DEVELOPMENTAL PROGRAMMING OF ENERGY BALANCE

Acute and long-term effects
of early postnatal food restriction by raising rats in large litters
on energy use and hypothalamic neuropeptide gene expression

VRIJE UNIVERSITEIT

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Energy (Floor Remmers)

1 | AN INTRODUCTION TO DEVELOPMENTAL PROGRAMMING, HYPOTHALAMIC ENERGY BALANCE REGULATION, AND PROGRAMMING OF ENERGY BALANCE

Introduction

The concepts of 'nutritional programming', 'foetal programming', 'foetal origins of adult disease', 'developmental origins of health and disease', 'developmental induction', and 'developmental programming' [27, 199, 203, 226, 382][*] were all conceived to explain the same phenomenon: a detrimental environment during a critical period of development has persistent effects, whereas the same environmental stimulus outside that critical period induces only reversible changes. Many epidemiological studies have shown an association between low birth weight and an elevated risk of developing several chronic diseases in adulthood [reviewed in e.g. 30, 140, 167, 203, 362]. It is assumed that low-birth weight infants, who are not small per se, but rather small for gestational age (SGA)[**], suffered from intra-uterine growth restriction (IUGR) due to a low availability of nutrients. As adults, these subjects have an increased risk for insulin resistance, hypertension, and cardiovascular disease; collectively called the 'metabolic syndrome' or 'syndrome X' [103]. How early malnutrition should lead to conditions normally related with affluent environments has been the subject of much debate. It is now believed that adaptations that aided these IUGR- or SGA infants to survive during pregnancy may become detrimental in later life when nutrients are no longer scarce. It is said that these individuals are 'programmed' for the metabolic

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[*]   Cited literature is listed in the References, from page 127.

[**] Abbreviations are explained at their first occurrence in each chapter and are listed in the Abbreviations, from page 177.

syndrome. Whether the obesity that is part of the metabolic syndrome is also programmed has long been a matter of debate [109, 400, 455, 467]. Therefore, we are interested whether the early environment can permanently alter energy balance and its regulation. In this chapter, developmental programming will be discussed, followed by energy balance, its regulation, its normal development, and what is known about its programming.

Developmental programming

The concept of developmental programming implies that characteristics of the environment encountered during early development can permanently alter physiology in later life [227]. The perinatal level of nutrition has been proposed to be a particularly important feature [231]. In early development, there is a window of plasticity, a period in which the developing organism has a large potential to adapt to its environment. Once the window of plasticity has closed, many of these adaptations will become fixed. Although epidemiological studies have mainly concentrated on the detrimental consequences of programming, it is not principally a harmful phenomenon. Instead, it is a well-known process in developmental biology [413, 646]. This developmental plasticity allows the growing organism to choose one from a range of possible phenotypes before the window of plasticity closes. Being able to adjust your phenotype to the environment encountered in early life can be 'evolutionary' adaptive if the environment is relatively stable. If the environment changes within the lifetime of the organism, and hence there is a mismatch between the environment in early life and that in adult life, inappropriate adaptations may lead to problems.

This is thought to be the case for SGA infants in developed countries. The low nutrient availability during intra-uterine development that causes these babies' growth restriction is usually not due to a low maternal energy intake, but to other causes such as placental insufficiency [245], drug use (including caffeine, alcohol and smoking), and stress or illness [181], and hence does not give an adequate prediction of nutrient availability in postnatal life. SGA subjects that develop the metabolic syndrome in later life are a good example of 'developmental programming gone bad'; the problems that can occur if you are adapted to the wrong environment [203]. It is now well established that the perinatal environment can programme similar changes in experimental animals [reviewed in 47, 255, 439, 465].

Energy balance regulation

Energy homeostasis, or the process whereby stable energy reserves are maintained over long periods of time, is tightly regulated. The mechanisms used to attain energy balance are very similar in many mammals, including humans. This is done by closely balancing the input and output of the energy stores in the body. In other words: energy intake must equal energy expenditure. Whereas energy intake is simply all the energy that is obtained by

eating, energy expenditure contains different components: the energy that is needed to maintain body functions at rest or basal metabolic rate (biochemical processes, heart beat, etc.), energy that is used to increase temperature or thermogenesis (in response to food intake or in a cold environment), energy needed for growth and development, and energy used for activities such as movement [645]. If energy intake exceeds energy expenditure, energy balance is positive and excess energy will be stored (e.g. in adipose tissue). If energy expenditure exceeds energy intake, energy balance is negative and energy is mobilised from the (adipose tissue) reserves. A disturbed long-term energy balance results in weight loss (negative balance) or weight gain (positive balance).

