeostatic and hedonic regulation of food intake, are affected by ES exposure, and v) mitochondria could be a subcellular entity possibly implicated in ES-induced programming. This thesis thereby contributes to a better understanding of how ES programs individuals for life, and provides new avenues for putative future prevention or intervention strategies.

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English summary

English summary

Exposure to stress during the sensitive period of early life (early-life stress; ES) increases the risk to develop metabolic diseases such as obesity, as well as cognitive disorders. Notably, these types of disorders often occur together. ES is very common: in the United States, approximately 61% of adults experienced some form of early-life adversity (e.g. parental separation, domestic violence and abuse). Prevention of ES is difficult, and currently no intervention strategies are available.

The concept that the early environment shapes individuals for life, is called 'programming'. During the period in which the brain is undergoing rapid development, also a metabolic 'setpoint' is established.
In this thesis, we investigated the elements in the early-life environment that could mediate such programming by ES, focusing on fatty acids in the maternal milk. In addition, we studied the (neuro)biological pathways that could be involved in the metabolic vulnerability following ES exposure and investigated whether there is a role for food choice. We furthermore set out to investigate if there are converging subcellular substrates by which ES affects both metabolic and cognitive functions, and finally, as ES may not affect males and females similarly, we focused on potential sex differences in the response to ES.

In **chapter 1**, we extensively reviewed the clinical and pre-clinical literature on the effects of early-life adversity (ELA; pre-and postnatal) on metabolic, mental and cognitive outcomes. We specifically focused on; i) whether ELA differently affects both sexes, ii) if this depends on the timing of ELA exposure (pre- versus postnatal), and iii) if clinical and preclinical literature converge in their findings. We found that ELA impacts on metabolic, mental and cognitive health in a sex-specific manner. While boys/males seemed more vulnerable for cognitive and externalizing behavioral problems, girls/females were more prone to develop overweight/adiposity. For internalizing behavioral problems, both boys and girls were negatively affected, depending on the timing of ELA. Furthermore, clinical and pre-clinical studies largely converged in their findings, indicating rodent studies can be helpful in obtaining a better understanding of the (neuro)biological substrates involved in programming by ELA.

In **chapter 2**, we aimed to increase our understanding in the elements in the early-life environment that mediate early-life stress (ES)-induced programming, focussing on fatty acids (FAs). We used a mouse model for ES in which the bedding and nesting material was limited, resulting in an impoverished environment and fragmented maternal care. FAs are critical for proper (brain) development, and alterations in the early-life dietary FA composition can affect the risk for developing later obesity and cognitive dysfunction, comparable to the ES-induced phenotype. We had previously shown that ES alters the offspring brain FA status, and that altering the early dietary FA composition could prevent ES-

induced cognitive impairments. Since maternal milk is the sole dietary source of fatty acids early in life, we hypothesized that ES alters the milk FA composition. We found that stress affected FA status in milk and in the offspring. Moreover, there were sex differences in the offspring FA status, with females more often being affected by ES compared to males, and milk and offspring FA status (partly) correlated. We therefore suggest that stress-induced changes in milk composition could, at least in part, mediate programming by ES, and this should be further investigated in future studies.

In **chapter 3**, we further explored the concept of an interaction between ES and early nutrition by providing ES-exposed mice with an early-life dietary intervention. Maternal milk provides the optimal nutrition during early development and has many health benefits. The physical properties of the lipid droplets in milk might contribute to these benefits. Nuturis® is an infant milk formula that has been developed to closely mimic the physical properties of mammalian milk. We had previously shown that ES-exposed mice accumulate more fat in response to a western-style diet (WSD), while Nuturis® could prevent such WSD-induced fat accumulation. Peripheral and central inflammation are implicated in diet-induced obesity development. Therefore, in chapter 3, we studied the effects of ES and Nuturis® on inflammation and found that both ES and Nuturis® affected peripheral and central inflammatory profiles, both early and later in life. Such changes might be key in the later life risk for developing metabolic derangements.

In **chapter 4**, we explored the effects of ES on an important, yet under-investigated, element contributing to the development of obesity in humans: food choice. We investigated if ES exposure affected food choice with a free choice high-fat high-sugar diet, known to induce prolonged overeating and snacking behaviour, in male and female mice. We reported that specifically ES-exposed females overeat the fat component in response to an acute stressor. In addition, we found that, physiologically, males and females responded differently to the diet as well as to ES, and that the brain reward circuits that regulate palatable food intake were affected by ES, partly in a sex-specific manner. Our data highlight sex-specific effects of ES on metabolic function and food choice.

