Perinatal essential fatty acid deficiency in mice - effects on metabolism and behaviour

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A Doctoral Thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in manuscript).
ABSTRACT

Maternal nutritional status during pregnancy and lactation influences the health of the adult offspring and an adequate supply of essential fatty acids is important for foetal and postnatal growth and development. The overall aim of this thesis was to study short- and long-term effects of perinatal essential fatty acid deficiency in mice on metabolism and behaviour.

An essential fatty acid deficient (EFAD) diet or a control diet was given to mouse dams during the latter half of pregnancy (prenatal EFAD) or 4 days before delivery and throughout lactation (postnatal EFAD). The pups were weaned to standard diet (STD) and were later subdivided into two groups, receiving high fat diet (HFD) or STD. Body weight, body composition, food intake, energy expenditure, glucose tolerance and plasma leptin were analyzed in the adult offspring in both the prenatal and postnatal EFAD studies. In the postnatal EFAD males, lipids, fatty acids and gene expression in the liver and plasma lipids were also analyzed. In addition, the short- and long-term effects of postnatal EFAD on brain fatty acids together with long-term effects on behaviour were studied in the female mice.

Prenatal EFAD resulted in sex-specific long-term effects with lower body weight and leptin levels in the adult female mice and higher fasting glucose and lower insulin sensitivity in the adult male mice compared to controls. Mice of both gender with postnatal EFAD exhibited lower body weight, reduced body fat and lower plasma leptin and insulin concentrations compared to controls. The postnatal EFAD mice were resistant to HFD-induced obesity, liver steatosis and hypercholesterolemia during adult life. Finally, postnatal EFAD had long-term effects associated with decreased anxiety and increased risk behaviour in adult female mice. In conclusion, these results suggest that both the sex and the period of exposure (prenatal or postnatal) modulate the long-term effects of EFAD in mice.
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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-IV.

I  Prenatal essential fatty acid deficiency in mice results in long-term sex-specific effects on body weight and glucose metabolism.  
Palsdottir V, Wickman A, Strandvik B, Gabrielsson BG and Olsson B.  

II  Postnatal deficiency of essential fatty acids in mice results in resistance to diet-induced obesity and low plasma insulin during adulthood.  
Palsdottir V, Wickman A, Andersson N, Hezaveh R, Olsson B, Gabrielsson BG and Strandvik B.  

III  Postnatal essential fatty acid deficiency in mice affects lipoproteins, hepatic lipids, fatty acids and gene expression.  
Palsdottir V, Olsson B, Borén J, Strandvik B and Gabrielsson BG.  
Submitted manuscript

IV  Long-term effects on anxiety-related behaviour by postnatal essential fatty acid deficiency.  
Palsdottir V, Månsson JE, Blomqvist M, Egecioglu E and Olsson B.  
Submitted manuscript
<table>
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<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ACAT</td>
<td>acetyl-coA acetyltransferase</td>
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<tr>
<td>ALA</td>
<td>$\alpha$-linolenic acid</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>DGLA</td>
<td>dihomo-$\gamma$-linolenic acid</td>
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<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DXA</td>
<td>dual-energy X-ray absorptiometry</td>
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<tr>
<td>EFA</td>
<td>essential fatty acid</td>
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<tr>
<td>EFAD</td>
<td>essential fatty acid deficiency/deficient</td>
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<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
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<td>ETA</td>
<td>eicosatrienoic acid</td>
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<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
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<tr>
<td>GC</td>
<td>gas chromatography</td>
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<td>HDL</td>
<td>high density lipoprotein</td>
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<td>high fat diet</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>IDL</td>
<td>intermediate density lipoprotein</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>ipGTT</td>
<td>intraperitoneal glucose tolerance test</td>
</tr>
<tr>
<td>LA</td>
<td>linoleic acid</td>
</tr>
<tr>
<td>LCPUFA</td>
<td>long-chain polyunsaturated fatty acid</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PGC1$\alpha$</td>
<td>PPAR-$\gamma$ coactivator 1-$\alpha$</td>
</tr>
<tr>
<td>PLTP</td>
<td>phospholipid transfer protein</td>
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<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
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<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
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<tr>
<td>RER</td>
<td>respiratory exchange ratio</td>
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<td>sterol regulatory element-binding protein</td>
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<td>standard diet</td>
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<td>UCP1</td>
<td>uncoupling protein 1</td>
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<tr>
<td>VLDL</td>
<td>very-low density lipoprotein</td>
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<tr>
<td>VCO$_2$</td>
<td>CO$_2$ production</td>
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<tr>
<td>VO$_2$</td>
<td>$O_2$ consumption</td>
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INTRODUCTION

Essential fatty acids

Structure and function
Fatty acids are lipids with many important biological functions. They are divided into saturated, monounsaturated and polyunsaturated fatty acids (PUFA) reflecting the number of unsaturated double bonds (0, 1 or ≥2, respectively). The dietary fatty acids often have a trivial name, but can also be written in a shorter notation based on the number of carbon atoms and the number of double bonds in the carbon chain with a colon in between. The position of the double bond closest to the methyl end of the fatty acid is notated as “n-“ followed by the number of the first carbon involved in the double bond [1].

Some fatty acids are essential because they cannot be synthesized in mammals and must therefore be obtained from the diet. The primary essential fatty acids (EFA) are linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3). LA is abundant in many vegetable oils, such as corn oil and safflower oil while ALA is found in walnuts and rape-seed oil. Several long-chain polyunsaturated fatty acids (LCPUFA) can be produced from these two essential fatty acids by elongation and desaturation steps (Figure 1). The rate-limiting enzymes in this pathway are the Δ5- and Δ6-desaturases, which are encoded by the genes fatty acid desaturase 1 and 2, respectively [2, 3]. The fatty acids from the n-3, n-6 and n-9 series compete for the same enzymes in the elongation and desaturation steps. For example the affinity of fatty acids to Δ6-desaturase is ALA (18:3n-3) > LA (18:2n-6) > oleic acid (18:1n-9) [4]. In the absence of n-6 and n-3 fatty acids eicosatrienoic acid (ETA, 20:3n-9; also called mead acid) is produced from oleic acid (18:1n-9) (Figure 1), and thus ETA is a marker of EFA deficiency [5].

The n-3 LCPUFA eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are abundant in marine food, such as oily fish. Functions of LCPUFA in the body are energy provision, membrane structure, cell signalling and regulation of gene expression. They are components of all cell membranes and affect the membrane fluidity and thickness. The supply of EFA can also affect the production of eicosanoids, which are biologically active lipid derived mediators important for intercellular signalling, inflammation and proliferation [6]. The main families of eicosanoids are prostaglandins, prostacyclins, tromboxanes and leukotrienes which are derived from arachidonic acid (AA, 20:4n-6), dihomo-γ-linolenic acid (DGLA, 20:3n-6) and EPA (20:5n-3).
Figure 1. Metabolism of polyunsaturated fatty acids.

n-3 Fatty acids
- α-Linolenic acid (18:3n-3)
  ▼
  Stearidonic acid (18:4n-3)
  ▼
  Eicosatetraenoic acid (20:4n-3)
  ▼
  Δ5-Desaturase
  Eicosapentaenoic acid (20:5n-3)
  ▼
  Elongase
  Docosapentaenoic acid (22:5n-3)
  ▼
  Δ6-Desaturase
  Tetracosapentaenoic acid (24:5n-3)
  ▼
  Elongase
  Docosahexaenoic acid (22:6n-3)

n-6 Fatty acids
- Linoleic acid (18:2n-6)
  ▼
  γ-Linolenic acid (18:3n-6)
  ▼
  Dihomo-γ-Linolenic acid (20:3n-6)
  ▼
  Arachidonic acid (20:4n-6)
  ▼
  Eicosatrienoic acid (20:3n-9)

n-9 Fatty acids
- Oleic acid (18:1n-9)
  ▼
  Octadecadienoic acid (18:2n-9)
  ▼
  Docosatetraenoic acid (22:4n-6)
  ▼
  Tetracosatetraenoic acid (24:4n-6)
  ▼
  Tetracosapentaenoic acid (24:5n-6)
INTRODUCTION

LCPUFA and eicosanoids can affect gene expression either through modulating G-protein activation or by acting as ligands for transcription factors [7]. Recently, the G protein-coupled receptor 120 was found to mediate the anti-inflammatory and the insulin-sensitizing effects of n-3 fatty acids [8]. Stearoyl-CoA desaturase 1 is the rate-limiting enzyme catalyzing the synthesis of monounsaturated fatty acids and converts palmitic (16:0) and stearic (18:0) acids to palmitoleic (16:1n-7) and oleic (18:1n-9) acids, respectively. PUFA from both n-3 and n-6 series can inhibit the gene expression of stearoyl-CoA desaturase [9].

Essential fatty acids during pregnancy and lactation

Essential fatty acids are important for normal growth and development early in life [10, 11]. Thus, foetuses and newborn infants need an adequate supply of these fatty acids from the placenta and after birth from breast milk or formula. During the last trimester of pregnancy, there is a selective transport of LCPUFA from the mother to the foetus [12]. An increased supply of these fatty acids are necessary to support the growing brain [13]. The order of preference for transplacental transport of fatty acids is DHA (22:6n-3) > AA (20:4n-6) > ALA (18:3n-3) > LA (18:2n-6) in humans. The composition of fatty acids on the maternal side regulates which fatty acids are transferred to the foetal side [14, 15]. The transfer of fatty acids from the mother through the placenta to the foetus is mediated by several membrane proteins including plasma membrane associated fatty acid binding protein, fatty acid translocase and a family of fatty acid transport proteins [16]. The plasma membrane fatty acid binding protein is located exclusively on the maternal-facing microvillous membranes of the placenta, and data suggests that it is the most important transport protein for LCPUFA to the foetus [16].