Each individual uses a more or less fixed amount of energy for the basal metabolic rate, which depends on body size and body composition [e.g. 606, 648]. In adults, the amount of energy expended for growth is obviously negligible. Therefore, in order to maintain neutral energy balance; energy intake, thermogenesis, and activity need to be regulated. These components of energy balance are regulated by at least two separate, but interrelated systems: 1) a short-term system that controls the initiation and termination of meals, depending on the contents of the gastro-intestinal tract, and 2) a long-term system that defends the stability of the energy reserves and thereby that of body weight [e.g. 566]. In addition, higher brain functions such as motivation and reward, as well as environmental factors such as social influences and food availability alter our food intake and activity levels [45, 532]. It is the task of the short-term and long-term regulatory systems to balance the energy reserves in the face of a changing environment. Manipulations of the gastro-intestinal peptides involved in the short-term regulation of hunger and satiety were mostly shown to have little effect on food intake and body weight over a longer period, and therefore we rely predominantly on the long-term system to maintain neutral energy balance.

The first indications that an important part of the long-term regulatory system resides in the hypothalamus of the brain came from early studies reporting severe anorexia or obesity after lesions of distinct areas of the hypothalamus [13, 249]. The hypothalamus is located below the thalamus and above the pituitary gland, in the ventral part of the diencephalon. It extends from the optic chiasm to the caudal end of the mammillary bodies and forms the inferior and lateral walls of the third ventricle. It regulates homeostasis with respect to temperature, water, energy, sleep, reproduction, and other functions. The hypothalamus consists of several distinct nuclei that produce specific neuropeptides and perform specific tasks. In the brain, there are extensive connections to, within, and from the hypothalamus [387]. In **Figure 1**, the location of the hypothalamus in the brain and some of its nuclei are shown. For the purpose of this review, we are especially interested in the arcuate nucleus (ARC), the paraventricular nucleus (PVN), and the lateral hypothalamic area (LHA).
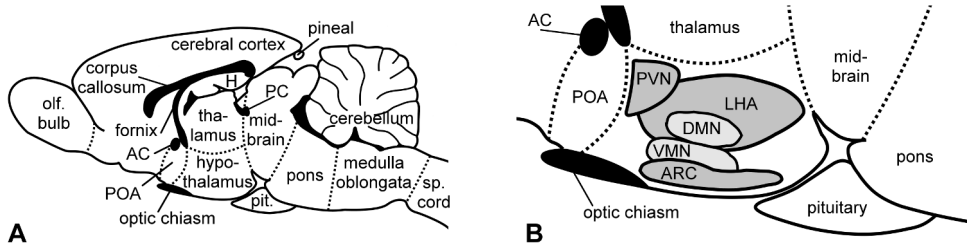
Figure 1. Schematic representations of the hypothalamus. **A**, the location of the hypothalamus within the rat brain. **B**, several important nuclei of the hypothalamus. AC, anterior commissure; ARC, arcuate nucleus; DMN, dorsomedial nucleus; H, hippocampus; LHA, lateral hypothalamic area; olf. bulb, olfactory bulb; PC, posterior commissure; pit., pituitary; POA, preoptic area; PVN paraventricular nucleus; sp. cord, spinal cord; VMN, ventromedial nucleus. Adapted with permission from [472].

The regulation of energy balance by the hypothalamus is a complicated process and has not yet been fully elucidated. This chapter focuses on those substances and connections that are relevant to the research described in this thesis. Therefore, it is important to bear in mind that the following explanation is a simplified summary. Furthermore, it is important to keep in mind that in addition to energy balance, these nuclei and peptides are involved in other hypothalamic functions as well. Although the majority of the literature cited concerns research in rodents (i.e. rats and mice), most of this discussion is also applicable to humans [e.g. 139, 145, 146, 333, 411, 416].

