Chapter 1

General introduction
EDCs and obesity: setting the stage

Exposure to endocrine disrupting compounds (EDCs) is inevitable and could harm humans, especially the vulnerable, unborn baby (Haugen et al., 2014). Interference by EDCs during pregnancy could leave a ticking time bomb in the newborn which may ‘explode’ years later in the form of adult diseases such as infertility and certain (endocrine) cancers (Haugen et al., 2014). A well-known example is the synthetic estrogen, diethylstilbestrol (DES), previously used for treatment of miscarriage, resulting in reproductive dysfunction, including vaginal cancer in the DES daughters (Newbold, 2004). The Developmental Origins of Health and Disease (DOHaD) paradigm links any early in life exposure, including EDCs, to chronic adult diseases, which have expanded in recent years to include more diseases than only reproductive dysfunction and cancer. Obesity and related metabolic disorders, a growing major health concern worldwide, were first hypothesized to be related to EDC exposure by Heindel in 2003 (Heindel, 2003). Heindel was familiar with DOHaD and reproductive dysfunction, but had also learned about postnatal weight gain after early exposure to nicotine. He proposed his idea about obesity and EDCs after reading a paper published by Baillie-Hamilton linking the increase in obesity to the increase in chemical production in the last few decades (Baillie-Hamilton, 2002). Industrial chemicals end up in the environment resulting in ubiquitous exposure of all living creatures. While it is clear that modern society promotes an obesogenic lifestyle in the form of an excess availability of (energy-dense) food and a decreased need to exercise due to modern technology, interventions targeted at reducing obesity are ineffective, since the prevalence of obesity is still high. The ‘environmental obesogen’ hypothesis, coined by Blumberg in 2006, provided a plausible case for a role for chemicals in stimulating fat cell differentiation and/or predisposing or sensitizing individuals to obesity (Grun and Blumberg, 2006). Epigenetics is proposed as an underlying mechanism (Ruchat et al., 2013). The scientific evidence to support the obesogen hypothesis, however, was very limited in 2009, both in terms of laboratory animal and human observational studies. Therefore, after a research call issued by the European Commission, our research group started an international multidisciplinary project named ‘OBELIX’ to comprehensively study the hypothesis that prenatal exposure to EDCs in food is associated with the development of obesity and related metabolic diseases later in life (Legler et al., 2011). OBELIX is an abbreviation for OBesogenic Endocrine disrupting chemicals: LIking prenatal eXposure to the development of obesity later in life and a metaphor for the cartoon character Obelix. Obelix fell
into a cauldron of magic potion as a baby and was programmed towards a specific adult phenotype; an obese person with superhuman strength. In order to study this hypothesis, this project combined in vitro, animal and epidemiological studies, of which the animal studies are described in this thesis.

Endocrine disrupting compounds

Exposure to certain environmental chemicals early in life has been associated with adult diseases later in life. Research in the delayed effects of early life exposure to chemicals first comprised metals (primarily lead and mercury) and later expanded to persistent organic pollutants (POPs), with a focus on pesticides (Haugen et al., 2014). Subsequently, less persistent and less bioaccumulative, but ubiquitous, environmental chemicals were also studied. Some of these environmental chemicals have endocrine disrupting properties, in this thesis referred to as endocrine disrupting compounds (EDCs). EDCs have received much attention due to increasing evidence that early life exposure plays a role in lifelong increased risk of disease. The definition of an EDC, although still under discussion, in this thesis is: ‘An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations’ (WHO, 2002; WHO, 2013). EDCs can interfere with signaling pathways of natural hormones by acting as agonists (mimicking the endogenous hormone), antagonists (blocking the endogenous hormone activity), or indirectly via disruption of natural hormone synthesis or metabolism, or other mechanisms such as altering expression and regulation of genes.