During the early postnatal period breast milk or formula is the only source of essential fatty acids for the infant. The fatty acid composition of the breast milk is dependent on the mother’s diet, the de novo synthesis of fatty acids in the mammary glands and the adipose tissue stores of the mother [17]. In clinical experiments with ingestion of isotope labelled fatty acids, about 30% of LA (18:2n-6) in the milk originates from the diet [18]. Newborn infants are not as efficient as adults in converting LA (18:2n-6) and ALA (18:3n-3) to LCPUFA, and therefore breast milk or formula are important sources of AA (20:4n-6) and DHA (22:6n-3) [19]. The de novo synthesis in the mammary glands mainly produces medium chain fatty acids (C8-12), and consequently the proportion of these fatty acids in milk reflects the contribution of mammary fatty acid synthesis to total milk fat content [20]. The remaining fat in the breast milk comes from adipose tissue lipolysis, which has been estimated in studies in rats to contribute with 10-20 % of milk fat production [20, 21]. In well-nourished women the requirement for mobilization of adipose tissue lipid for lactation is
small and thus the fat from adipose tissue lipolysis is a minor component of the milk fat in humans [22, 23].

The optimal intake of n-3 and n-6 fatty acids has been discussed for a long time, but all recommendations are estimates. Crawford et al. calculated that a minimum requirement of EFA during pregnancy and lactation is around 5 energy % [24]. According to the Swedish nutritional recommendations at least 3 energy % should come from n-6 and n-3 fatty acids and at least 0.5 % should come from n-3 fatty acids. Pregnant and lactating mothers are recommended a higher intake with a total of 5 energy % from n-6 and n-3 fatty acids including 1 energy % of n-3 fatty acids [25]. The guidelines of World Association of Perinatal Medicine, specifies that the fatty acid composition of formula given to infants should contain at least 0.2 % of total fatty acids as DHA (22:6n-3) and a minimum amount of AA (20:4n-6) equivalent to the content of DHA (22:6n-3) [26].

**Essential fatty acids in the brain**

The solid matter in brain contains about 50-60 % lipids [27]. Lipids are also important as components in myelin. Myelin is produced by oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. It covers axons and increases the speed of neuronal signalling. The importance of myelin is illustrated in multiple sclerosis where myelin is targeted by the immune system and destroyed, which may result in impaired vision, motor weakness and disability. AA (20:4n-6) and DHA (22:6n-3) are independent determinants of brain growth and development, and these long-chain EFA derivatives are preferentially incorporated into the developing brain over the parent fatty acids LA (18:2n-6) and ALA (18:3n-3) [28]. During the early postnatal period several important developmental processes like neurogenesis, migration and axonal projection occur in the central nervous system. EFA are important for the myelination process in the brain during the lactation period [29] and the second postnatal week in the mouse is important for developing behavioural responses to emotional stress [30].

A low maternal intake of fish and seafood, rich in n-3 fatty acids, during pregnancy is reported to increase the risk of low IQ and a suboptimal neuro-developmental outcome in the offspring [31]. Conversely, supplementation with DHA (22:6n-3) and AA (20:4n-6) during lactation has been shown to improve mental development in children [31-33]. Children with attention-deficit-hyperactivity disorder have often lower plasma levels of AA (20:4n-6), DHA (22:6n-3) and EPA (20:5n-3) [27]. However, randomized controlled trials with LCPUFA supplements have generally been unsuccessful to demonstrate treatment effects on behavioural outcomes [27].
Essential fatty acid deficiency (EFAD)
EFAD was first described in 1929 by George and Mildred Burr as a new deficiency disease produced by the exclusion of fat from the diet of rats [34]. The symptoms observed in the rats maintained on the fat-free diet were scaly skin, inflamed and swollen tail, hair loss, sores in the skin and cessation of growth. In the subsequent papers they showed that this deficiency could be prevented or cured by adding LA (18:2n-6) or ALA (18:3n-3) to the diet [35, 36]. However, the first clinical study designed to evaluate the role of EFA in infant feeding was published in 1958 [37]. The infants from the group given the lowest level of fat developed symptoms of EFAD with frequent stools, perianal irritation, dryness and desquamation of the skin and abnormal serum fatty acid composition [37]. Since then EFAD has been described in a number of different conditions, such as protein malnutrition [38-42], cystic fibrosis [43, 44], inflammatory bowel disease [45, 46], fat malabsorption [47] and during fat-free parenteral nutrition in infants [48, 49] and adults [50, 51].

Since Burr’s report [34], several other animal models of EFAD have been developed. The purpose of the first animal experiments with EFAD was to study the function and metabolism of EFA in mammals, but later EFAD models were set up to study autoimmune disorders [52], cystic fibrosis [53, 54], hepatic steatosis [55], cholestasis [56], malabsorption [57] and malnutrition [58]. From both clinical and experimental studies, it became obvious that exposed infants and young animals showed marked effects on growth and that the symptoms of EFAD developed faster in infants than in adults. Therefore, EFAD early in life is an important issue that has been studied in several animal models [11, 54, 59]. However, the long-term effects of early EFAD are not well understood.

Korotkova et al. investigated the effect of EFAD during the latter half of pregnancy and throughout 3 weeks of lactation in rat pups. The pups had lower serum leptin levels, lower gene expression of leptin and lower amount of adipose tissue compared to control pups [60, 61]. Furthermore, the pups had modified immunological tolerance to dietary antigens [62]. The male rat offspring of the EFAD dams had increased body weight, decreased trabecular bone mineral density and increased cortical bone mineral content in femur compared to controls during adult life [63].

Maternal EFAD during pregnancy and lactation influence the fatty acid composition in the brain of the offspring [64] and especially the n-3 fatty acids have been shown to be important for brain development and brain function [65-67]. Previous studies in rodents have investigated the effect of dietary EFAD during the last week of pregnancy [68, 69], during lactation [70] or during both pregnancy and lactation [71, 72] on memory and learning. These studies show that perinatal EFAD results in a long lasting impairment of learning and memory performance later in life.
Lipid and lipoprotein metabolism

Lipids
Fatty acids belong to the lipid class called fatty acyls, but they are also present in various larger lipid molecules. This chapter starts with a brief description of the lipid classes mentioned in this thesis.

The glycerolipids include almost all lipids containing a glycerol backbone. The largest group of glycerolipids are the mono-, di- or triglycerides that have one, two or three fatty acids attached to the glycerol backbone, respectively. The triglycerides (Figure 2) are the main storage form of fat in animal tissues [1]. Although glycerophospholipids also contain a glycerol backbone, they are not included in the glycerolipid group, but form a separate category.

The glycerophospholipids, also called phospholipids, are the primary components of biological membranes. Additionally, some phospholipids, such as phosphatidic acid, act as membrane-derived second messengers. A phospholipid contains one polar head and two fatty acyl chains, often one saturated fatty acid and one PUFA (Figure 2). Phospholipids may be divided into different classes depending on the polar head group. Examples of abundant phospholipids in biological membranes are phosphatidylcholine, phosphatidyl-
ethanolamine and phosphatidylserine. Although phospholipids constitute only a small fraction of the total dietary fat, they are an important source of EFA [1].

Sphingolipids are a complex family of compounds with the common sphingoid base, synthesized from the amino acid serine and a long chain fatty acid linked to coenzyme A. Examples of sphingolipid classes are ceramides, phosphosphingolipids and glycosphingolipids. Ceramides are generally precursors to more complex sphingolipids. The most common phosphophingolipids in mammals are sphingomyelins (Figure 2). The glycosphingolipids have one or more sugar residues linked to the sphingoid base. Some glycosphingolipids are important components of myelin in the nervous system, e.g. cerebrosides, gangliosides and sulfatides [1].

The sterol lipids have the common steroid core with a fused 4-ring structure, a hydrocarbon side chain and an alcohol group. The alcohol group can be esterified with a fatty acid to form a sterol ester (Figure 2). Cholesterol is the primary sterol in animals and act also as a precursor for steroids such as oestrogen, testosterone and cortisol and as a precursor for bile acids synthesized in the liver [1].

**Lipoproteins**

The lipid and lipoprotein metabolism is regulated in a complex manner in the organism. In the circulation, insoluble lipids are transported in spherical lipoprotein particles. Lipoprotein particles consist of a hydrophobic core with triglycerides and cholesterol esters and a surface composed by a monolayer of phospholipids, cholesterol and an apolipoprotein molecule. The main phospholipid in the lipoproteins is phosphatidylcholine, but other phospholipids can also be found in lesser amounts such as phosphatidylethanolamine, sphingomyelin and phosphatidylinositol. The apolipoproteins are crucial for the interaction between lipoprotein particles and enzymes, transfer proteins and surface receptors. The lipoproteins are divided into five major classes depending on the density of the particles; chylomicrons, very-low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). The first four lipoproteins are involved in delivery of lipids to peripheral tissues. HDL is involved in the reverse cholesterol transport and the transport of cholesterol to steroidogenic tissues.

After a meal dietary lipids are absorbed into the enterocytes in the intestinal mucosa where they form triglyceride rich chylomicrons, which are secreted into the lymph and enters the blood stream via the thoracic duct [73]. The triglycerides in the chylomicrons are hydrolyzed by lipoprotein lipase, which is present on the endothelial surface [74]. The chylomicron remnant particles are then taken up by the liver via LDL-receptor and the LDL-receptor like protein
The liver in turn synthesises triglyceride-rich VLDL particles containing apolipoprotein B, which are secreted into the circulation. The VLDL particles are converted into IDL by lipoprotein lipase [74] and further into LDL by lipoprotein lipase and hepatic lipase [76]. LDL is removed from the circulation by both the liver and peripheral tissues via LDL receptor mediated endocytosis [77]. HDL precursors containing apolipoprotein A are produced by the liver and after cholesterol uptake they form spherical HDL particles. These are involved in the reverse cholesterol transport i.e. the transport of cholesterol from peripheral tissues to the liver. Furthermore, HDL deliver cholesterol to peripheral tissues, especially those producing steroid hormones [78].