In short [e.g. 544], neurons in the mediobasally located ARC receive information about the status of the energy reserves (e.g. adipose tissue) through peripheral hormones that circulate in amounts related to body fat stores; the information is integrated and passed on to several other hypothalamic nuclei, including the PVN and the LHA, and from there on to output functions. The whole process is also influenced by satiety hormones from the gastro-intestinal tract, see **Figure 2**.
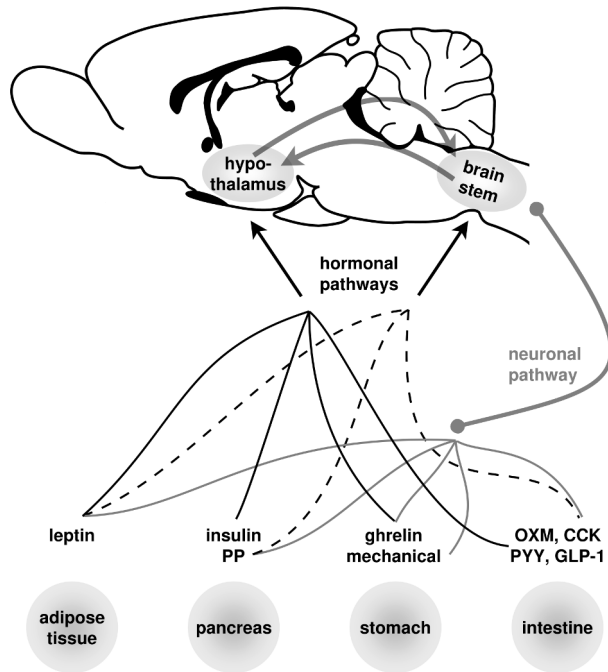
Figure 2. A simplified overview of the regulation of energy balance. Peripheral signals of energy reserves reach the hypothalamus and brain stem via hormonal and neuronal pathways. The former pathway is more important in transferring long-term adiposity signals, whereas the latter handles the rapid transmission of short-term satiety signals, mainly through the vagus nerve. Several brain areas, including the hypothalamus and brain stem, then interact with each other to regulate intake and expenditure of energy. This thesis will focus on the interaction between long-term adiposity signals and the hypothalamus. See the text for further information. CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; OXM, oxyntomodulin; PP, pancreatic polypeptide; PYY, peptide YY. The upper part of the figure was adapted with permission from [472].

Peripheral signals

Several hormones provide the brain with information on the status of energy balance. These can be divided into two categories: 1) hormones that are produced by the gastro-intestinal tract and signal short-term information on satiety and hunger and 2) hormones that signal the status of fat reserves of the body, the long-term signals. Leptin is the major peripheral hormone involved in long-term energy homeostasis.

Leptin

Leptin was first identified as the product of the gene that is defect in obese *ob/ob* mice and was named after 'leptos', the Greek word for 'thin' [225, 680]. In both humans and rodents, it is produced by adipose tissue, in proportion to the body fat content [102, 183, 346, 389, 463]. Therefore, leptin is regarded as an adiposity signal [42]. The leptin receptor (Ob-Rb) is highly expressed in the ARC and is also found in some other hypothalamic areas

[147, 159, 219]. In addition, short forms of the Ob-R (a, c–f), which probably act as leptin transporters, exist in the choroid plexus and other areas where substances may cross the blood brain barrier [55, 147, 159, 219].

Leptin gene expression and serum levels were shown to decrease with fasting and food restriction in both humans and rodents [58, 102, 184, 389, 420, 463]. This reduction was larger than would be expected by the reduction in fat mass [642], indicating that although leptin levels mainly indicate the levels of the energy reserves, its levels are disproportionately reduced during acute depletion, to initiate immediate action to restore these reserves. The central effects of leptin were investigated using intracerebroventricular injections in both rats and mice, and include reductions in food intake and body weight gain and increased energy expenditure [419, 543, 549].

Other peripheral signals

Insulin, produced by the β-cells of the pancreas, controls blood glucose availability. An additional function is as an adiposity signal, similar to leptin [42]. Its circulating levels are directly correlated with fat reserves, it also reacts to acute changes in energy levels such as meal ingestion, it enters the brain, where it acts on receptors in the ARC for example, and when injected into the brain it reduces food intake and increases energy expenditure [reviewed in 42].

Ghrelin, produced by the stomach, indicates negative energy balance [111]. Its levels rise before meal onset and decrease with feeding. Over longer time-periods, ghrelin levels are inversely correlated to energy stores. Ghrelin crosses the blood-brain barrier and receptors are expressed in the hypothalamus where it influences ARC neuron activity. Upon peripheral or central injection, ghrelin stimulates food intake and decreases energy expenditure [reviewed in 111].

Other peripheral signals, which are predominantly involved in the short-term regulation of the initiation and termination of meals, are produced in different regions of the gastro-intestinal tract, and include cholecystokinin, glucagon-like peptide 1, oxyntomodulin, pancreatic polypeptide, and peptide YY [436, 571]. These are mainly secreted upon eating, and inhibit further food intake. Their actions on food intake are exerted via the vagus nerve, the brainstem, as well as via the hypothalamus. It has also been shown that several of these peripheral signals interact with each other to regulate food intake. Another important function of these gut peptides is to control the proper processing of the nutrients ingested in a meal [reviewed in 436, 571].