The developmental toxicity of EDCs has been well established. For example, early life exposure to EDCs has been linked to reproductive abnormalities in wildlife such as feminization of male fish, and in humans, such as testicular dysgenesis syndrome (WHO, 2013). This reproductive dysfunction in males is supported by experimental studies that show effects on testosterone producing cells in the testis (Leydig cells) after early life exposure to phthalates (Hallmark et al., 2007). Therefore, safe exposure levels have been determined by different agencies such as the European Food Safety Authority (EFSA) and the Environmental Protection Agency (EPA) of the United States. Exposure to low levels of EDCs occurs worldwide since they are ubiquitously present in our environment (WHO, 2013). Man-made EDCs are found in everyday consumer products including plastic bottles, metal cans, cookware, toys,
and cosmetics. Examples of EDCs present in these products are bisphenol A (BPA), perfluoroalkylated substances (PFASs), phthalates, dioxins and dioxin-like (DL) and non-dioxin-like (NDL) polychlorinated biphenyls (PCBs). Due to environmental contamination, EDCs are present in soil and water and therefore, have ended up in livestock, agriculture and wildlife (Annamalai and Namasivayam, 2015). The main route of exposure for humans is oral via food and drinks, but other routes, such as dermal and inhalation, can also play a role, depending on the EDC and the product it is in. Studies measuring levels of EDCs (e.g. BPA) in human sera and urine have indicated that these compounds are indeed present in our bodies. Importantly, perinatal exposure occurs, during which, organisms are highly vulnerable. Although the placenta functions as a transport barrier and both the pregnant mother and the placenta metabolize EDCs, a portion of the EDCs (parent compounds and/or active metabolites) still passes the placenta. The EDCs in the umbilical vein then enter the fetus via two vessel branches. One smaller branch supplies oxygen to the liver and after hepatic circulation enters the heart. The larger well-oxygenated branch traverses the liver and directly enters into the heart, circumventing any (hardly developed) fetal liver detoxification system (Cunningham et al., 2010). Subsequently, EDCs distribute through the fetal circulation. In addition, EDCs are present in mother milk, especially the lipophilic POPs, exposing the newborn postnatally (WHO, 2013).

**Obesity**

Worldwide, prevalence of overweight (body mass index (BMI) of ≥ 25 < 30 kg/m²) and obesity (BMI of ≥ 30 kg/m²) has reached epidemic proportions and is still increasing (Ng et al., 2014). In 1980 versus 2013, the proportion of adults worldwide with a BMI ≥ 25 kg/m² was 28.8% versus 36.9% in males and 29.8% versus 38.0% in females. Sexes taken together, 11% of adults were obese in 2013. These numbers indicate an increase of 27.5% in the prevalence of overweight and obesity combined, over a period of 33 years. Importantly, prevalence of overweight and obesity in children is increasing rapidly (47.1% between 1980 and 2013) in both developed and developing countries (Ng et al., 2014). In 2013, 42 million children (< 5 years of age) were overweight or obese (WHO, 2015a). In addition to health problems as a child, these children are at risk to become obese adults. Moreover, new statistics available for adults in 2014 indicate a halt in the rise of obesity is not in sight. In 2014, 39% of adults worldwide were overweight and 13% were obese (WHO, 2015b).
Obesity is a major risk factor for noncommunicable diseases, such as diabetes and other metabolic disorders, cardiovascular disease and certain cancers (WHO, 2015a). There also appears to be an association between obesity and a variety of neurological impairments, such as mild cognitive decline, Alzheimer’s disease, and depression (Nguyen et al., 2014). In addition, functions of components of both the innate and adaptive immune system are altered in obesity (Patel et al., 2013). For three European countries, including The Netherlands, the calculated contribution of overweight to health care costs of nine diseases (ischemic heart disease, stroke, hypertension, type 2 diabetes mellitus, colorectal cancer, postmenopausal breast cancer, endometrial cancer, kidney cancer and osteoarthritis) was about 25% (Lette et al., 2014). Regarding total health care expenses, disease costs attributable to overweight range from 2% to 4%. Although more and more is known about obesity, many of the factors involved in its etiology are still not completely understood.

Pathophysiology of obesity

Obesity occurs due to a continuous imbalance of energy intake and expenditure. Normally, the body maintains a homeostasis of energy (Bouret et al., 2015). When intake (e.g. food) exceeds expenditure (e.g. exercise), energy is primarily stored as fat in adipose depots. However, energy homeostasis is a complicated process that can be influenced by many factors.