**Figure 3.** Lipoprotein metabolism. Chylomicrons are secreted from the intestine and very-low density lipoproteins (VLDL) are secreted from the liver. These lipoproteins are metabolized by lipoprotein lipase (LPL) and hepatic lipase (HL) into chylomicron remnants, intermediary density lipoproteins (IDL) and low density lipoproteins (LDL). High density lipoproteins (HDL) are formed by HDL precursors and modified by phospholipid transfer protein (PLTP) and in humans by cholesteryl ester transfer protein (CETP).

HDL is modified by several proteins including phospholipid transfer protein (PLTP) that transfer surface lipids from VLDL to HDL [79]. In humans, the cholesteryl ester transfer protein (CETP) transfer cholesterol esters from HDL to VLDL and triglycerides in the opposite direction. This transfer results in triglyceride-rich HDL particles that are easily removed through an increased
activity of hepatic lipase [80]. In mice, the levels of HDL are higher than in humans, partly because the mice lack the gene for CETP [81, 82].

**Dyslipidemia**

Dyslipidemia is a state of imbalance in the lipoprotein metabolism. The most common form is characterized by increased secretion of triacylglycerol-rich VLDL particles from the liver, formation of small dense LDL particles and reduced amount of HDL particles. These changes are often observed in patients with type 2 diabetes and the metabolic syndrome and are associated with increased risk of cardiovascular disease [83].

**Cholesterol metabolism**

The cholesterol content in the cell presents a balance between endogenous synthesis, exogenous LDL-derived cholesterol and cholesterol esterified by acetyl-coenzyme A acetyltransferase (ACAT). ACAT2 is the main enzyme esterifying cholesterol in the human and mouse liver, catalyzing the intracellular synthesis of cholesterol ester from free cholesterol and acetyl-coenzyme A [84, 85]. A rise in intracellular cholesterol activates ACAT to convert the cholesterol to its storage ester form.

**Transcription factors involved in lipid metabolism**

Endogenous cholesterol synthesis is limited by the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase and the expression of this enzyme is controlled by the transcription factors sterol regulatory element-binding protein 1a (SREBP1a) and 2 (SREBP2) [86]. The two isoforms of SREBP1, SREBP1a and SREBP1c, are encoded by the same gene and are both active in lipogenesis. However, SREBP1c is mainly involved in the regulation of fatty acid synthesis. Hepatic SREBP1 transcription factors are suppressed by PUFA [87]. SREBP2 regulates the expression of the LDL-receptor gene and of several other genes needed for the cholesterol biosynthesis in the cell [88, 89].

There is another well-known family of transcription factors regulating lipogenesis and lipid storage, and they are called peroxisome proliferator-activated receptors (PPAR). PPAR are activated by PUFA [90] and the three major subtypes are PPARα, PPARβ/δ and PPARγ. PPARα is abundant in the liver where it regulates genes involved in the uptake, transport and metabolism of fatty acids [91]. Fibrates are hypolipidemic drugs that act as ligands for PPARα, and the hypolipidemic effect is in part due to decreased VLDL secretion from the liver [92]. PPARβ/δ is ubiquitously expressed in the body and is involved in several biological pathways, such as fatty acid transport, fatty acid oxidation, and thermogenesis [93]. There is no PPARβ/δ agonist available on the market yet, but recently published studies show that a synthetic PPARβ/δ agonist have beneficial effects on the lipid and lipoprotein metabolism in
INTRODUCTION

normal weight [94] and overweight men [95]. PPARγ is highly expressed in adipose tissue, where it regulates lipid storage [96]. The insulin sensitizing drugs thiazolidinediones are PPARγ agonists that are widely used in the treatment of type 2 diabetes [96]. These PPARγ agonists also decreases the hepatic fat content [97].

Carbohydrate metabolism

Metabolic actions of insulin and glucagon

The blood glucose level in the circulation is mainly regulated by the pancreatic hormones insulin and glucagon, but other hormones and factors do also affect the regulation. Insulin is a peptide hormone produced by the beta-cells in the pancreas. It is an anabolic hormone that stimulates glucose uptake into peripheral tissues and promotes the storage of glycogen and triglycerides. On the opposing side we have the catabolic hormone glucagon, which stimulates gluconeogenesis and thereby increases blood glucose levels. A balance between these hormones is necessary to maintain glucose homeostasis. [98]

After a meal, the increase in blood glucose concentration stimulates the release of insulin into the circulation. Starting with the uptake of glucose in the beta-cells by glucose transporter 2, the glycolytic phosphorylation of glucose causes a rise in the adenosine triphosphate/adenosine diphosphate ratio. This rise inactivates potassium channels that depolarises the membranes, causing calcium channels to open up. The inward flow of calcium ions leads to exocytotic release of stored insulin. The first phase of insulin secretion comes from release of preformed insulin vesicles that are stored in the beta-cells. The second phase starts 10-15 minutes later, when new insulin has been produced by the beta-cells as a response to the increase in blood glucose levels. [98]

The insulin in the circulation lowers blood glucose levels mainly through the translocation of glucose transporters to the cell surface of the peripheral tissues such as skeletal muscle and adipose tissue. The glucose transporters are then able to transport the glucose into the cells where it is phosphorylated into glucose-6-phosphate. In the liver, insulin stimulates the metabolism of glucose and the glucose excess is converted into the storage form glycogen. Insulin also promotes the accumulation of glycogen in skeletal muscle. In adipose tissue insulin stimulates the enzyme fatty acid synthase leading to formation of fatty acids, which are stored as triglycerides. Insulin also inhibits lipolysis i.e. the hydrolysis of triglycerides. The triglyceride stores in adipose tissue are the most important reserve of energy in the body and can be released by lipolysis in times of need. [98]

PPAR-γ coactivator 1-α (PGC1α) was originally identified as a coactivator of PPARγ and was shown to play a critical role in the control of adaptive
thermogenesis [99]. PGC1α is involved in energy expenditure and expressed in several tissues, including the liver [99]. PGC1α has also been shown to be a key modulator of hepatic gluconeogenesis [100]. Glucagon increases hepatic PGC1α while insulin inhibits PGC1α expression and activity [100]. PGC1α in hepatocytes decreases the triglyceride production and increases fatty acid β-oxidation [101].

**Type 2 diabetes**

The World Health Organization diagnostic criteria for diabetes is elevated fasting plasma glucose levels ($\geq 7.0$ mmol/l) and/or elevated plasma glucose levels ($\geq 11.1$ mmol/l) 2 hours after oral ingestion of 75 g of glucose [102]. The total number of people with diabetes was in the year 2000 estimated to be 171 million and this is projected to increase to 366 million by the year 2030 with the largest increase in the developing countries with ongoing urbanization [103].

The development of type 2 diabetes often starts with a decrease in peripheral insulin sensitivity leading to an increased production of insulin in order to maintain a normal blood glucose level. When the insulin resistance progresses, the pancreatic beta-cells reach their maximal capacity to produce and release insulin, which is insufficient to compensate for the increased insulin resistance resulting in elevated blood glucose levels. When the body is not capable to regulate the blood glucose levels the type 2 diabetes is fully developed. [104]

**Energy balance**

**Energy intake and energy expenditure**

![Figure 4. Energy balance regulation.](image)

The first law of thermodynamics states that energy can neither be created nor destroyed. Thus, if energy intake exceeds the energy expenditure the remaining energy will be stored. Both energy intake and energy expenditure are tightly regulated in order to achieve long-term energy balance and stable body weight (Figure 4.). Energy intake is regulated by appetite and satiety signals from the
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Feeding behaviour is regulated by the integrated information from metabolic, endocrine and neuronal factors, which are processed in the central nervous system. The cognitive, visual and olfactory cues as well as the taste of food contribute to the complex regulation of appetite and satiety [106]. The feeding behaviour may be divided into short- and long-term control systems. The short-term regulation pathways include the appetite and satiety signals. Appetite signals such as neuropeptide Y, Orexin A and ghrelin are important for meal initiation, while alpha-melanocyte-stimulating hormone activates the melanocortin-4 receptor and thereby inhibits feeding [107]. Gastric distension and the release of the gut factor cholecystokinin are satiety signals that are important for meal termination [108]. Long-term signals identified in food intake regulation are leptin and insulin, and these factors provide information on the status of energy stores to the central nervous system [106].

Leptin is a circulating peptide hormone secreted from adipose tissue that participates in the regulation of food intake and energy expenditure. The leptin receptor is highly expressed in the nuclei in hypothalamus that regulates energy balance. When fat mass decreases, the circulating concentrations of leptin decrease, which in turn stimulate appetite and suppress energy expenditure until fat mass is restored. The opposite occur when the fat mass increases followed by increased leptin concentrations. Then leptin suppresses appetite until the weight is lost [109, 110]. However, in the obese state this regulation is lost due to increased leptin resistance.

Obesity

Although the energy balance is tightly regulated as described above, sometimes there is an imbalance between energy intake and energy expenditure. A prolonged excess in food intake and/or a deficit in energy expenditure results in increased storage of body fat. An excess storage of body fat over a long time results in obesity. A body mass index of $\geq 30$ kg/m$^2$ is classified as obesity and 25-30 kg/m$^2$ as overweight. Obesity is one of the most common non-communicable diseases in the world today, affecting approximately 500 million adults globally in 2008, which means that more than 10 % of the world’s adult population is obese [111]. Obesity is also a major risk factor for hypertension, type 2 diabetes, cardiovascular disease, depression and some forms of cancer [112-114].
The reasons for the increase in obesity during the last decades are most likely caused by an increased living standard associated with excess food intake and decreased physical activity. A hypothesis of why humans are prone to obesity called “The Thrifty Genotype Hypothesis” was published in 1962 by Neel [115]. According to this hypothesis there has been a positive selection of genetic variants during the evolution that promote efficient energy intake and storage due to a survival advantage during periods of famine. But this hypothesis has been criticized and an alternative hypothesis of “Drifty Genes” has been proposed, suggesting that random genetic drift is responsible for the mutations increasing the susceptibility to obesity [116]. In addition to genetic inheritance, imbalance in early nutrition during gestation and lactation has been proposed to cause adaptations that increase the risk of obesity and metabolic disorders in adult life. This hypothesis was formulated by Hales and Barker, who called it “The Thrifty Phenotype Hypothesis” [117].