Peptides from the arcuate nucleus (ARC)

The ARC is located mediobasally in the hypothalamus (see **Figure 1**), close to the median eminence. This is a circumventricular organ where the blood-brain barrier is incomplete and blood borne signals can easily reach the ARC neurons [156, 480]. In addition, leptin, insulin, and ghrelin are actively transported across the blood-brain barrier [24, 25, 542]. Apart from

these direct inputs from the periphery, the hypothalamus also receives information concerning energy balance from brainstem areas [571]. The ARC integrates this information and drives other hypothalamic areas such as the PVN and the LHA [544].

The ARC contains two populations of neurons that are strongly involved in the regulation of energy balance. These two populations express different neuropeptides. A medial population co-expresses the orexigenic, or feeding stimulating, peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) [75, 222]. The anorexigenic, or feeding inhibiting, peptides α-melanocyte-stimulating hormone (α-MSH) and cocaine- and amphetamine-regulated transcript (CART) are produced by a more lateral population of ARC neurons [144]. Both these populations co-express receptors for peripheral signals, including those for leptin [33, 96, 145], insulin [41, 327], and ghrelin [618, 657].

Neuropeptide Y (NPY)

NPY is one of the most abundant peptides in the brain [11]. It is expressed in many brain regions, including the hypothalamus, and especially in the ARC [195, 348, 428]. High concentrations of NPY peptide are found in cell bodies of the hypothalamus, most notably the ARC, whereas elaborate fibres containing NPY can be seen throughout the hypothalamus and the rest of the brain [11, 75, 98, 125]. In the rat, four subtypes of the NPY receptor have been identified; NPY receptor 1 (Y_1R), Y_2R, Y_4R, and Y_5R [172]. Two more subtypes do exist [414]: for the putative Y_3R there is as yet only pharmacological evidence [354] and y_6R is only functional in mice and rabbits, not in rats and humans [573]. The four receptors in the rat have all been found in many different brain areas, including most hypothalamic nuclei [172, 468]. Within the ARC, Y_1R and Y_5R mRNA and protein are found in many α-MSH/CART neurons [76, 171, 173, 188], whereas the mRNA and protein for Y_2R, which is believed to be an autoreceptor [319], was found in most NPY/AgRP neurons [76, 171].

Situations characterised by negative energy balance, such as fasting and food restriction, have been shown to increase NPY peptide and expression levels [5, 46, 48, 70, 299, 329, 533]. Mimicking positive energy balance by leptin injections, on the other hand, has been reported to lower NPY expression [5, 329, 543, 576]. Leptin has also been shown to decrease the activity of NPY neurons [618], NPY secretion by the hypothalamus [576], and NPY levels in the PVN [110]. Intracerebroventricular and intrahypothalamic injections of NPY stimulate food intake and body weight gain [100, 361, 568-570], increase white fat lipid storage, and reduce brown fat thermogenesis [51, 52, 142]. In contrast, NPY injections in most areas outside the hypothalamus did not have any effect on food intake [568].

Agouti-related protein (AgRP)

AgRP was discovered because of its resemblance to agouti, which in mice causes severe obesity when overexpressed [453, 554]. In rodents, AgRP gene expression is found solely in the ARC, where it is co-expressed in almost all NPY neurons [75, 222, 236, 554]. The protein is colocalised with NPY and projections are found throughout the hypothalamus [23, 74, 75,

146, 236]. AgRP is an inverse agonist of the constitutively active melanocortin (MC) receptors [237, 444], which are discussed below.

Like with NPY, levels of AgRP expression, peptide, and activity are increased by fasting and decreased by leptin [46, 48, 329, 365, 421, 533, 594]. Likewise, injections of AgRP or other antagonists of MC receptors were shown to elevate food intake, body weight, and body fat and to reduce energy expenditure and brown fat thermogenesis [268, 296, 563]. In contrast to the relatively short-lived effects of NPY, a single injection of AgRP will increase food intake for up to a week [220, 379, 523]. These long-lasting feeding effects of AgRP are proposed to be mediated by other routes than the MC receptors [220, 501].