Energy homeostasis is regulated by an integrated system residing in different sites in the brain. These sites contain metabolic sensing neurons that respond to both hormones (e.g. ghrelin, insulin, leptin) and substrates (e.g. glucose, free fatty acids), which are transported across the blood-brain barrier (Bouret et al., 2015). For example, the hypothalamus contains sites for satiety and feeding control. Feeding is not only controlled by homeostatic systems to maintain energy balance, but is also regulated by the hedonic (‘reward’) system (Meye and Adan, 2014). This reward system is connected with the mesolimbic system, both centered in the brain, and in this way food can give a pleasurable experience (palatability of food). Emotions and cues (e.g. food advertisements) induce the desire to eat by influencing the mesolimbic system. This effect on feeding behavior occurs especially in obese persons. The metabolic hormones (e.g. leptin, ghrelin, insulin) can modulate the reward system and therefore our motivation to eat (Meye and Adan, 2014). Thus,
the neurological system is involved in the **pathophysiology of obesity** via several levels.

The brain controls energy homeostasis, but it receives its information about the metabolic and physiological state of the body from peripheral organs, such as the gastrointestinal tract, pancreas, skeletal muscle, liver and (white) adipose tissue (Bouret et al., 2015). The gastrointestinal tract determines the amount of energy that is taken up by the body from food. In addition, cells from the gastrointestinal tract produce several metabolic hormones, for example ghrelin, the ‘hunger hormone’. Ghrelin promotes feeding and suppresses energy expenditure. The pancreas produces insulin and glucagon which are important for glucose homeostasis. Insulin and glucagon have complementary functions. Insulin increases uptake of glucose from the circulation into the cells, such as skeletal muscle. In contrast, glucagon stimulates the liver to transform stored glycogen into glucose to prevent a hypoglycemic state. Skeletal muscle is a major energy consumer and therefore important for energy expenditure. In addition, both skeletal muscle and liver play a role in storage of lipids. Finally, adipose tissue (mainly white), the main storage site for lipids, previously seen as its only function, has now been accepted as a relevant endocrine organ that produces adipocytokines, for instance leptin and adiponectin (Blüher and Mantzoros, 2015). Leptin, the ‘satiety hormone’, has a complementary function to ghrelin; it provides a satiety signal to the brain and increases energy expenditure. Adiponectin is involved in glucose homeostasis and lipid catabolism.

**Developmental Origins of Health and Disease**

Although energy-dense food consumption and a sedentary lifestyle are involved in the development of obesity, evidence indicates that more factors play a causal role (McAllister et al., 2009). Genetic factors (single gene mutations or polygenic mode of inheritance) have been discovered to play only a minor role (Bouret et al., 2015). Therefore, attention to other potential contributing factors has grown. One of these factors is the early life period, i.e. *in utero* and early postnatal or ‘perinatal’ period. In the eighties, Barker and colleagues discovered the link between low birth weight and an increased risk for ischemic heart disease (Barker et al., 1989) and metabolic disorders (e.g. diabetes type 2, metabolic syndrome) (Barker et al., 1993) in later life. This association was independent of adult lifestyle (e.g. smoking, diet), but there was
a geographical link with areas with high neonatal mortality. Babies that survived in these areas likely grew up under poor living conditions. These findings generated the hypothesis that undernutrition \textit{in utero} and early postnatal, leading to an impairment in growth and development, was a risk factor for cardiovascular and metabolic disease in later life (Barker, 2007). At that time, the existing framework supposed positive selection of a thriving genotype responsible for the increased risk for adult metabolic disease in an environment with excess availability of food, in addition to adult life style factors (Neel, 1962). Data of Barker and colleagues countered this paradigm and they proposed the thrifty phenotype hypothesis (Hales and Barker, 1992). Research into this phenomenon gained interest. Particularly, the Dutch famine studies have shown the importance of nutrition in early life (Painter et al., 2005). In this special setting, humans were well nourished, but very suddenly, due to the Second World War and conditions of scarcity in 1944–1945, a famine begun. This famine only lasted for a few months since food availability quickly returned to normal after the liberation on May 5\textsuperscript{th}, 1945. Therefore, a specific cohort of adults exists who have been underfed for only a short period during pregnancy and/or early postnatal. A higher proportion of these individuals compared to non-exposed controls have impaired glucose tolerance, irrespective of a low birth weight (Ravelli et al., 1998). The exposed individuals also have an increased incidence in schizophrenia (Susser et al., 1996), obstructive airways disease, microalbuminuria, coronary heart disease, and obesity (Painter et al., 2005). Already in 1976, a study with 19-year old conscripts showed that those exposed to undernutrition during the Dutch famine had an increased risk for obesity (Ravelli et al., 1976). A later Dutch famine study with both men and women 50 years of age, showed obesity only in women (Ravelli et al., 1998). Other human famine studies have shown similar results of a sex-specific association of prenatal famine with an increase in body weight (Lumey et al., 2011).