**Developmental origins of adult health and disease**

**Epidemiological studies**

The first epidemiological studies proposing a link between infant conditions and adult disease were presented by Forsdahl in 1973 and 1977 [118, 119]. Forsdahl also suggested that a combination of early poverty and later exposure to affluence is a risk factor for arteriosclerotic heart disease [119]. The concept that the foetal and early life environment results in irreversible metabolic alterations affecting health and disease in adult age was tested in several epidemiological observational studies by Barker and his colleagues. They found associations between low birth weight and increased risk of diseases in adult life, including type 2 diabetes, hypertension and cardiovascular heart disease [120-123].

Most of the early studies were based on correlations between low birth weight and adult disease, using the low birth weight as a surrogate marker for foetal growth. Later on also the postnatal growth during the first 1-2 years of life was implicated as a factor affecting adult disease [124]. It became clear that the timing of malnutrition was important for the risk of adult disease, and that prenatal and postnatal exposure sometimes gave different results. By studying birth cohorts from the period before, during and after the Dutch famine during World War II, the epidemiologists could separate the effects of early prenatal, late prenatal and early postnatal undernutrition. For example the risk of developing obesity in young men was increased in those exposed to undernutrition during early prenatal life, but decreased in those exposed to undernutrition during early postnatal life [125].
Growth during early life

Gestational and lactation are important periods in which genes and environmental conditions together affect the development of the foetus and infant. As shown above, poor foetal growth has been linked to increased risk of obesity, type 2 diabetes and cardiovascular disease during adult life. It has also been shown that the combination of a low birth weight followed by a rapid weight gain during early childhood results in the highest risk of developing type 2 diabetes [126] and coronary heart disease [127]. The adaptive changes during foetal life are supposed to prepare the child for the outside world. Hence, a shortage of nutrition during foetal life primes the child for a life with limited access to food. However, when the opposite occur postnatally i.e. an excess in food availability and intake, it results in obesity. Therefore, a mismatch between foetal and postnatal conditions aggravates the long-term consequences of intrauterine growth restriction [128]. An alternative hypothesis to explain the correlation between low birth weight and type 2 diabetes in adult life was proposed by Hatterslay and Tooke in 1999 and is called the “foetal insulin hypothesis”. They suggested that that genetically determined insulin resistance results in impaired insulin-mediated growth in the foetus as well as insulin resistance in adult life [129].

Insulin is the most important growth factor in the mouse during foetal and early postnatal life. During the third postnatal week, growth hormone dependent growth is initiated and becomes the dominant growth factor for the longitudinal growth up to the adult age [130]. There are also insulin-like growth factors (IGF) mediating some of the effects of insulin and growth hormone. IGF-I regulates growth hormone secretion through feed-back in the pituitary [131]. IGF-II mainly acts during foetal and neonatal life, and thereafter decreases to undetectable levels. IGF-I on the other hand, is present in low levels at birth, but increases 10-fold to reach its highest circulating concentrations at 4 weeks of age, and remains high throughout adulthood [132]. In the circulation IGF-I is bound to soluble IGF-binding proteins, of which IGF-binding protein 3 is the most abundant [133]. In mice, IGF-I levels appear to be regulated by caloric intake and weight gain [132]. Other hormones important for early growth are the thyroid hormones. The active form is triiodothyronine which is less abundant but 3-5 fold more potent than the thyroxine form. Thyroxine can be peripherally converted into triiodothyronine by deiodinases. An illustration of the importance of thyroid hormones comes from children that are hypothyroid from before or at birth. These children develop cretinism that is characterized by mental retardation and stunted growth [134].

Experimental studies

In parallel with the epidemiological studies, a large effort has been made to study the developmental origin hypothesis in more detail by using experimental
animal models. Maternal overnutrition serves as a model for the dietary habits in the Western World, while maternal undernutrition followed by adult overnutrition serves as a model for developing countries, immigrant populations and foetal growth restriction due to placental disorders [135]. The most common models of nutritional manipulation during gestation and lactation are maternal energy restriction [136, 137] and maternal protein restriction [138] but also maternal deficiencies in micro-nutrients such as zinc, iron and EFA have been studied. The animal models have been useful in understanding the physiological and molecular mechanisms behind the programming of metabolic diseases.

These experimental models of malnutrition during early life have revealed several structural changes in different tissues that can contribute to the development of adult disease. Maternal energy restriction during gestation resulted in reduced beta-cell mass and insulin secretion in the pancreas of rat offspring [137], which could increase the risk of developing diabetes. Decreased expression of glucose transporters in peripheral tissues have been reported as a consequence of intrauterine growth retardation in rats [139], and could result in peripheral insulin resistance. Maternal malnutrition during gestation also results in decreased nephron numbers in the kidney of rat offspring, which is associated with renal disease and a risk factor for hypertension [140].

Endocrine systems, such as the hypothalamic-pituitary-adrenal axis, and several hormones have been implicated in the experimental studies of nutritional manipulations during early life. For example, energy restriction leading to an excess of glucocorticoids during foetal life has impact on both stress-tolerance and adipose tissue in adulthood [141]. Maternal energy restriction results in reduced IGF-I levels, which affects the growth hormone axis and the postnatal growth of the offspring [142]. Furthermore, during early life plasma leptin levels are predominantly regulated by nutrition. In the developing brain leptin is necessary to stimulate the neural projections in hypothalamus, which are important for appropriate appetite regulation [143].
The overall aim of this thesis was to investigate the effects of EFAD during development on adult metabolism and behaviour, by examining the offspring of mice given EFAD diet during gestation (prenatal EFAD) or lactation (postnatal EFAD).

The specific aims of the different studies were:

I. To study the long-term effects of prenatal EFAD on anthropometry, energy expenditure and glucose metabolism in mice.

II. To investigate the long-term effects of postnatal EFAD in mice on anthropometry, energy expenditure, glucose metabolism and fatty acids in plasma and skeletal muscle.

III. To study the short- and long-term effects of postnatal EFAD on hepatic lipid metabolism and hepatic gene expression in mice.

IV. To explore the short- and long-term effects of postnatal EFAD on brain fatty acids and the long-term effects on behaviour in mice.
METHODOLOGICAL CONSIDERATIONS

Animals and diets

The advantages of using animals in nutritional studies are that you have a strict control of the nutrient intake including the composition of fatty acids and that an extreme deficiency can be induced. Furthermore, long-term and life-time studies can be performed, confounding environmental, genetic and disease variables can be avoided and all tissues are available for analysis. The limitations of animal studies are of course that they are not humans with inherent differences in for instance lipid metabolism. Moreover, differences in nutrient requirements and differences in development stages and maturation may not be comparable across species [144].

Mice have several advantages as model animals, in addition to those mentioned above. Practical advantages include short generation time, short lifespan and small size making them relatively inexpensive and easy to house. Since mice have been used in experiments for decades, their genome and physiology have been extensively studied and several in vivo equipment systems and experimental protocols are available. The mouse and human share many of the basic physiological functions and have most of the genes in common [145]. However, since there are also differences in physiology and metabolism between mice and humans, one has to be careful when extrapolating findings in mice into the human situation. The strain chosen for the studies included in this thesis was C57BL6J. This strain is prone to develop obesity and diabetes and thus suitable for studies of these conditions.

An overview of the experimental design in this thesis is shown in Figure 5. Time-mated pregnant C57BL/6J mice were received on the 9th day of gestation and housed in individual cages until the end of lactation. In Paper I, the dams received either an EFAD diet or an isoenergetic balanced diet (control) from the 11th day of gestation and until the day of delivery. Prior to the 11th day of gestation and after delivery the dams had free access to a standard diet (STD). In Paper II-IV the dams and offspring had free access to the STD prior to the 16th day of gestation and after weaning. From the 16th day of gestation and throughout 3 weeks of lactation, the dams received either the EFAD or the control diet. In Paper II-III the groups were subdivided at 15 weeks of age and randomly assigned to continue on STD or change to a high fat diet (HFD) until the end of the experiment. The composition of the four diets EFAD, control, STD and HFD is found in Table 1, Paper II. The fatty acid compositions of these four diets are shown in Table 2, Paper II.
Figure 5. Experimental design. The Roman numerals refer to the Papers I-IV in this thesis. Cont., Control diet; EFAD, essential fatty acid deficient diet; HFD, high fat diet; STD, standard diet.

Physiological measurements

Anthropometric measurements and food intake
The anthropometric measurements used were body weight, body length (nose-anus) and body composition (Paper I-II). The growth of the mice was followed by weekly weighing. To evaluate if the body weight gain in the mice was due to increases in fat mass, lean body mass or both, body composition was analyzed. Dual energy X-ray absorptiometry (DXA) is a non-invasive method that can be used for determining body composition in live animals. The absorbance of X-ray energy is different in tissues with different densities, and the radiation absorbed when the X-rays pass through the body is analyzed. We analyzed the body composition by DXA in a PIXImus Imager (GE Lunar Corp., Madison, WI) [146]. The heads were excluded from all analyses, since the entire mouse did not fit into the PIXImus scan area. The % of fat measured by DXA has been shown to correlate to the amount of dissected fat [147]. In addition, when the mice were anaesthetized for DXA the body length was analyzed providing data regarding longitudinal growth.

The food intake of the offspring (3-5 mice per cage) was recorded in their home cages by weighing the pellets in each cage three times a week (Paper I-II). Average food intake was expressed as kJ per g body weight per day. The advantage of measuring the food intake in their home cages is that the mice are
not stressed, which is often the case when analysis of food intake is performed in metabolic cages where the mice are housed individually on a metal grid instead of a normal floor.