In **chapter 5**, we increase our understanding of the neurobiological substrates that mediate the effects of ES on metabolism, and focus on nutrient sensing in the hypothalamus. The hypothalamus is a key brain region that regulates food intake and energy expenditure, by sensing hormones (leptin, insulin, ghrelin) and nutrients (glucose, fatty acids). We investigated if and how ES altered hypothalamic nutrient sensing directly after stress exposure and in adulthood, and if such effects were sex specific. We discovered strong sex differences in the hypothalamic nutrient sensing pathways as well as a targeted modulation by ES early in life.

In **chapter 6**, we focus on the comorbidity of ES-induced metabolic and cognitive alterations. Mitochondrial function is impaired upon chronic stress exposure and is associated with both metabolic diseases and cognitive dysfunction. However, if and how mitochondria are affected by ES remained elusive. We found that ES impacts mitochondria throughout life, thereby uncovering a converging mechanism that might contribute to the ES-induced vulnerability for both metabolic diseases and cognitive dysfunction.

In **chapter 7**, we continue our study on mitochondria as a possible subcellular substrate for programming by ES. It had previously been shown that mitochondria in hypothalamic microglia drive high-fat diet (HFD)-induced inflammation, and subsequently HFD-induced weight gain. We therefore investigated mitochondrial structure, density and function in microglia of ES-exposed male and female mice. In this preliminary report, we show age-, diet- and sex-dependent effects of ES on microglial mitochondrial structure and density, and report that ES affected mitochondrial functions more so in females.

Chapter 8 integrates the results from this thesis and discusses i) the role for early-life nutrition in programming by ES, ii) metabolic programming by ES exposure, focussing on food choice, the adipose tissue and the underlying brain circuits, iii) factors that could underlie sex-specific programming by ES focussing on sex steroids, iv) the comorbidity between metabolic and brain disorders, and v) the clinical relevance of our work.

In **summary,** our data shows that i) stress alters the milk fatty acid status; ii) ES affects food choice, the adipose tissue and the neurobiological substrates regulating food intake and energy expenditure, partly in a sex-specific manner; and iii) that mitochondria are affected by ES in both peripheral and central tissues, making them an interesting subcellular target by which ES could program both the body and the brain. As such, this thesis contributes to a better understanding of how ES shapes individuals for life, and could aid the future development of targeted therapeutic strategies.

Nederlandse samenvatting

Nederlandse samenvatting

Blootstelling aan stress vroeg in het leven verhoogt de kans op het ontwikkelen van zowel metabole ziekten, zoals overgewicht, als ook cognitieve (leer en geheugen-) problemen later in het leven. Het idee dat omstandigheden vroeg in het leven de latere gezondheid beïnvloedt, noemen we 'programmeren'. Ons brein is in deze vroege periode niet alleen nog volop in ontwikkeling, ook wordt er vroeg in het leven een metabool 'setpoint' ingesteld, wat de latere regulatie van het lichaamsgewicht lijkt te bepalen.

Helaas komt vroege stress veel voor. In Amerika heeft ongeveer 61% van de volwassen enige vorm van vroege stress ervaren, zoals bijvoorbeeld fysiek, emotioneel of seksueel misbruik, huiselijk geweld of gescheiden ouders. Voorkomen van vroege stress is lastig, en momenteel zijn er geen goede behandelingen beschikbaar.

In dit proefschrift hebben we onderzocht welke factoren uit die vroege omgeving nu bijdragen aan de effecten van vroege stress op nakomelingen. We richten ons daarbij op een rol voor voeding. Daarnaast onderzochten we welke (neuro)biologische en gedragsveranderingen ten grondslag kunnen liggen aan de vergrote kans op metabole ziekten na blootstelling aan vroege stress, en of er hierbij een rol is voor (ongezonde) voedselkeuze. Verder heeft ons onderzoek zich gericht op mogelijke veranderingen in de cel, die zowel de metabole als cognitieve consequenties van vroege stress zouden kunnen helpen verklaren. Tot slot, aangezien vroege stress mogelijk niet dezelfde effecten heeft op jongens en meisjes maar dit onvoldoende onderzocht is, hebben we ook seks verschillen onderzocht.