α-Melanocyte-stimulating hormone (α-MSH)

α-MSH is cleaved from the precursor polypeptide pro-opiomelanocortin (POMC), together with other peptides like β-endorphin and adrenocorticotropic hormone [391]. POMC is expressed in a more lateral population of neurons within the ARC than NPY and AgRP [194, 222]. α-MSH axons are found in many hypothalamic nuclei and in other brain regions [23, 279]. The MC3 and MC4 receptors, two of the five MC receptors that have been identified, are both highly expressed in hypothalamic nuclei [430, 520]. The MC4 receptor is more widely expressed throughout the brain [430]. Both are implicated in the regulation of energy balance [94, 235, 267, 396], although some debate previously existed over the involvement of the MC3 receptor [303].

As an anorexigenic peptide, POMC gene expression is reduced by fasting and stimulated by leptin injections and overfeeding [5, 46, 48, 70, 221, 329, 533, 619]. In addition, leptin stimulates activity of POMC neurons and α-MSH release [106, 144, 318, 488]. Central administration of α-MSH or its agonist melanotan II (MTII) decreases food intake, weight gain, and adiposity and increases energy expenditure, brown adipose tissue activity, and body temperature, but not locomotor activity [240, 268, 296, 363, 435, 609]. Intrahypothalamic injections reduced food intake in the ARC, PVN, and LHA among others [317]. In contrast to α-MSH, β-endorphin has been shown to increase food intake [609].

Cocaine-and amphetamine-regulated transcript (CART)

CART was identified when its expression levels were shown to be increased after administration of cocaine or amphetamine [136], although it was sequenced as a peptide with unknown function long before that [567]. CART mRNA and peptide can be found in several brain areas, including the hypothalamic ARC, LHA, and PVN [136, 336, 337, 637]. The peptide is co-expressed and colocalised in ARC POMC cells, and is also found in the PVN and LHA [73, 144, 145, 637]. A receptor for CART has not been identified yet [626], but specific CART binding has been reported in cell lines [308, 627] and more recently also in cultured cells from the hypothalamus, hippocampus, and especially the nucleus accumbens [294].

ARC CART mRNA levels are decreased by fasting and increased by leptin and overfeeding [5, 46, 342, 533, 667]. These results identify CART peptide as an anorexigenic peptide. Most

studies using CART injections are in accordance with this view. Intracerebroventricular CART was shown to decrease food intake [2, 342, 572], with chronic injections also reducing body weight gain and increasing lipid oxidation [349, 518]. Injections into distinct hypothalamic nuclei, however, have produced conflicting results, either increasing or decreasing food intake [2, 326, 572, 640]. Intrahypothalamic injection did reliably increase gene expression of a peptide involved in thermogenesis of brown adipose tissue [326, 640], which is again in line with an anorexigenic function. These contradictory results, and its colocalisation with both orexigenic and anorexigenic neuropeptides, have been interpreted to suggest that CART may play a modulatory role, with different effects depending on its localisation [626].

Anorexigenic peptides from the paraventricular nucleus (PVN)

Another important nucleus in the regulation of energy balance is the PVN. In this nucleus, the anorexigenic peptides corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) are expressed in two distinct cell populations. The PVN receives innervation from ARC NPY/AgRP, POMC, and CART terminals [23, 77, 342]. In addition, receptors for these peptides are expressed in the PVN [430, 468], providing all the 'machinery' for signalling from the ARC to the PVN. The PVN also receives some input from the LHA, dense projections from the dorsomedial nucleus, and indirect input from the amygdala [387].

Corticotropin-releasing hormone (CRH)

CRH is known for its role in the hypothalamic-pituitary-adrenal (HPA) axis, which is responsible for stress responses, but it is also involved in the regulation of energy balance [515]. CRH peptide is widely distributed in the rat brain, but can mainly be found in PVN parvocellular neurons [534, 590]. PVN CRH neurons project to the median eminence, where CRH regulates the release of adrenocorticotropic hormone and β-endorphin from the pituitary [295, 590], and to some cells groups in the brainstem and spinal cord [534]. In the rat brain, its effects are mediated by two receptors, the CRH_1- and CRH_2-receptor, although the latter has a low affinity for CRH [378, 479]. The CRH_1-receptor is widely expressed in the brain, most strongly in several cortical and hippocampal areas, amygdala, and hypothalamus [498, 665], while the CRH_2-receptor is highly expressed in the hypothalamus, but is also found in the amygdala, hippocampus, and some brainstem areas [377, 621]. Additionally, CRH activity is modulated by the CRH-binding protein, which controls its availability to the receptors [647].