**Indirect calorimetry**

Energy expenditure was measured by indirect calorimetry using the OxyMax system (Columbus Instruments, Columbus, OH) during a 2 h period at thermoneutrality (30 °C) as previously described [148] (Paper I-II). The indirect calorimetry method is based on analysis of oxygen consumption (VO₂), which originates from oxidation of nutrients. Since oxygen is not stored in the body and its consumption keeps pace with immediate needs, the amount of oxygen consumed has proven to be a highly accurate estimate of energy expenditure [149]. The mouse is placed in an enclosed chamber and is exposed to air that has a constant gas composition. The changes in oxygen and carbon dioxide percentages in the outgoing compared to the ingoing air reflect the metabolic rate. The respiratory exchange ratio (RER) is calculated as the ratio between CO₂ production (VCO₂) and VO₂ (RER=VCO₂/VO₂), and the value differs depending on which macronutrient is consumed. When only carbohydrates are used as energy substrate, RER rises to 1, whereas it falls to around 0.7 when only fats are being used. Energy expenditure (kJ/kg/min) is calculated from the RER and VO₂ by using the formula energy expenditure = (3.815+1.232·RER)·VO₂·4.1868. The minimum of energy expenditure in an animal is called basal thermogenesis or resting metabolic rate. To do a proper estimate of the resting metabolic rate, the energy expenditure should be measured at 30 °C, because then there are minimum disturbance of the non-exercise and exercise associated thermogenesis.

**Glucose tolerance test**

The glucose homeostasis and insulin sensitivity can be measured by different methods. Measuring fasting blood glucose and plasma insulin concentrations, and calculating surrogate indexes for insulin resistance and beta-cell function, such as homeostasis model assessment or quantitative insulin-sensitivity check index has been evaluated in both human [150] and mouse studies [151]. However, the predictive accuracy of these surrogate indexes are not as good in mice as in humans [151]. In mice, the preferred tests are glucose tolerance test, insulin tolerance test and hyperinsulinemic glucose clamp. Hyperinsulinemic glucose clamp is the golden standard technique to study insulin sensitivity. This is however a terminal experiment in mice, while both glucose and insulin tolerance tests are not.

Glucose tolerance test is the most commonly used test to screen differences in glucose metabolism in mice, and this test was used in Paper I and II. The glucose is given orally, intravenously or intraperitoneally. Then there are
repeated measurements of blood glucose and plasma insulin during a follow-up during 2 hours. In addition to the administration route, the fasting duration, the dose of glucose and the state of consciousness are factors that have to been considered when planning the test. In this thesis the glucose was given by intraperitoneal injection in conscious mice fasted overnight (14-16 h; Paper I) or fasted for 6 h (Paper II). A recent paper, evaluating the glucose tolerance test in mice during different conditions, showed that 6 h of fasting resulted in the largest differences in glucose tolerance between chow-fed and high fat diet-fed C57BL/6J mice [152]. Since approximately 70% of the daily food intake of a mouse is ingested during the night [153] and the metabolic rate is much higher than in humans, an overnight fast is a long time for a mouse. The time points for blood glucose measurements were chosen to get a curve over the increase and decrease in blood glucose with tighter intervals during the first hour (0, 15, 30, 60 and 120 min). For the insulin measurements the early time points were chosen at 0, 2 and 5 min after glucose injection to try to catch the first phase of insulin secretion.

Lipid and fatty acid analysis

Lipid extraction
The first steps of most lipid analyses are homogenisation and extraction of lipids from the tissue sample. In this thesis lipids were extracted from liver (Paper I and III), plasma (Paper II), skeletal muscle (Paper II) and brain (Paper IV). Moreover, the lipids from the different diets were extracted and analyzed (Paper II). The lipid extraction is performed by organic solvents, most commonly a mixture of chloroform and methanol based on the method of Folsch et al. [154]. Then an aqueous buffer with KCl is added to remove non-lipid components. The resulting lipid extract is evaporated under nitrogen and prepared for separation and analysis.

Lipid separation
Separation of lipid extracts into different lipid classes can be performed by chromatographic methods. All chromatographic methods are based on the differences in distribution of the compounds you want to separate between a mobile phase (gas or liquid) and a stationary phase fixed inside a column or on a plate. The tissue glycerophospholipids and neutral lipids were separated by high-performance liquid chromatography (HPLC) in Paper I-III and by thin-layer chromatography in Paper IV. In Paper III, the total glycerophospholipids were further separated into different glycerophospholipid classes and the neutral lipids were separated into triglycerides, free cholesterol, and cholesterol esters by HPLC and quantified according to a method described by Homan and
The lipid fractions were collected by split post-column enabling further analysis of the fatty acids.

**Analysis of fatty acids**

The fatty acid composition of the lipid fractions obtained was determined by gas chromatography (GC). In GC the sample is vaporized and carried by a gas through the stationary liquid phase of the column and is only suitable for easily vaporized and thermally stable compounds. Therefore, the lipid fractions are methylated to form volatile fatty acid methyl esters before injection into the GC. The fatty acid methyl esters are detected by a flame ionization detector and can be identified by comparison with retention times of reference substances.

**Lipoprotein profiles**

The lipoprotein profiles in plasma were obtained by fast protein liquid chromatography (FPLC) gel filtration and subsequent analysis of the FPLC fractions (Paper III). The FPLC is a form of liquid chromatography performed under lower pressure and the stationary phase is composed of gel beads. The different lipoprotein particles are separated due to their different sizes, where the largest particles are eluted first and the smallest are eluted last. The eluted fractions were collected for analysis of triglyceride and cholesterol distribution.

**Gene expression analysis**

Real-time polymerase chain reaction (PCR) technique was used to quantitatively analyse the messenger ribonucleic acid (mRNA) levels of genes from white and brown adipose tissue (Paper II), and liver (Paper III). Total RNA is extracted and then reverse transcribed into complementary deoxyribonucleic acid (cDNA). The cDNA is then used as a template in the real-time PCR together with a primer pair and a probe. The primers are designed to span an exon junction to allow amplification of cDNA, but avoid the amplification of genomic DNA. The probe binds to the sequence between the forward and reverse primer and contains a fluorescent reporter dye at the 5’ end and a non-fluorescent quencher at the 3’ end. The quencher suppresses the fluorescent dye from the reporter as long as they are in proximity to each other. During the extension phase of the PCR reaction the quencher is released from the probe and the fluorescent signal increases. The fluorescent signal is determined in each PCR cycler step and the cycle in which the fluorescent signal reaches a specified threshold is directly related to the number of starting copies of the target transcript. The amount of target gene and reference genes is determined from the corresponding standard curve. Then the amount of the target gene is related to the mean amount of the reference genes to obtain a normalized value. The reference genes are used to adjust for differences in
sample variations such as different amount of starting mRNA and different efficiency of cDNA synthesis. The expression of an ideal reference gene should not vary in the tissues or cells included in the study, or in response to experimental treatments. Since there is no perfect reference gene, the use of two or more reference genes is preferable to normalize the target gene.

**Behavioural tests**

In Paper IV, anxiety-related behaviour was investigated using four widely used tests in rodents: the open field, the light-dark transition, the elevated plus maze and the social interaction test [156-158]. Memory was assessed using the object recognition test [159]. The open field, light-dark transition and elevated plus maze tests all belong to a category of tests called exploratory-based approach-avoidance tests and will be discussed together.

**Figure 6.** Tests for assessing anxiety responses in mice. a. Open field b. Light-dark transition c. Elevated plus maze. Adapted by permission from Macmillan Publishers Ltd: Nat Rev Drug Discov [158], copyright 2011.

**Exploratory-based approach-avoidance tests**

The state of anxiety is a normal adaptive response to danger and mice tend to avoid open, well-lit spaces, probably as a defence against predators. The mice have also an innate explorative behaviour, which facilitates the search for food. The approach-avoidance tests are based on the conflict between approaching versus avoiding a potentially dangerous area. The exposed and protected zone takes different forms in different tests (Figure 6). In the open field test the box is divided into an exposed central zone and a protected peripheral zone along the walls of the box (Figure 6a). In the light-dark transition test there is a large light compartment and a smaller black compartment connected by an opening (Figure 6b). Finally, the elevated plus maze is divided into two exposed open arms and two protected closed arms (Figure 6c). Untreated wild-type mice are expected to avoid the exposed zones and remain in the protected zones for most
of the testing period. Decreased avoidance of the exposed zones in treated mice compared to wild-type mice is interpreted as a reduced anxiety-like behaviour and an increased avoidance of the exposed zones is interpreted as augmented anxiety-like behaviour. However, these tests do not dissociate decreased anxiety-related avoidance from increased explorative or impulsivity-related approach behaviour, since both behaviours result in increased time spent in the exposed zone [158].

Other aspects of behaviour that can be measured in the above mentioned tests (open field, light-dark transition and elevated plus maze) are ambulatory activity and ethological parameters such as grooming and rearing. The ambulatory activity in the open field test was measured as number of line crossings, in the light-dark transition as the number of transitions between the compartments and in the elevated plus maze as the number of arm entries.

**Social interaction**

Another type of anxiety test is the social interaction test originally developed for rats [160], and later adapted to mice [161]. The test is based on the observation that when pairs of rats or mice of the same sex but unfamiliar to each other are placed together the time spent performing social interactions is dependent on the environment. Active social interactions such as sniffing, following, grooming, chasing and playing are increased in dim light and decreased in bright light conditions. When comparing two groups of mice under the same conditions, the group spending less time in active social interactions is interpreted as more anxious than the group spending more time interacting socially.

By repeating the social interaction test with the same pairs of mice the next day, a second parameter called social memory can be measured. Social memory is defined as a time-delayed recognition of a familiar mouse. An untreated wild-type mouse has the ability to distinguish a familiar and an unfamiliar mouse. Two unfamiliar mice placed together spend more time investigating each other by social interactions, while two familiar mice spend less time in active social interactions. Thus, when placing a pair of unfamiliar mice in the same environment on two consecutive days, less time performing social interactions during the second day (when the mice are familiar) compared to the first day (when the mice are unfamiliar) is interpreted as an intact social memory.