In **hoofdstuk 1** geven we een samenvatting van het reeds bestaande klinische (humane) en preklinische (dier) onderzoek naar de effecten van vroege stress op de metabole, cognitieve en mentale maten in zowel jongens/mannetjes als meisjes/vrouwtjes. We richtten ons met name op; i) of vroege stress dezelfde effecten had op beiden seksen; ii) of dit afhing van de timing van de vroege stress (tijdens de zwangerschap versus vlak na de geboorte); en iii) of klinische en preklinische literatuur hetzelfde beeld geeft. We vonden dat vroege stress een ander effect heeft op de metabole, mentale en cognitieve gezondheid van jongens en meisjes. Terwijl jongens een grotere kans hebben op cognitieve en zogenoemde externaliserende gedragsproblemen na blootstelling aan vroege stress, lopen meisjes meer kans om overgewicht te krijgen. Voor internaliserende gedragsproblemen vonden we dat zowel jongens als meisjes beïnvloed worden door vroege stress, maar dat dit afhangt van de timing van stress. Zowel klinische als preklinische literatuur lieten grotendeels hetzelfde patroon zien. Dit duidt erop dat we door preklinisch onderzoek goed kunnen begrijpen hoe vroege stress het functioneren van het volwassen brein en lichaam verandert.

In **hoofdstuk 2** onderzochten we vetzuur-samenstelling van de moedermelk als mogelijke factor die bij zou kunnen dragen aan het programmeren door vroege stress. We maakten gebruik van een diermodel voor vroege stress, waarbij de huisvesting van de moeder en haar pups wordt versobert. De kooien krijgen een minimale hoeveelheid zaagsel en nestmateriaal tijdens dag 2 tot dag 9 na de geboorte. Dit zorgt voor stress en gefragmenteerde zorg van de moeder. Vetzuren zijn belangrijk voor het goed ontwikkelen en functioneren van het brein en een verstoorde balans in de vetzuur-samenstelling vroeg in het leven kan, net zoals vroege stress, de kans op obesitas en cognitieve problemen vergroten. We hadden eerder aangetoond dat de vetzuur-samenstelling in het brein van dieren die vroege stress hebben gehad, veranderd is, en dat een dieet met een gunstige vetzuur samenstelling de cognitieve problemen na vroege stress juist kon voorkomen. Omdat moedermelk de voornaamste bron van vetzuren is in het vroege leven, veronderstelden we dat vroege stress mogelijk leidt tot een andere vetzuur-samenstelling in de melk. We vonden effecten van stress op i) de melk vetzuur-samenstelling, ii) de vetzuur-samenstelling in de nakomelingen, en iii) dat deze deels aan elkaar gerelateerd konden worden. We veronderstellen daarom dat de effecten van stress op de melk compositie een belangrijke bijdrage zou kunnen leveren aan het programmeren door vroege stress.

In **hoofdstuk 3** hebben we het concept van een interactie tussen vroege stress en voeding verder onderzocht door middel van een dieet interventie. Naast het feit dat de samenstelling van moedermelk belangrijk is, kunnen de fysieke eigenschappen van de vetdruppels in melk mogelijk ook bijdragen aan de positieve effecten van borstvoeding. Nuturis® is ontwikkeld om de deze fysieke eigenschappen van humane melk na te bootsen. We hadden eerder gezien dat vroege stress de vet accumulatie juist vergroot wanneer muizen worden blootgesteld aan een ongezond, western-style dieet (WSD), en dat een vroege dieet interventie met Nuturis® zulke vet accumulatie door een WSD kon beperken. Het was echter nog onbekend hoe vroege stress en Nuturis® de vet accumulatie beïnvloeden. Omdat ook inflammatie in het lichaam en brein betrokken is bij het ontstaan van obesitas, onderzochten we ook de effecten van vroege stress en Nuturis® op inflammatie. We vonden dat vroege stress en Nuturis® beiden invloed hadden op markers voor inflammatie in het vetweefsel en het brein, wat mogelijk bijdraagt aan de effecten van zowel vroege stress als Nuturis® op het latere metabolisme.