CRH expression and peptide levels are decreased by food deprivation and increased by overfeeding and leptin injections [70, 543, 548, 586, 611, 619]. Furthermore, CRH expression is increased by injections of both α-MSH and CART [162, 565]. Intracerebroventricular CRH has been shown to decrease food intake and body weight gain and to induce both locomotor activity and activity of brown adipose tissue [16, 72, 83, 141, 355]. These data all point towards an anorexigenic and catabolic role for CRH.

Thyrotropin-releasing hormone (TRH)

TRH is well known for its role in the hypothalamic-pituitary-thyroid (HPT) axis, in which it stimulates thyroid-stimulating hormone release from the pituitary. Via thyroid hormone, which stimulates energy expenditure and thermogenesis, this axis plays an important role in maintaining energy homeostasis [351]. Like CRH, TRH is produced in parvocellular PVN neurons [352, 353]. Unlike CRH cells, many TRH cells co-express CART [73, 145]. TRH terminals are found throughout the hypothalamus, in the median eminence, and the pituitary [e.g. 352]. Its effects are mediated by two receptors, the TRH_1- [583] and TRH_2-receptor [87, 277]. The former is mainly expressed in the hypothalamus, whereas the TRH_2-receptor is more widely expressed in the brain, in the thalamus and several cortical areas among others [250].

TRH expression levels have been shown to be reduced by fasting and increased by leptin injections [161, 165, 356]. In addition, TRH expression and release are reduced by NPY and AgRP and stimulated by α-MSH and CART [160, 161, 163, 164, 318, 445]. Central and peripheral injections of TRH were shown to decrease food intake and increase body temperature [97, 589, 635]. Despite increased food intake, which may have been a compensatory response, chronic oral TRH caused a reduction in body weight [273]. These data point towards an anorexigenic and catabolic role for TRH.

Orexigenic peptides from the lateral hypothalamic area (LHA)

Another important nucleus in the regulation of energy balance is the LHA. Lesions of this area were shown to result in anorexia and an increased metabolic rate, and it was recognised early on as a 'feeding centre' [13, 249]. The orexigenic peptides melanin-concentrating hormone (MCH) and the orexins (ORX) are expressed in two distinct cell populations within the LHA, which receive innervation from nerve terminals containing NPY, AgRP, and α-MSH [74, 146]. In addition, receptors for these peptides are expressed in the LHA [430, 468, 520]. Apart from this input from the ARC, the LHA also receives input from the PVN, the hypothalamic dorsomedial nucleus, and from some higher brain areas including anterior limbic cortical areas, the nucleus accumbens, and indirectly from the hippocampus [387].

Melanin-concentrating hormone (MCH)

MCH was first discovered in fish, as the peptide that causes melanosomes to contract and thereby has a skin-lightening effect [306]. Thereafter, a similar peptide was found in rat brain [562] and the homologous peptide was identified [623]. MCH is heavily expressed in the LHA [54, 74]. Some LHA MCH cells also express and contain CART [73, 145, 637]. From the cell bodies in the hypothalamic area, MCH fibres project to many different brain areas, including cortical areas, the amygdala, and the brainstem [54, 562, 677]. MCH was identified as the ligand for an orphan receptor by several groups simultaneously [21, 93, 359, 528, 553]. The distribution of this MCH_1-receptor corresponds well with that of the MCH peptide

[248, 527]. In humans, an additional receptor had been identified, but this MCH_2-receptor has not been found in rodents [596].

MCH expression was either decreased (as would be expected for an orexigenic peptide) or increased by injections of leptin [260, 607]. Fasting has been shown to increase MCH expression [46, 499, 504, 607]. In contrast to this orexigenic profile, MCH neurons appear to be inhibited by NPY [615]. Most studies have found that MCH injections stimulate feeding [1, 385, 504, 521], although the opposite has also been reported [499]. When injected chronically, the orexigenic effect of MCH faded, and body weight was not affected [522]. Mice that lack MCH or its receptor show hyperactivity [398, 550]. This suggests that MCH suppresses activity, which would be in accordance with an orexigenic, anabolic role.

The orexins (ORX-A and ORX-B)

ORX-A (or hypocretin 1) and ORX-B (hypocretin 2) were discovered and named simultaneously by two groups [122, 531]. Both are produced from the precursor peptide prepro-ORX, which is expressed predominantly in the LHA [122, 531], especially in its perifornical area (PFA) [74]. The orexins project throughout the hypothalamus and the rest of the brain [116, 438, 482], and the ORX_1- and ORX_2-receptors are expressed in the same areas that receive dense ORX innervation [394, 608].