Over time, new insights have been gained, resulting in the currently used concept: \textbf{Developmental Origins of Health and Disease} (DOHaD) (Gluckman and Hanson, 2006). One of the new insights is the discovery that low birth weight is not a prerequisite for an increased risk to develop chronic noncommunicable diseases in later life (Newnham and Ross, 2009). Studies have shown effects in adult life across the complete range of birth weight (Hanson and Gluckman, 2014). Subsequently, small for gestational age in combination with postnatal catch-up growth was proposed as the precondition for increased risk to obesity and related metabolic diseases later in life (Martin-Gronert and Ozanne, 2012). In particular, studies showing formula-
fed infants gain more weight over the first year of life than breast-fed infants, and are at higher risk for obesity in later life, were the basis for this hypothesis. However, inconsistencies in the catch-up growth literature suggest that growth is also not a causal factor (Kuzawa, 2005). Both birth weight and postnatal catch-up growth are likely proxy parameters for underlying causal influences. Moreover, the range of chronic noncommunicable diseases involved with DOHaD expanded (e.g. cancer, mental disorders) and researchers recognized that, for example, also functioning of immune system, behavior, and reproduction could be affected (Hale et al., 2014; Heindel and Vandenberg, 2015). Furthermore, not undernutrition per se, but malnutrition, including overnutrition and inadequate diet composition, was found to be a risk factor.

DOHaD research has extended further and many other environmental risk factors, including EDCs, have been found to play a role in various adult diseases (Hale et al., 2014; Hanson and Gluckman, 2014). The DES daughters, mentioned earlier, with an increase in reproductive abnormalities including vaginal cancer, are an example (Newbold, 2004). Another example is the finding that maternal care (i.e. licking and grooming) in rodent pups determines the behavioral and endocrine responses to stress in adulthood (Meaney and Szyf, 2005). Early life factors, next to EDCs, specifically implicated in adult obesity and/or metabolic disorders are: smoking (Tounian, 2011), maternal overweight/obesity (Symonds et al., 2013), gestational diabetes, stress, circadian rhythm, sleep, and gastro-intestinal tract microbiome (Milagro et al., 2013).

Programming

DOHaD can be explained by a concept named ‘programming’ (Dörner, 1975; Lucas, 1991). This term was originally defined as the ability of an environmental factor to disrupt the development of an organism during early life, resulting in disease in later life. However, this definition is not sufficient since ‘programming’ also occurs in the normal situation. For evolutionary purposes, the normal physiological process of establishing tissues and homeostatic systems during development is not completely laid down in advance. The mammalian fetus is able to adapt its phenotype to the future environment by responding to environmental cues provided by the mother. This phenomenon is called developmental plasticity (West-Eberhard, 1989) and
exists throughout the complete life course, but decreases with age (Hanson and Gluckman, 2014). Developmental plasticity is high during early life, causing this phase to be very sensitive to exposure to environmental factors. During this developmental period, pluripotent stem cells are differentiating into specific cell types to form tissue, and physiological and regulatory systems. Systems are formed within different time windows. Hormones and other signaling molecules control these developmental events and their timing. Therefore, the timing of environmental factors determines their effects on the phenotype, and environmental factors that can alter functioning of the endocrine system, like EDCs, have high potential to interfere with these developmental processes. This developmental plasticity of the fetus has two goals: 1) to survive on the short term, and 2) to provide itself with the best opportunity for survival in the long term. However, these environmental factors may act disruptively when the established phenotype is a mismatch for the living conditions later in life. The consequence of this mismatch is an increased risk for developing adult diseases. The Dutch famine cohort is a useful example, since their early life prepared for a scarce environment while these people grew up in an obesogenic environment. In short, current insights have led to the following definition of *programming*: the process through which the translation from genotype to phenotype is tuned towards optimal adaptation to the environment. Disrupted programming, for example by early life exposure to EDCs, may lead to a maladapted or adverse phenotype with an increased risk for diseases later in life. However, in general use, like in this thesis, the short use is ‘programming’, encompassing both normal and disrupted variations.