In **hoofdstuk 4** hebben we de effecten van vroege stress op een belangrijk, maar tot dusver zeer beperkt onderzocht aspect van obesitas onderzocht: voedselkeuze. Door muizen bloot te stellen aan een ‘free choice high-fat high-sugar’ dieet, dat bekend staat te leiden tot snack gedrag en langdurig overeten, kon voedselkeuze onderzocht worden. We deden dit in zowel mannetjes als vrouwtjes muizen, en onderzochten hun fysiologische respons op het dieet, als ook de hersencircuits die voedselinname reguleren. We vonden dat, alleen in vrouwtjes, vroege stress de vetinname verhoogd na een kortdurende, milde stressor in volwassenheid.

Daarnaast vonden we ook dat mannetjes en vrouwtjes fysiek anders reageerden op zowel het dieet als de blootstelling aan vroege stress. We identificeerden verder ook veranderingen in het beloningssysteem van het brein (betrokken bij de inname van voedsel dat we lekker vinden) geïnduceerd door de vroege stress, die mogelijk betrokken kunnen zijn bij de effecten van vroege stress op latere voedselkeuze.

In **hoofdstuk 5** gaan we dieper in op de werking van de circuits in het brein die de energie balans reguleren, in dit geval de hypothalamus. De hypothalamus reguleert voedsel inname en energie verbruik door middel van het detecteren van metabole hormonen (leptine, ghreline, insuline) en nutriënten (suiker, vetzuren). We hebben onderzocht of vroege stress deze 'nutrient sensing pathways' in de hypothalamus verandert, in zowel mannetjes als vrouwtjes, en zowel vroeg als later in het leven. We vonden sterke seksverschillen, en, vroeg in het leven, een gerichte modulatie van de nutrient sensing pathways door vroege stress.

In **hoofdstuk 6**, focussen we op het vaak samen voorkomen van de metabole en cognitieve veranderingen na vroege stress. Mitochondriën zijn de organellen in onze cellen die energie produceren. Het functioneren van mitochondriën wordt beperkt door chronische stress, en mitochondriën zijn ook betrokken bij zowel metabole ziekten als bij leren en geheugen functies. Of mitochondriën ook worden aangetast door vroege stress is nog onbekend. In deze studie vonden we dat vroege stress invloed heeft op mitochondriën, zowel perifeer als centraal (hippocampus en hypothalamus), afhankelijk van weefsel en leeftijd. Of mitochondriën onderliggend zouden kunnen zijn aan zowel de metabole als cognitieve problemen na vroege stress moet in toekomstige studies nog verder onderzocht worden.

In **hoofdstuk 7** zetten we onze studie naar mitochondriën voort, maar nu specifiek in relatie tot de effecten van vroege stress op overgewicht. Het is eerder aangetoond dat mitochondriën in microglia (de immuun cellen in het brein) in de hypothalamus obesitas kunnen stimuleren. We onderzochten daarom of vroege stress ook de structuur en functie van mitochondriën in microglia verandert. In dit voorlopige rapport laten we zien dat vroeg stress de mitochondriën in microglia op een leeftijd-, dieet- en seks-specifieke manier beïnvloedt. Met name de functie van mitochondriën lijkt sterker beïnvloedt door de vroege stress in vrouwtjes.

Hoofdstuk 8 integreert de resultaten die verkregen zijn in deze scriptie, en bediscussieert; i) de rol voor voeding in het programmeren door vroege stress, ii) welke factoren bij kunnen dragen aan de vergrote kans op metabole ziekten na vroege stress, waarbij we ons richten op voedsel keuze, vetweefsel en de hersencircuits betrokken bij voedsel inname, iii) geslachtsverschillen in de response op vroege stress, en wat daar de oorzaak van zou kunnen zijn, iv) de comorbiditeit tussen metabole ziekten en cognitieve dysfunctie, en v) de klinische relevantie van het werk gepresenteerd in deze thesis.

Samengevat laten we zien dat; i) vroege stress de vetzuur-samenstelling van moedermelk verandert; ii) dat de effecten van vroege stress op voedselkeuze, het vetweefsel en de neurobiologische circuits die metabolisme reguleren (deels) seks-specifiek zijn; en iii) dat mitochondriën worden beïnvloed door blootstelling aan vroege stress. Dit maakt mitochondriën een interessant onderwerp voor verder onderzoek naar effecten van vroege stress op metabole en cognitieve problemen. Dit proefschrift draagt bij aan een beter begrip van hoe vroege stress nakomelingen voor het leven vormt, en kan in de toekomst mogelijk bijdragen aan het ontwikkelen van gerichte behandel methoden.