Negative energy balance has been shown to increase LHA ORX expression and peptide levels [84, 370, 422, 531], although unchanged expression levels were also reported [46, 591, 607]. Injections of leptin, mimicking positive energy balance, have been shown to lower LHA ORX expression and ORX-A levels [37, 370, 672]. Although AgRP injections were shown to increase activity of ORX neurons [681] as might be expected, NPY either decreased or failed to affect their activity in two different paradigms [86, 186]. Nevertheless, Y_1R and Y_5R antagonists were shown to suppress ORX-induced feeding, which is again more in line with the orexigenic role of the orexins. Intracerebroventricular and intrahypothalamic ORX injections stimulated feeding [137, 239, 434, 531], although results for ORX-B have been less consistent than for ORX-A, and the outcome generally depended on the time of the day [330]. These findings support an orexigenic role, but ORX does not seem to fit the profile for a truly anabolic protein. Metabolic rate and activity seem to be stimulated, rather than decreased, by ORX [272, 320, 331, 381] and continuous infusion of ORX altered the circadian rhythm of feeding without affecting total food intake or body weight [239, 673]. Apart from its complicated role in the regulation of energy balance, ORX is also involved in the regulation of arousal and vigilance, and its prime function in energy balance may be to synchronise feeding behaviour with other essential behaviours and the environment [530, 588, 660].

Downstream events

Although this thesis does not deal specifically with the processes downstream of the LHA and PVN, it is important to have a general idea of how these signals in the hypothalamus can

eventually alter the intake and expenditure of energy. There are basically three output pathways. The hypothalamus can drive behaviour that is aimed at obtaining, sparing, or spending energy; and modify energy-saving or energy-spending processes by a neuroendocrine and an autonomic neural pathway. The first pathway influences behaviour through integration of signals from many brain areas, and ultimately, the activation of motor neurons [e.g. 45]. A final effect of increased hypothalamic orexigenic activity may be the initiation of a meal. The second pathway, the neuroendocrine route, influences energy balance through the secretion of hormones. The HPA- and HPT axes are part of this pathway. In the HPA axis, CRH from the PVN stimulates the release of adrenocorticotropic hormone from the pituitary, which in turn induces glucocorticoid release from the adrenal glands [387]. These can indirectly influence feeding behaviour and energy expenditure [reviewed in 442]. In the HPT axis, TRH from the PVN, via the release of thyroid-stimulating hormone release from the pituitary, induces the release of thyroid hormone by the thyroid gland, which stimulates energy expenditure and thermogenesis [reviewed in 351]. The third pathway is via the autonomic nervous system. Several hypothalamic nuclei, especially the PVN, innervate neurons in the brainstem and the spinal cord that are part of the autonomic nervous system, both sympathetic and parasympathetic [387]. Via these sympathetic and parasympathetic pathways, energy expenditure can be regulated, for example by influencing the heart rate and thermogenesis by adipose tissue and skeletal muscle [reviewed in 178, 476, 566].

Mutual connections

When reflecting on the regulation of energy balance, it is important to bear in mind that the pathways and processes described above are much simplified. The main route for information indeed is from the periphery to the ARC and then via the PVN or LHA to the output systems (**Figure 3**), but this is not the only direction in which information is moving. Other brain areas (hypothalamic and otherwise) are involved as well, and there is ample feedback between the different brain areas. In several respects, the regulation of energy balance is much more complex than the relatively straightforward pathways described above. A few examples are given below.
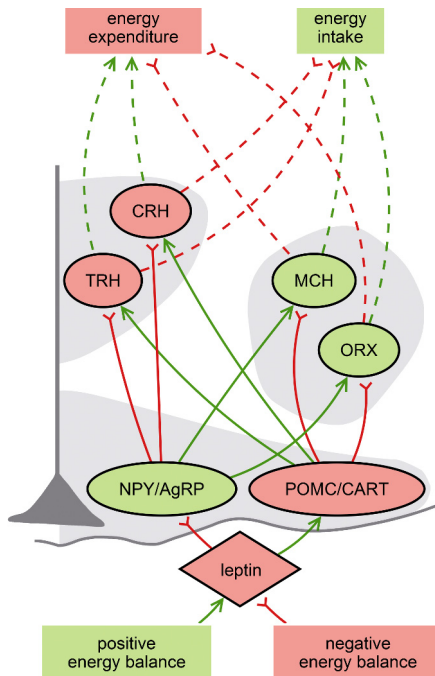
Figure 3. A simplified diagram of the hypothalamic regulation of energy balance. The main pathway for energy balance regulation is from peripheral input (leptin) to the ARC (with NPY, AgRP, POMC, and CART), via the PVN (CRH and TRH) and LHA (MCH and ORX), to the output systems. The nuclei are shown in light grey, with the third ventricle depicted in darker grey. Green arrow, stimulating effect; red inverted arrow, inhibiting effect; solid line, direct connection; dashed line, indirect connection; activity of green (red) nodes leads to more positive (negative) energy balance. See the text for details.