Disrupted programming in any of the stem cells, tissues and regulatory systems involved with energy homeostasis (i.e. endocrine, neural and metabolic) can increase the risk for obesity (McMillen and Robinson, 2005). Adipogenesis is the process by which progenitor cells differentiate into adipocytes, and occurs mostly pre- and postnatally, although some differentiation continues throughout adulthood (Symonds et al., 2011). Alterations in adipocyte functioning, for instance in their lipid storage capacity, can influence the risk for adult obesity. Moreover, remodeling of appetite control centers in the hypothalamus, for example, could result in an energy regulating state which favors increased energy intake (de Gusmao Correia et al., 2012). Furthermore, hormones like insulin and leptin, but also glucocorticoids, play an important role in brain development during the neonatal period (Martin-Gronert and Ozanne, 2012). Glucocorticoids can influence the activity of the
hypothesis-pituitary-adrenal axis (Bouret et al., 2015). Thus, environmental factors, e.g. EDCs, can increase the risk for the development of obesity later in life by disrupting programming of energy homeostasis, including eating (e.g. food preference) and stress-related behavior.

Epigenetics

Epidemiological studies form the basis of the concepts of DOHaD and programming. Animal studies have provided evidence and insights into the underlying mechanisms. At present, both types of studies have yielded plausible evidence for epigenetics to be mechanistically involved in programming (van Dijk et al., 2015). Its special feature – it can explain how early events can be memorized by the cells in an organism and effects result after a latent period – is key in this respect. Epigenetics, a phenomenon first described by Waddington in 1940, literally means above the genetics (Waddington, 1940). Without changes in DNA sequence, alterations in the epigenome change gene expression and in this way, one genotype can give rise to multiple phenotypes. The epigenetic profile varies across different cell types and endures specific, coordinated changes during a life span (Desai et al., 2015). Environmental factors interacting with establishment of the epigenetic profile early in life have been hypothesized as a mechanism underlying the developmental origins of noncommunicable diseases, including obesity, presenting in adult life.

The epigenome consists of three different layers: alterations in DNA methylation, post-translational modifications of histones (acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination), and changes in non-coding RNA-mediated signaling pathways (Stein, 2012). DNA methylation is the most studied epigenetic mechanism. Methylation of DNA in cells rebuilds and fixes into a pattern during embryogenesis and this is crucial for developmental plasticity (Seisenberger et al., 2013). Simplistically, hypomethylation of DNA is associated with gene transcription and hypermethylation of DNA is associated with gene silencing (Jones, 2012). Abnormal DNA methylation has been linked to human diseases. Cancer was the first disease for which this link was firmly established (Jones and Baylin, 2002). Importantly, as proposed by Holliday (1987), programming can even be transmitted across generations (e.g. from parents onto children and grandchildren) via epigenetic mechanisms that have not yet been clearly elucidated (Vickers, 2014).
In the last years, research interests for obesity and the role of epigenetics in its development have grown (van Dijk et al., 2015). In contrast to the obvious effects on DNA methylation in cancer, observed DNA methylation differences associated with obesity have generally been small. Maternal malnutrition rodent studies have shown evidence that changes in DNA methylation in several tissues (e.g., liver, brain, adipose, whole blood) are linked to adult obesity (Lillycrop and Burdge, 2011). Unfortunately, human research in this respect is limited, but the Dutch famine studies provide useful insights. Tissue availability is restricted to blood samples at adult age. However, assuming that DNA methylation profiles are established early in life and are static, even adult biomaterials provide an important cohort for epigenetic research. One research group of the Dutch famine cohort showed no association between DNA methylation of four candidate genes (e.g., peroxisome proliferator-activated receptor gamma (PPARγ)) and metabolic diseases later in life (Veenendaal et al., 2012). In contrast, another Dutch famine research group focusing on the imprinted gene, insulin-like growth factor 2, and 15 other loci implicated in growth and metabolic disease, showed differential DNA methylation for some of these loci (e.g., IL-10, leptin) (Heijmans et al., 2008; Tobi et al., 2009; Tobi et al., 2012). Moreover, a genome-scale analysis showed multiple differentially methylated regions along pathways related to growth and metabolism (Tobi et al., 2014). Although other epigenetic mechanisms than DNA methylation are less extensively studied, research suggests histone modifications can also play a role in the development of obesity (Ge, 2012; Jufvas et al., 2013).