**Object recognition**

Mice naturally tend to approach and explore novel objects and they tend to prefer novel over familiar objects, similar to the preference of investigating an unfamiliar compared to a familiar mouse described above. This behaviour is utilized to study recognition memory in the object recognition test described in
rat [162] and evaluated in mice of different strains [163]. The one-trial object recognition test consists of one sample trial, during which the mice explore two identical objects in a familiar arena, and one test trial, in which one of the familiar objects is exchanged for a novel object. Exploration is defined as closely looking at, sniffing or touching the object. An untreated wild-type mouse is expected to recognize the familiar object and therefore spend more time exploring the novel object. If a mouse does not discriminate between the novel and familiar object it is interpreted as a decreased recognition.
SUMMARY OF RESULTS

Paper I

Prenatal essential fatty acid deficiency in mice results in long-term sex-specific effects on body weight and glucose metabolism

The purpose of this study was to investigate the effects of an EFAD diet given to mice during late gestation on anthropometry and metabolism in the adult offspring.

The body weight of the dams did not differ on the 11th day of gestation, when they were divided to receive either an EFAD or a control diet, but the dams given the EFAD diet gained less weight during the remaining gestation. There were no differences in length of gestation, litter size or litter sex distribution between EFAD and control mice. The total hepatic phospholipids from newborn EFAD pups contained a higher proportion of monounsaturated fatty acids and a lower proportion of PUFA compared to controls. Additionally, the EFAD pups had a higher proportion of ETA (20:3n-9) compared to control pups, demonstrating that a maternal EFAD-diet during the last 10 days of gestation resulted in EFAD in the offspring.

The body weight of the EFAD pups was lower than that of the controls during the first 4 weeks and the body weight continued to be lower in the female EFAD offspring throughout the experiment. There was also a trend (P=0.08) towards lower fat mass in the EFAD females compared to controls and plasma leptin concentrations were lower in the EFAD females. The lean mass was also lower in the females at 15 weeks of age, but not in proportion to body weight. Body weight and body composition did not differ between the EFAD and control males at 15 weeks of age. The food intake and energy expenditure did not differ between EFAD and control offspring of either gender during adult life. Furthermore, plasma triglyceride and cholesterol concentrations did not differ between EFAD and control offspring.

Fasting blood glucose and plasma insulin levels were higher in EFAD males compared to controls, but did not differ in the females. There were no differences in blood glucose levels between EFAD and controls of either sex during the intraperitoneal glucose tolerance test. However, plasma insulin levels during the intraperitoneal glucose tolerance test (ipGTT) were higher in the EFAD males compared to controls whereas there were no differences between the females. Furthermore, in the females there was a clear first insulin peak in both groups 2 min after glucose injection, but in the males this peak was less pronounced.
SUMMARY OF RESULTS

In conclusion, exposure to an EFAD diet during the last 10 days of gestation resulted in lower body weight and leptin levels in the adult female offspring and higher fasting glucose and decreased insulin sensitivity in the adult male offspring. These results indicate sex-specific long term effects of prenatal exposure to EFAD in mice.

Paper II

Postnatal deficiency of essential fatty acids in mice results in resistance to diet-induced obesity and low plasma insulin during adulthood

In this paper we studied the consequences of a maternal EFAD diet given during the last four days of gestation and 3 weeks of lactation on anthropometry and glucose metabolism in the adult mouse offspring.

There were no differences in length of gestation or litter size between the EFAD and control dams. After 3 weeks of lactation the plasma phospholipid concentration of ETA (20:3n-9) was elevated in both EFAD dams and their 3-week-old pups indicating EFAD, whereas it was undetectable in the controls. The EFAD pups of both gender also had lower body weight (~45% of control pups) and body length (~80% of control pups) at 3 weeks of age. Although the EFAD offspring had a catch-up phase during the first 2 weeks after weaning, their body weight remained lower than that of the controls throughout the experiment. However, the body length was normalized in the male offspring and was almost normalized in the female offspring at 15 weeks of age. From that time point half of the offspring was fed HFD, and the other half continued on STD. The body weight of the controls increased markedly after HFD, whereas there was little effect on the body weight of the EFAD offspring.

At 15 weeks of age, the total fat mass was lower and the lean tissue mass was higher in proportion to body weight in the EFAD offspring compared to the controls. Between 15 and 20 weeks of age, the total fat mass increased in control mice of both gender fed STD, but was not affected at all in the EFAD offspring. Moreover, after 5 weeks of HFD, the increase in fat mass was not as pronounced in the EFAD offspring as in controls. The retroperitoneal and epididymal white adipose tissue depots weighed less in absolute measures and in proportion to body weight in the male EFAD offspring than in controls fed STD. This pattern was maintained for the retroperitoneal fat depots after HFD, whereas there was no difference in the weight of the epididymal depots. The plasma leptin concentrations were lower in the EFAD pups compared to controls at 3 weeks of age and continued so throughout the experiment in both sexes and irrespective of offspring diet. The plasma concentrations of leptin increased over time in controls as well as EFAD males, but not in the females.
SUMMARY OF RESULTS

The food intake was similar in adult EFAD and control offspring and there were no differences in energy expenditure between EFAD offspring and controls of either sex fed STD, as measured by indirect calorimetry. In contrast, energy expenditure was higher in EFAD males and females fed HFD compared to controls. The relative gene expression of uncoupling protein 1 (Ucp1) in brown adipose tissue was not different between the males receiving STD, but after HFD the expression was higher in EFAD males than in controls.

There was no difference in blood glucose concentrations between 20-week-old EFAD males and controls at baseline, irrespective of offspring diet. Glucose concentrations during ipGTT were lower in the male EFAD offspring fed STD than in controls. There was also a trend towards lower glucose concentrations in the EFAD males receiving HFD compared to controls. Irrespective of offspring diet, plasma concentrations of insulin at baseline were lower in EFAD males than in controls, as well as insulin during ipGTT. There were no differences in blood glucose concentrations at baseline between female EFAD offspring and controls fed STD. However, the blood glucose concentrations were lower in the EFAD females fed HFD compared to controls. Glucose concentrations during ipGTT did not differ between the females fed STD, but were lower in the EFAD females fed HFD compared to controls. Plasma concentrations of insulin at baseline and during ipGTT were lower in EFAD females than in controls, irrespective of offspring diet.

The concentration of LA (18:2n-6) was higher, while the DGLA (20:3n-6) concentration was lower in plasma phospholipids from EFAD offspring compared to controls, irrespective of offspring diet. In phospholipids from skeletal muscle DGLA (20:3n-6) was significantly lower in EFAD offspring fed HFD compared to controls.

In conclusion, adult offspring of mice fed an EFAD diet during lactation exhibited lower body weight, reduced body fat and lower plasma leptin and insulin concentrations compared to controls. The observed resistance in EFAD offspring to HFD-induced obesity during adult life may in part be explained by higher energy expenditure and greater expression of Ucp1 in brown adipose tissue.

Paper III

Postnatal essential fatty acid deficiency in mice affects lipoproteins, hepatic lipids, fatty acids and gene expression

In Paper III we investigated the short- and long-term effects of early postnatal EFA deficiency on lipids, lipoproteins and hepatic gene expression in mice.
The livers of 3-week-old pups from dams given an EFAD diet during the 4 last days of gestation and throughout 3 weeks of lactation were smaller in proportion to body weight than the livers of the control pups. In the 25-week-old EFAD offspring fed HFD for 10 weeks the livers were also smaller in proportion to body weight compared to controls, but the relative liver weight did not differ between the EFAD and control offspring fed STD.

There was no difference in hepatic triglyceride levels between the 3-week-old EFAD and control pups (Fig 1A) whereas both cholesterol and cholesterol ester levels were markedly increased in the livers of the EFAD pups compared to controls. The hepatic levels of cholesterol and cholesterol esters were still higher in the adult EFAD offspring fed STD whereas the hepatic triglyceride levels were unchanged. Moreover, the hepatic triglyceride levels in the EFAD offspring fed HFD remained low, while the triglyceride levels increased markedly in the controls fed HFD.

At weaning, the fatty acid composition of the total hepatic phospholipids was greatly affected by the maternal EFAD diet with lower concentrations of PUFA and higher concentrations of monounsaturated fatty acids. The concentration of LA (18:2n-6), AA (20:4n-6) and DHA (22:6n-3) were markedly lower in the total hepatic phospholipids of EFAD pups compared to controls. However, when analyzing the different phospholipids separately, the AA and/or DHA levels were well preserved in diphosphatidylglycerol, phosphatidylserine and phosphatidylethanolamine fractions. The ETA (20:3n-9) levels were higher in all hepatic phospholipids of EFAD pups than in controls, which had almost undetectable levels. Analysis of total hepatic phospholipids from the adult offspring showed primarily higher concentrations of LA (18:2n-6) in EFAD offspring compared to controls, irrespective of adult diet. These differences were most pronounced in diphosphatidylglycerol and phosphatidylcholine in the offspring fed STD. The estimated fatty acid Δ5-desaturase activity (AA/DGLA) was higher and the Δ6-desaturase activity (DGLA/LA) was lower in the EFAD offspring compared to controls.

Total levels of triglycerides in plasma did not differ between 3-week-old EFAD and control pups, but the amount of triglycerides in the VLDL fraction were lower in EFAD compared to controls. The 3-week-old EFAD pups also had higher plasma levels of total cholesterol levels compared to controls primarily due to higher levels in the VLDL and the HDL fractions. However, the cholesterol levels in the IDL/LDL fraction was slightly lower in the EFAD pups. In the adult offspring, the total triglyceride levels were slightly higher in the EFAD mice fed STD compared to controls, mainly due to effects in the VLDL fraction. There was no difference in the total levels of plasma cholesterol between the offspring fed STD, but in the HFD fed mice, there was a marked
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increase in IDL/LDL cholesterol levels in the controls fed HFD that was not observed in the EFAD offspring.