Publications

Peer-reviewed publications

Ruigrok, S.R., Stöberl, N., Yam, K.Y., de Lucia, C., Lucassen, P.J., Thuret, S. *et al*. (2021) Modulation of the hypothalamic nutrient sensing pathways by sex and early-life stress. *Frontiers in Neuroscience, Neuroenergetics, Nutrition and Brain Health,* 15, 695367.

Ruigrok, S.R., Yim, K., Emmerzaal, T.L., Geenen, B., Stöberl, N., den Blaauwen, J.L., *et al.* (2021) Effects of early-life stress on peripheral and central mitochondria in male mice across ages. *Psychoneuroendocrinology,* 132, 105346.

Ruigrok, S.R., Kotah, J.M., Kuindersma, J.E., Speijer, E., van Irsen, A.A.S., la Fleur, S.E., *et al.* (2021). Adult food choices depend on sex and exposure to early-life stress: underlying brain circuitry, adipose tissue adaptations and metabolic responses. *Neurobiology of Stress,* 15, 100360.

Du Preez, A., Lefèvre-Arbogast, S., Houghton V., de Lucia, C., Low, D.Y., Helmer, C., … **Ruigrok, S.R.,** *et al.* (2021). The serum metabolome mediates the concert of diet, exercise and neurogenesis, determining the risk for cognitive decline and dementia. *Alzheimer's and Dementia,* 1-22

Lefèvre-Arbogast, S., Hejblum, B.P., Helmer, C., Klose, C., Manach, C., Low, D.Y., … **Ruigrok, S.R.**, *et al.* (2021) Early signature in the blood lipidome associated with subsequent cognitive decline in the elderly: A case-control analysis nested within the Three-City cohort study. *EBioMedicine*, 64, 103216.

Ruigrok, S.R., Abbink, M.R., Geertsema, J., Kuindersma, J.E., Stöberl, N., van der Beek, E.M., *et al.* (2021). Effects of Early-Life Stress, Postnatal Diet Modulation and Long-Term Western-Style Diet on Peripheral and Central Inflammatory Markers. Nutrients, 13(2), 288.

Houghton, V., Du Preez, A., Lefevre-Arbogast, S., De Lucia, C., Low, D., Urpi Sarda, M., … **Ruigrok, S.R.**, *et al.* (2020). Caffeine compromises proliferation of human hippocampal progenitor cells. *Frontiers in Cell and Developmental Biology*, 8, 806.

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Rajani, R.M., Quick S., **Ruigrok S.R.**, Graham D., Harris S.E., Verhaaren B.F.J., *et al.* (2018). Reversal of endothelial dysfunction reduces white matter vulnerability in cerebral small vessel disease in rats. *Science Translational Medicine*, 10.

Hoeijmakers, L., Meerhoff, G. F., de Vries, J. W., **Ruigrok, S. R.,** van Dam, A-M., van Leuven, F., *et al.* (2018). The age-related slow increase in amyloid pathology in APP.V717I mice activates microglia, but does not alter hippocampal neurogenesis. *Neurobiology of Aging*, 61, 112-123.

Hoeijmakers, L., **Ruigrok, S.R.,** Amelianchik, A., Ivan, D., van Dam, A-M., Lucassen, P.J., *et al*. (2017). Early-life stress lastingly alters the neuroinflammatory response to amyloid pathology in an Alzheimer's disease mouse model. *Brain, behavior, and immunity,* 63, 160-175.

Yam, K.Y., **Ruigrok, S. R**., Ziko, I., De Luca, S.N., Lucassen, P.J., Spencer, S. J., *et al.* (2017). Ghrelin and hypothalamic NPY/AgRP expression in mice are affected by chronic early-life stress exposure in a sex-specific manner. *Psychoneuroendocrinology,* 86, 73-77.

van der Harg, J.M., Eggels, L., Bangel, F.N., **Ruigrok, S.R.,** Zwart, R., Hoozemans, J.J.M., *et al.* (2017). Insulin deficiency results in reversible protein kinase A activation and tau phosphorylation. *Neurobiology of Disease,* 103, 163-173.

van der Harg, J.M., Eggels, L., **Ruigrok, S.R.,** Hoozemans, J.J.M., la Fleur, S.E., & Scheper, W. (2015). Neuroinflammation is not a Prerequisite for Diabetes-induced Tau Phosphorylation. *Frontiers in Neuroscience*, 9, 432.