**Obesogens**

The obesogen hypothesis (Grun and Blumberg, 2006) has evolved and *obesogens* are currently defined as ‘chemicals that promote obesity by increasing the number of fat cells and/or the storage of fat into existing adipocytes’ (Janesick et al., 2014). The authors also state: ‘Obesogens can also act indirectly to promote obesity by changing basal metabolic rate, by shifting energy balance to favor calorie storage, by promoting food storage via gut microbiota, and by altering hormonal control of appetite and satiety’ (Janesick et al., 2014). The potential of certain EDCs as obesogens has been shown in *in vitro*, *in vivo* and human studies. *In vitro* studies provide support via for example the observation of an increase in adipocyte differentiation of mouse pre-adipocytes after compound exposure, such as to the flame retardant 2,2,4,4-tetrabrominated diphenyl ether (BDE-47) (Bastos
Sales et al., 2013). In addition, in this pre-adipocyte model with BDE-47, the key adipogenic transcription factor PPARγ showed an increase in gene expression and demethylation of its promoter’s DNA (Kamstra et al., 2014). In another study, human peripheral blood mononuclear cells displayed compound-specific metabolic and inflammatory gene expression changes after exposure to several EDCs (Wens et al., 2013). However, in vivo studies researching effects of EDCs in a multifactorial system are necessary. For example, in vitro and in vivo studies have shown that EDCs can influence adipocyte differentiation and/or function by disrupting signaling pathways involved in the differentiation of multipotent mesenchymal stem cells into adipocytes (Chamorro-Garcia et al., 2013; Kirchner et al., 2010). For tributyltin (TBT), an organotin pesticide, this mechanism has been suggested as the explanation for the observed obese phenotype in prenatally exposed mice (Grun et al., 2006; Kirchner et al., 2010). In a follow-up study, this effect even persisted up to the F3 generation (Chamorro-Garcia et al., 2013). However, studies linking early life exposure to EDCs to both an adult obese phenotype and epigenetic changes are limited (Inadera, 2013). In the TBT study described above, for example, adipose derived stem cells (ADSCs) from in utero exposed animals showed an increase in adipocyte differentiation compared to control animals after ex vivo adipogenic induction, displayed by an increase in expression of adipogenesis marker genes, including fatty acid-binding protein 4 (Fabp4). This effect was accompanied by decreased methylation levels in the promoter/enhancer region of Fabp4 in the undifferentiated ADSCs (Kirchner et al., 2010). Another example is a study showing perinatal BPA exposure resulted in an increased fat/lean mass ratio in male, not female, F1 rats, at postnatal day (PND) 90. This phenotype was associated with changes in hepatic expression, DNA methylation and histone marks of the Cpt1a gene, the key β-oxidation enzyme, at PND1 (Strakovsky et al., 2015).

Animal studies of programming of obesity by EDCs
Evidence of programming by EDCs early in life for adult diseases is provided by experimental studies (animal and cellular). However, most rodent studies that have been published up to now which examine effects specifically on body weight show divergent results and were performed under different or unrealistic experimental conditions. A wide perinatal window of exposure is important since the timing of development of organs varies throughout gestation. Also, the route of exposure influences toxicokinetics and could be relevant in the induction of health effects. Furthermore, most studies are short-term instead of long-term while follow-up of
offspring into adulthood is necessary to test the DOHaD hypothesis. Moreover, studies that focus on obesity and related metabolic parameters are scarce. Finally, studies that combine an extensive long-term follow-up with research into epigenetics as the underlying mechanism are even more rare.