The mRNA expression of Pparγ and Pgc1α was higher in the livers of the 3-week old EFAD pups compared to controls. Furthermore, the hepatic expression of Srebp1c was higher in the EFAD pups whereas Acat2 was lower. In the adult offspring, the hepatic expression of Pparγ was decreased in EFAD mice irrespective of diet. In addition, Pgc1α and Pparβ/δ mRNA expressions were higher in the EFAD mice fed STD compared to controls.

In conclusion, early postnatal EFAD resulted in both short- and long-term alterations of hepatic lipid levels and protection against diet-induced steatosis and hypercholesterolemia during adult life.

Paper IV

Long-term effects on anxiety-related behaviour by postnatal essential fatty acid deficiency

In this paper we investigated the long-term effects of 3 weeks of postnatal EFAD on anxiety, risk behaviour, activity, memory and social behaviour in adult female mice. Furthermore, the fatty acid composition in the brain at 3 and 19 weeks of age was also investigated. Anxiety-related behaviour was analyzed using the open field, the light-dark transition, the social interaction and the elevated plus maze test. Memory was assessed using the object recognition test.

In the light-dark transition test, the EFAD mice spent more time in the white box and less time in the black box compared to controls. Furthermore, there was a trend of longer latency to the first entry into the black box and increased number of rearing in the EFAD mice compared to controls, but the latency to enter the white box again after the first entry into the black box and the total entries into black box did not differ between the groups. In the elevated plus maze, the EFAD mice spent more time in the open arms and less time in the closed arms than the control mice. The number of total head dips and head dips from the open arm was higher in the EFAD group compared to controls, and consequently the proportion of protected head dips was lower in the EFAD group. The number of entries into the open and closed arms did not differ between EFAD and control mice.

There was a trend of higher number of total line crossings in the EFAD mice compared to controls in the open field test, but no other spatiotemporal or ethological parameters differed between the groups. In the social interaction test, there were no differences in the number and time of social interactions between EFAD and control mice. The interaction time was decreased the second day in both groups and the decrease in interaction time and frequency of
interactions was also similar between EFAD mice and controls. In the object recognition test, there was no difference in the proportion of time spent on exploring the new object between the groups. However, there was a trend that the total time exploring both old and new objects was shorter in EFAD offspring compared to controls.

The analysis of brain fatty acids showed lower levels of DHA (22:6n-3) and LA (18:2n-6) but higher levels of 20:2n-6 and docosapentaenoic acid (DPA, 22:5n-6) in the EFAD pups compared to controls. The ratio of DPA/DHA was increased seven-fold in the EFAD pups, while the ratio of n-6/n-3 was only increased by 60% at 3 weeks of age. However, the fatty acid composition of the adult prefrontal cortex was normalized with only minor differences.

In conclusion, postnatal EFAD had short-time effects on the fatty acid composition in brain and long-term effects on behaviour associated with decreased anxiety and increased risk behaviour during adult life.
EFAD and effects on fatty acid composition

The foetus and newborn infant need an adequate supply of essential fatty acids from the placenta and breast milk or formula. The fatty acid composition of the breast milk is dependent on the mother’s diet, the \textit{de novo} synthesis of fatty acids in the mammary glands and the adipose tissue stores of the mother [17]. A previous study of rat dams given an EFAD diet from 10 days before delivery show that the fatty acid composition of the breast milk was clearly affected at 1 and 3 weeks after delivery, which resulted in deficiency of n-3 and n-6 fatty acids in the serum phospholipids of the pups [61]. Low supply of essential fatty acids to the offspring was also found in our mouse studies as demonstrated by the fatty acid composition of plasma and hepatic phospholipids (Paper I-III). The fatty acid composition in the liver of our newborn pups showed that an EFAD diet during the latter half of gestation was enough to induce a large reduction of n-3 and n-6 PUFA in the pups (Paper I). The levels were even lower in the offspring of the dams given an EFAD diet during the last 4 days of gestation and throughout 3 weeks of lactation (Paper III). The reduction in n-3 and n-6 fatty acids led to higher proportions of oleic acid (18:1n-9), palmitoleic acid (16:1n-7) and ETA (20:3n-9) in both prenatal and postnatal EFAD pups.

During starvation or deficiency of certain nutrients, some tissues are more prioritized than others. The supply of essential fatty acids to the developing brain seems to be the most important since the changes in fatty acid composition were smallest in that compartment compared to liver, skeletal muscle and plasma. This is in line with an earlier study of perinatal EFAD in rats, where the fatty acid composition in brain was more resistant to EFAD than other tissues [164]. In contrast to the liver and plasma, the total n-6 fatty acids in the brain were higher in the EFAD pups than in the control pups in our study. This was due to an increase in DPA (22:5n-6) and eicosadienoic acid (20:2n-6) in the brain of the EFAD pups. Further differences between the brain and the other tissues were that AA (20:4n-6) was unaffected and ETA (20:3n-9) was only slightly increased in the brain of the EFAD pups. These data together with those from liver and plasma indicate that there is a redistribution of n-6 fatty acids from liver and possibly other tissues to the brain in order to maintain brain levels of AA (20:4n-6) intact. Thus, the AA (20:4n-6) probably plays an important role in the developing brain.

It has previously been proposed that ETA (20:3n-9) can substitute for AA (20:4n-6) and that DPA (22:5n-6) can substitute for DHA (22:6n-3) because of their similar biochemical structure [164]. In the same manner, the increase in oleic acid (18:1n-9) might compensate for the decreased level of LA (18:2n-6)
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in the livers of the EFAD pups. The substitution of DHA (22:6n-3) by DPA (22:5n-6) was only observed in the brain, indicating the importance of incorporating LCPUFA in the brain. The substitution of AA (20:4n-6) by ETA (20:3n-9) and LA (18:2n-6) by oleic acid (18:1n-9) was observed in both liver and plasma of the pups. These substitutions confirm the earlier established desaturase order of fatty acids to be n-3 > n-6 > n-9 [4]. In the absence of n-3 fatty acids, n-6 fatty acids are desaturated and in the absence of both n-3 and n-6 fatty acids, n-9 fatty acids are desaturated.

In plasma, liver, muscle and brain from adult EFAD offspring the fatty acid composition was mainly normalized with some exceptions. In both plasma (Paper II) and liver (Paper III) the proportion of LA (18:2n-6) was increased and DGLA (20:3n-6) was decreased in adult male EFAD offspring compared to controls. In the muscle from HFD-fed male EFAD mice the proportion of DGLA (20:3n-6) was also slightly lower than in the controls and in the muscle from STD-fed EFAD mice the proportion of palmitoleic acid (16:1n-7) was lower and oleic acid (18:1n-9) higher than in STD-fed controls (Paper II). The smallest changes were observed in the brain fatty acids from adult female EFAD offspring, where there was a minor decrease in the proportion of oleic acid (18:1n-9) in the EFAD mice compared to controls (Paper IV). In conclusion, the fatty acid compositions were most affected in plasma and liver in the 3 week-old pups and these tissues also showed the largest differences in fatty acid composition in the adult mice. It is also interesting that LA (18:2n-6), which was largely reduced at 3 weeks of age in the plasma and liver from EFAD pups, was increased in the same tissues in the adult EFAD mice. The reason for the low levels of LA (18:2n-6) at an early age is probably due to the need for transforming LA (18:2n-6) to LCPUFA in the EFAD pups. A decreased activity of Δ6-desaturase indicated by the decreased ratio of DGLA/LA could play a role in the altered fatty acid composition in adult mice.

**EFAD and effects on metabolism**

The male mice exposed to prenatal EFAD had lower insulin sensitivity compared to controls, despite similar weight and body fat (Paper I). The underlying mechanisms for the decreased insulin sensitivity in the EFAD exposed males are not fully understood. According to the foetal origin of adult disease hypothesis, malnutrition at critical periods of intrauterine development causes permanent changes in the structure and or function of the developing endocrine status of the foetus [165]. This reprogramming consists mainly of the development of insulin resistance, which in turn enables sparing use of energy in times of nutritional deprivation, but may in long-term facilitate the development of glucose intolerance and diabetes [166].
The postnatal EFAD mice, irrespective of sex and diet, had lower plasma concentrations of insulin than controls at baseline and during ipGTT, indicating improved insulin sensitivity (Paper II). Furthermore, the fatty acid composition in plasma, liver and muscle of the adult postnatal EFAD mice was in line with an increased insulin sensitivity profile (Paper II-III). Lower concentrations of DGLA (20:3n-6) along with higher concentrations of LA (18:2n-6) in serum have previously been associated with an increased insulin sensitivity in humans [167, 168]. Associations between different types of dietary fat and insulin sensitivity have also been observed in experimental studies of rats [169, 170].

The postnatal EFAD offspring fed HFD had lower liver weights and lower hepatic triglyceride content compared to controls, indicating that postnatal EFAD may protect against diet-induced liver steatosis. The hepatic gene expression of Ppar\(\gamma\) in the EFAD offspring was 50% lower than in the control offspring, indicating a reduction in lipogenesis and fatty acid synthesis (Paper III). Increased triglyceride accumulation in liver is associated with metabolic complications e.g. insulin resistance [171, 172], and this may be related to the higher insulin concentrations seen in the control offspring compared to EFAD offspring (Paper II). The lower plasma cholesterol levels observed in the adult postnatal EFAD offspring fed HFD were associated with lower LDL-cholesterol. This indicates that postnatal EFAD also may be beneficial from a cardiovascular point of view, since elevated LDL-cholesterol in plasma is a known risk factor for cardiovascular disease.