PhD Portfolio

PhD portfolio

Oral presentations

2019 | Meeting of the European Brain and Behavior Society | Prague, The Netherlands
Early-life stress increases vulnerability to develop cognitive and metabolic dysfunction: a cross talk between stress, inflammation and nutrition

2019 | Dutch Neuroscience Meeting | Lunteren, The Netherlands
Effects of early-life stress on peripheral and central energy metabolism

2019 | Dutch Neuroscience Meeting | Lunteren, The Netherlands
Investigating the role of the hypothalamus in early-life stress-induced metabolic vulnerability

2019 | Dutch Endocrine Meeting | Noordwijkerhout, the Netherlands
Effects of early-life stress on metabolism, the hypothalamic feeding circuitry and food preferences

2018 | Dutch Neuroscience meeting | Lunteren, the Netherlands
Metabolic programming by early-life stress exposure

Poster presentations

2018 | ECNP congress | Barcelona, Spain
2018 | Int. Congress of Neuroendocrinology | Toronto, Canada
2018 | Brain SIN-posium | Amsterdam, The Netherlands
2017 | Munich Winter Conference on Stress | Garmisch-Partenkirchen, Germany
2017 | Dutch Neuroscience meeting | Lunteren, the Netherlands

Teaching and organization experience

2019 | Chairing a parallel session at the Dutch Neuroscience Meeting 2019
2018 | Organizing and chairing at the brain SIN-posium Amsterdam

Grants

2020 | Travel grant for an exchange with Yale University
2019 | Travel grant to visit the EBBS conference in Prague
2018 | Travel grant Amsterdams Universiteitsfonds to visit the Int. Congress of Neuroendocrinology in Toronto

Public outreach

2017-2020 | Volunteer at Brein in Beeld, an organization aiming at bringing neuroscience to the public

About the author

About the author

Silvie Rosalie Ruigrok was born on October 18th 1991, in Leiden, The Netherlands. She grew up in Lisse, where she obtained her pre-university degree at Fioretti College and her fascination about the functioning of the brain started. She went on to study 'Psychobiologie' (BSc) at the University of Amsterdam, from which she graduated in 2013. She continued her studies at the University of Amsterdam, and obtained her Master's degree in Biomedical Sciences – track Neurobiology in 2015.

As part of her training, she performed several research internships. During her Bachelor studies, she worked on the effects of exercise on neurogenesis in mouse models for Alzheimer's disease under the supervision of Prof. dr. Paul J. Lucassen and Gideon Meerhoff at the Swammerdam Institute for Life Sciences. For her Master's degree, she worked on tau phosphorylation in a rat model for diabetes mellitus type 1 under the supervision of dr. Wiep Scheper and dr. Judith M. van der Harg at the Center for Neurogenomics and Cognitive Research. Finally, to complete her Master's degree, she moved to Edinburgh, where she studied the upstream and downstream effects of endothelial dysfunction in small vessel disease, supervised by Prof. dr. Anna Williams and dr. Rikesh M. Rajani.

In 2016, Silvie started her PhD trajectory at the Swammerdam Institute for Life Sciences, University of Amsterdam, under the guidance of dr. Aniko Korosi and Prof. dr. Paul J. Lucassen. She investigated how early-life stress programs offspring for life, focusing on the metabolic alterations that follow such exposure, with a specific attention on sex differences. Moreover, she studied which aspects in the early postnatal environment could be involved in programming by early-life stress, as well as the comorbidity between metabolic and brain dysfunction. Silvie furthermore spent 6 months in the lab of Prof. dr. Sabrina Diano at Yale University, USA, to investigate potential subcellular substrates of early programming by stress. For her studies, Silvie collaborated with researchers from Nutricia Research, Radboud University Nijmegen, Yale University and Amsterdam Medical Center, among others. The findings reported in this thesis have been presented at multiple (inter)national conferences and are published in peer-reviewed journals.

Next to her research, Silvie was involved in teaching activities. She supervised 5 students during their research internships, and assisted in multiple Bachelor and Master courses. Moreover, Silvie volunteered at Brein in Beeld, and organization aiming at bringing neuroscience to the public. For this purpose, she gave workshops to primary and high school students about various topics in neuroscience.

From June 2021 onwards, Silvie works in the lab of Prof. dr. Carmen Sandi at École Polytechnique Fédérale de Lausanne in Switzerland to continue her career in neuroscience.

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