**Thesis objectives**

Given our hypothesis that perinatal exposure to EDCs plays a role in the onset of obesity, and the limitations of the animal studies published up to now to test this hypothesis, the objectives of this thesis were twofold (Figure 1):

1. To study in a mouse model whether early life exposure to EDCs programs obesity and metabolic alterations later in life

2. To determine whether epigenetics, specifically DNA methylation, is a mechanism by which early life exposure to EDCs programs obesity and metabolic alterations later in life

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Figure 1. Schematic overview of the main objectives of this thesis to study the hypothesis that early life exposure to EDCs programs obesity and metabolic alterations later in life (adapted from Bouret et al., 2015).
Chapter 1

Approach

The general study design for all in vivo studies discussed in this thesis is presented in Figure 2. Obesity-prone (Michel et al., 2005; Surwit et al., 1988) nulliparous female C57BL/6J mice were mated with male FVB mice to produce hybrid offspring for which comprehensive background information of phenotype and development is available in our lab (Dollé et al., 2011). To mimic the main human exposure route, F0 females were exposed to EDCs via the diet. Exposure started two weeks before mating to have a steady-state concentration of the EDCs present in the dams from mating and onwards. After these two premating weeks, exposure continued during mating (1 week), gestation (3 weeks), and lactation (3 weeks). At the age of weaning (postnatal day 21), offspring received a control diet and were group housed per sex. In this way, we included a broad perinatal window covering most developmental processes. Through termination of the exposure after weaning, we could test our hypothesis that any observed adult effects are not caused by direct exposure, but caused by permanent effects induced during exposure early in life.

In this study, one unexposed and seven exposed groups were included. Exposed groups covered a wide subtoxic dose range. Generally, the top dose was at or below the reproductive toxic dose calculated by regulatory agencies such as EPA or EFSA. Human exposure levels were approached or included in our dose range. Every group had four to six F0 females and two to three F0 females were mated with one F0 male. After weaning, according to availability, up to ten F1 males and ten F1 females were selected for follow-up. Follow-up of offspring was half a year or one year, to enable robust evaluation of programmed adult health effects. Follow-up included a broad range of metabolic parameters. Immune and neurobehavioral parameters were also examined, since the involvement of both the immune and neurobehavioral system in the development of obesity has been implicated. In vivo endpoints were: body weight (and the derivative, growth), glucose and insulin tolerance, food consumption and neurobehavior (physical activity, object recognition, sucrose preference). During the final weeks of each study, offspring were challenged with a high fat diet to test a possible change in response to energy-dense food. Ex vivo analyses included: testing function of adherent and non-adherent immune cells in the spleen (lymphoproliferative response), weighing and histopathology of organs and fat pads, determining parameters in the blood and serum, like endocrine
and lipid profile. In addition, to confirm exposure, external and internal dose measurements were performed. External doses were checked in diet and internal doses were determined in serum of dams and surplus pups at weaning age.

As female offspring exposed perinatally to BPA, one of our EDCs tested as described below, most prominently showed metabolic alterations in this study, their livers were investigated for DNA methylation changes. Both global DNA methylation and genome-wide DNA methylation analysis at specific CpG sites with the digital restriction enzyme analysis of methylation (DREAM) assay were assessed to identify associated epigenetic alterations, possibly suggesting mechanisms of action.

**Study design**

<table>
<thead>
<tr>
<th>Maternal exposure</th>
<th>Offspring not exposed</th>
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<tbody>
<tr>
<td>-6w</td>
<td>3w</td>
</tr>
<tr>
<td>-4w</td>
<td>23-28/53-57w</td>
</tr>
<tr>
<td>-3w</td>
<td></td>
</tr>
<tr>
<td>0w</td>
<td></td>
</tr>
<tr>
<td>Pre-mating</td>
<td>Juvenile/adult</td>
</tr>
<tr>
<td>Mating</td>
<td></td>
</tr>
<tr>
<td>Gestation</td>
<td></td>
</tr>
<tr>
<td>Lactation</td>
<td></td>
</tr>
</tbody>
</table>

mouse hybrid C57BL/6J x FVB

Mother | Father | F1

Dose range: subtoxic levels

Age ±17/42 wks: Challenge with high fat diet

- Body weight
- Fat pad weight
- Histopathology
- Food consumption
- Physical activity
- Serum lipid and endocrine profile
- Glucose homeostasis
- Epigenetic profile of liver DNA
- 2 Non-invasive neurobehavioral tests
- Immunotest (ex-vivo)
- Internal dose

Figure 2. Study design of animal studies described in this thesis.
Chapter 1

EDCs studied in this thesis

For the scope of this thesis four EDCs with different endocrine disrupting mechanisms will be discussed in more depth: bisphenol A (BPA), perfluorooctanoic acid (PFOA), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153). These compounds were selected because they have diverse mechanisms of endocrine action, are all found in food, and evidence exists for obesogenic effects in animal and/or epidemiological studies (Legler et al., 2011). Table 1 shows an overview of the compounds studied in this thesis with their known endocrine disrupting mechanisms.