Previous studies indicate that a maternal diet deficient in merely n-3 fatty acids does not decrease growth of the pups [173], in contrast to diets deficient in n-3 as well as n-6 fatty acids [29]. The growth pattern during the first postnatal weeks is a determining factor for adult body weight in mice [174]. This is also observed in children where an increased growth rate during the first 2 years of life leads to an increased risk of developing obesity [175]. An early postnatal catch-up growth is associated with increased risk of obesity and diabetes, while slow postnatal growth and late catch-up growth is associated with leanness and improved glucose tolerance in humans as well as in mice [176]. At least in rodent models, the intake of n-6 PUFA has been implicated as a factor influencing the size of fat depots and increasing the risk of obesity [177]. Higher LA intake raises tissue AA, which increases prostacyclin production and, in turn, stimulates signalling pathways implicated in adipogenesis [177]. Thus, the deficiency of n-6 fatty acids during the postnatal period could possibly contribute to the lower amount of body fat in the EFAD mice.
EFAD and effects on behaviour

Although exposure to EFAD postnatally seems to have beneficial effects on lipid and carbohydrate metabolism in adult mice, it presumably has negative effects on the developing brain. EFAD during early life has been shown to be detrimental for the developing brain and for the myelination of the nervous system in mouse pups [29], and especially n-3 fatty acid deficiency has been associated with decreased cognitive function and memory [178]. Our postnatal EFAD mice showed lower anxiety and higher risk behaviour during adult life, but there was no difference in memory analyzed by the object recognition test. However, the initial exploration time in the object recognition test was lower in the EFAD offspring and there was a trend towards lower exploration time during the second trial, which might indicate an attention deficit (Paper IV).

Increased serotonin levels in the brain are believed to be anxiolytic and may also be important for impulsivity. Postnatal deficiency of n-3 fatty acids has been shown to increase the basal serotonin release from the hippocampus of rat [179], indicating that this mechanism could also apply to our EFAD offspring. Rats kept on a continuous n-3 deficient diet had lower dopamine concentrations in the frontal cortex [180], but a compensatory upregulation of the dopamine receptors was seen in the cortex of offspring from n-3 deficient rats [181]. Generally, increased dopamine transmission would affect locomotor responses which could affect the behavioural outcome. However, since we did not see any significant differences in the locomotor activity in our study, the dopamine system might be less affected by the postnatal EFAD diet.

Prenatal versus postnatal EFAD

The comparison between our prenatal and postnatal EFAD studies reveals distinct differences, and in some aspects opposite outcomes in the adult offspring (Paper I-II). The prenatal EFAD male mice had lower insulin sensitivity compared to controls, while the postnatal EFAD mice of both gender had higher insulin sensitivity compared to controls. The prenatal EFAD females had lower body weight and plasma leptin concentrations compared to controls, but not as low as the body weight and leptin concentrations of the postnatal EFAD females. The EFAD diet in the prenatal study was only given during the last 10 days of gestation and in the postnatal study during the 4 last days of gestation and throughout 21 days of lactation. Thus, the time period of EFAD exposure was longer in the postnatal EFAD studies and the effects of the EFAD diet would be expected to be larger than in the prenatal EFAD study. However, organs developing mainly during foetal life could be more sensitive to a shorter prenatal deficiency than a longer postnatal deficiency.
Epidemiological studies of the Dutch famine at the end of the Second World War revealed that the timing of malnutrition is important for later outcome of health. Exposure to famine during the last trimester and during the first months of life resulted in decreased rate of obesity during adult life, whereas exposure to famine during the first half of foetal life was associated with increased rate of obesity [125]. It is known that fat depots are formed during the latter part of the third trimester in humans and during the second postnatal week in rodents [182, 183]. Furthermore, studies in humans have shown that the number of adipocytes is a major determinant for the fat mass in adults, and that the difference in their number between lean and obese individuals is established during childhood [184, 185]. Early postnatal undernutrition is known to decrease the number of adipocytes as well as adipocyte cell size in adult rats, probably by affecting proliferation of preadipocytes during the late suckling period [186]. Recently, a decreased size of adipocytes was also reported in mouse offspring from mothers with energy restriction during lactation, suggesting a decreased lipogenesis [187]. It is therefore possible that exposure to EFAD during this critical period in life may interfere with adipose tissue development, leading to fewer or smaller adipocytes and consequently a leaner phenotype.

**EFAD versus other models of malnutrition**

Similar to our postnatal EFAD mice, a lean phenotype in conjunction with enhanced glucose tolerance during an ipGTT was reported in adult male rat offspring, but not in female offspring, as a consequence of a maternal low protein diet during lactation [188, 189]. On the contrary, a maternal energy restriction (50% of normal intake) during lactation did not affect glucose tolerance in the male mouse offspring, despite lower body weight. [174]. However, adult offspring from energy restricted dams had normal body fat content [174], while our EFAD offspring had lower body fat compared to controls. Thus, it is likely that postnatal deficiency of specific nutrients, such as essential fatty acids or proteins, have different effects on the adult leanness and glucose tolerance, compared to a balanced postnatal food restriction.

The same pattern was found when comparing the effects of postnatal malnutrition on behaviour in rodents. In line with our results in postnatal EFAD mice, protein malnutrition during lactation in rats resulted in lower anxiety in the offspring, measured by the elevated plus maze test at 10 weeks of age [190]. However, no significant effects on anxiety was found in mouse offspring from dams with a 30% maternal energy restriction during lactation, when tested in the elevated plus maze at the same age [191]. These results indicate that nutritional deficits early in life can change the responsiveness to anxiety during adult life, and that the outcome differs depending on whether there is a general undernutrition or malnutrition of certain nutrients such as protein or essential
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fatty acids. Our study in mice (Paper IV) and a previous study in rats [70] show that a maternal EFAD diet during lactation decreases the proportion of the n-3 fatty acid DHA (22:6n-3) in the brain of the offspring. Previous studies of low protein maternal diets in rats during gestation [192, 193] or during both gestation and lactation [194], have also shown decreased proportion of DHA (22:6n-3) and/or total n-3 fatty acids in the brain of the rat offspring. On the contrary, a 40% maternal energy restriction during gestation did not affect the brain fatty acid composition in the newborn rat offspring [195]. Thus, both protein malnutrition and EFAD during the perinatal period result in an altered brain fatty acid composition, which may affect the responsiveness to stressful conditions later in life.

Gender differences

An epidemiological study of the Dutch famine showed that prenatal exposure to undernutrition, especially during the last trimester, was linked to decreased glucose tolerance during adult life [196]. A study investigating the association between small birth size and glucose tolerance showed that men, but not women, who were shorter or lighter at birth had decreased insulin sensitivity and a higher insulin secretion at 20 years of age [197]. The prenatal EFAD male offspring in our study had significantly higher plasma insulin levels during the glucose tolerance test compared to controls, indicating decreased insulin sensitivity. Additionally, the prenatal EFAD male offspring had higher fasting blood glucose and plasma insulin levels compared to controls (Paper I). Similar to our data, other experimental studies show sex-specific effects on glucose homeostasis in adult offspring as a result of prenatal malnutrition. In rats given a low protein diet during the entire pregnancy, the adult male, but not female, offspring had increased glucose and insulin levels [188, 189]. Mice that were exposed to a 50% calorie restriction during the last prenatal week showed a progressive decline in glucose tolerance with age in the males (significant at 6 months of age), which was less pronounced in the females [198]. Another study showed that male, but not female, C57BL/6 mice that were exposed to a 30% calorie restriction during prenatal life developed impaired glucose tolerance at 12 weeks of age [199]. These data suggest that male rodents are more sensitive to prenatal nutritional programming than females regarding insulin sensitivity.

The gender differences in insulin sensitivity in the mice exposed to prenatal EFAD might either be due to the protective effect of oestrogen in the females or the deleterious effect of testosterone in the males. 17-beta oestrogen has previously been shown to play a protective role in the development of insulin resistance in ovariectomised rats fed normal chow [200]. In contrast, testosterone has been shown to increase insulin resistance in sheep and rats. Female sheep treated with testosterone prenatally showed similar insulin
sensitivity later in life as untreated males [201], and female rats treated with testosterone prenatally displayed hyperinsulinemia in adult life [202]. Thus, sex hormone levels may mediate some of the differences in insulin sensitivity and possible mechanisms could be differential effects on the insulin sensitivity of peripheral tissues or the development of pancreatic differentiation and function. Since male mice are intrinsically more vulnerable to develop disturbed insulin sensitivity they are likely more sensitive to alterations in insulin sensitivity induced by foetal malnutrition.
CONCLUDING REMARKS

Prenatal EFAD resulted in lower body weight and leptin levels in the females but in higher fasting glucose levels and decreased insulin sensitivity in the males during adult life. In the postnatal EFAD mice we found the opposite results with increased insulin sensitivity, demonstrating that the period of exposure to malnutrition is important for the outcome.

Postnatal EFAD during suckling resulted in lower body weight, reduced body fat and lower plasma leptin and insulin concentrations in both gender compared to controls. The increased levels of LA (18:2n-6) and decreased levels of DGLA (20:3n-6) observed in the adult postnatal EFAD offspring have earlier been associated with increased insulin sensitivity. A decreased activity of Δ6-desaturase indicated by the decreased ratio of DGLA/LA could play a role in the altered fatty acid composition in adult mice.

Postnatal EFAD resulted in short-term alterations of hepatic lipids and gene expression associated with cholesterol accumulation in the livers of the 3-week-old male pups. In the adult males, postnatal EFAD resulted in protection against diet-induced steatosis and hypercholesterolemia. The postnatal EFAD mice were also resistant to HFD-induced obesity during adult life, which could in part be explained by higher energy expenditure and greater expression of Ucp1 in brown adipose tissue.

Postnatal EFAD had short-term effects on the brain fatty acid composition in females and long-term effects on behaviour associated with decreased anxiety and increased risk behaviour. The supply of essential fatty acids to the developing brain seems to be the most important since the changes in fatty acid composition were smallest in that compartment compared to liver and plasma.

The postnatal deficiency of essential fatty acids have similar effects on adult leanness, glucose tolerance and anxiety behaviour as postnatal protein deficiency, but these effects differ from those seen in a balanced postnatal food restriction. Both EFAD and protein deficiency early in life have effects on brain and liver fatty acid composition, which could be a common mechanism for some of the changes seen in metabolism and behaviour.
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