Table 1. EDCs tested in animal studies described in this thesis

<table>
<thead>
<tr>
<th>EDC</th>
<th>Endocrine disrupting mechanism</th>
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<tbody>
<tr>
<td>BPA</td>
<td>ER agonist</td>
</tr>
<tr>
<td>PFOA</td>
<td>PPAR agonist</td>
</tr>
<tr>
<td>TCDD</td>
<td>AhR agonist; antiestrogenic</td>
</tr>
<tr>
<td>PCB 153</td>
<td>CAR/PXR agonist</td>
</tr>
</tbody>
</table>

AhR, aryl hydrocarbon receptor;
CAR, constitutive androstane receptor;
PXR, pregnane X receptor;
ER, estrogen receptor;
PPAR, peroxisome proliferator-activated receptor

BPA

BPA, which is a widely studied EDC, is the first compound of investigation in this thesis (Chapter 2 and 3). BPA is mainly used in plastics, from which it can leach. Continuous background exposure of humans via food and drinks is reflected in detectable levels of BPA in serum, even though metabolism and excretion of BPA is highly efficient. Estrogenic EDCs can disrupt our endocrine system by activation of the estrogen receptors, which play a role in the programming of energy balance (Legler et al., 2011). However, for BPA, other pathways, are also suggested (Rubin, 2011). For example, interference with thyroid hormone pathways or acting as an antagonist for the androgen receptor, have been implied.

PFOA

PFOA, a commonly detected PFAS, is investigated in Chapter 4. PFASs are both hydrophobic and lipophobic (EFSA, 2012b). Due to these unique properties they repel both water and oils and are widely used as surfactants in commercial applications.
Although industry voluntarily committed to reduce emissions and product content of PFOA, it is still present in our environment due to its high persistence (EPA, 2015). Human exposure mainly occurs via the diet. Many PFASs, such as PFOA, are known agonists of the peroxisome proliferator-activated receptors (PPARs) (Abbott et al., 2012). PPARs are known to regulate processes involved in energy homeostasis such as fatty acid oxidation, lipid transport, and glucose metabolism (Abbott et al., 2012).

TCDD
TCDD, the most toxic dioxin used as a reference for the activity of other dioxins and dioxin-like compounds through the toxicity equivalency factor (TEF) system, is investigated in Chapter 5. The term ‘dioxins’ refers to the polychlorinated dibenzo-\(p\)-dioxins (PCDDs) and the polychlorinated dibenzofurans (PCDFs) (EFSA, 2012a). Dioxins are generated in industrial and combustion processes as unintended by-products. Humans are mainly exposed to dioxins via their diet (meat, fish and dairy products). Dioxins disrupt the endocrine system via binding and activation of aryl hydrocarbon receptor (AhR). AhR is suggested to play a role in energy homeostasis (Lu et al., 2015) and the immune response (Esser and Rannug, 2015). TCDD has also antiestrogenic effects (Safe et al., 1991).

PCB 153
PCB 153 is the most prominent NDL-PCB in our environment (EFSA, 2012a) and is investigated in Chapter 5. PCBs were used in a large number of industrial applications such as insulators and plasticizers, but their production was terminated already in the previous century. However, some PCBs are still produced unintentionally as by-products of manufacturing processes, like those used to make certain pigments used in dyes, inks, and paints (Grossman, 2013). In addition, PCBs are highly persistent and therefore, ubiquitously present in our environment. The main human route of exposure is via food (meat, fish and dairy products). Since PCBs are lipophilic, they can bioaccumulate in both animals and humans. NDL-PCBs, in contrast to DL-PCBs, do not activate AhR (EFSA, 2012a), but they have a variety of modes of action. PCB 153 is a potent agonist of constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Al-Salman and Plant, 2012). These xenobiotic receptors are involved in drug metabolism, but recent evidence has shown they are also involved in regulating energy homeostasis under both physiological and pathological (e.g. obesity, diabetes) conditions (Gao and Xie, 2012).
Chapter 1

References


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General introduction


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Chapter 1