Firstly, the different populations of cells in the ARC influence their own and each other's activity. Both NPY/AgRP and POMC/CART cells express receptors for NPY and MCs, albeit different subtypes: NPY/AgRP neurons express Y_2R and the MC3 receptor, whereas POMC/CART neurons express Y_1R, Y_5R, and the MC4 receptor [76, 171, 188, 429]. Through these receptors, NPY inhibits the POMC/CART cells [106, 519], NPY and AgRP stimulate each other's release and (at least *in vitro*) can be stimulated by α-MSH and CART [133], whereas CART reduces α-MSH release [572].

Secondly, the PVN and LHA may also receive peripheral input directly, through leptin receptors in these nuclei, although these receptors are not necessarily colocalised with the four peptides of our interest: CRH, TRH, MCH, and ORX [223, 224, 232, 358]. Leptin also influences motivational brain areas directly, via receptors in these areas [reviewed in 45, 174].

Thirdly, there is feedback within and between the PVN and LHA. CRH and TRH neurons are contacted by each other's axons [253], as are MCH and ORX neurons [218], and ORX stimulates both ORX and MCH neurons [366, 615]. Further, MCH and ORX have been shown to stimulate CRH neurons and release [309, 529], while MCH reduces TRH release [310]. CRH in turn, has been shown to activate ORX neurons [661].

Fourthly, besides this mutual influence of the PVN and LHA peptides, they also project back onto the ARC neurons. ORX axons, for example, terminate on both NPY and POMC neurons [217, 434]. Via these terminals, ORX stimulates NPY neurons and inhibits POMC neurons [434, 618]. MCH has been shown to have similar effects on the ARC as ORX [1].

Moreover, CRH receptors have been identified in NPY neurons in the ARC [85] and a CRH receptor agonist has been shown to inhibit medial ARC neurons [120].

Then, as mentioned before, there are many more brain areas involved in the regulation of energy balance than the three hypothalamic nuclei discussed above. Among these are other hypothalamic nuclei such as the ventromedial nucleus, the dorsomedial nucleus, and the medial preoptic area [53, 305, 415, 561, 577]. Further, the caudal brainstem is not only a relay station between the periphery and the hypothalamus; it is known to be capable of performing part of the regulation of food intake independently of the forebrain [208]. In addition, the higher brain areas that deal with the reward, cognitive, and social aspects of food intake, are not only output areas for the hypothalamus, but also send information back to the hypothalamus [45].

Lastly, although the peptides that have been mentioned here do play an important role in the regulation of energy balance, there are many other substances involved. Some of these are hypothalamic neuropeptides, such as galanin, galanin-like peptide, malonyl-CoA, neurotensin, and neuromedin U [43, 229, 340, 526, 663]. Naturally, the classical neurotransmitters, glutamate and γ-aminobutyric acid, are also present and functional in the hypothalamus [22, 246, 617]. Moreover, the hypothalamic nuclei and peptides discussed here are involved in many other processes besides energy balance. These include the immune system (leptin, the MCs), bone formation and -remodelling (leptin, NPY, CART, MCH), blood pressure and cardiovascular regulation (leptin, NPY), kidney function (NPY), reproduction (NPY, MCH), stress (NPY, CART, CRH, MCH), pigmentation and pain sensation (the MCs), reward and addiction (CART, MCH), anxiety (MCH), and the wake-sleep cycle (MCH and ORX) [12, 101, 244, 302, 432, 474, 489, 515, 530, 626, 654, 678].

This section is not meant to be exhaustive, but is intended merely to give an impression of the complexity of the regulation of energy balance (summarised in **Figure 4**). However, despite the many interconnections, the main pathway is believed to be from the peripheral input to the ARC (with NPY, AgRP, POMC, and CART), via the PVN (CRH and TRH) and LHA (MCH and ORX), to the output systems (as depicted in Figure 3). Although at first sight these peptides all seem to fulfil one of two functions (orexigenic or anorexigenic), a closer inspection reveals subtle differences between these peptides. In this section, we have concentrated on evidence from rodents. The regulation of energy balance is very similar in different animal species, including humans. However, since there is some variation in the details [e.g. 139, 412], it is important to always take care when extrapolating information from one species to another.
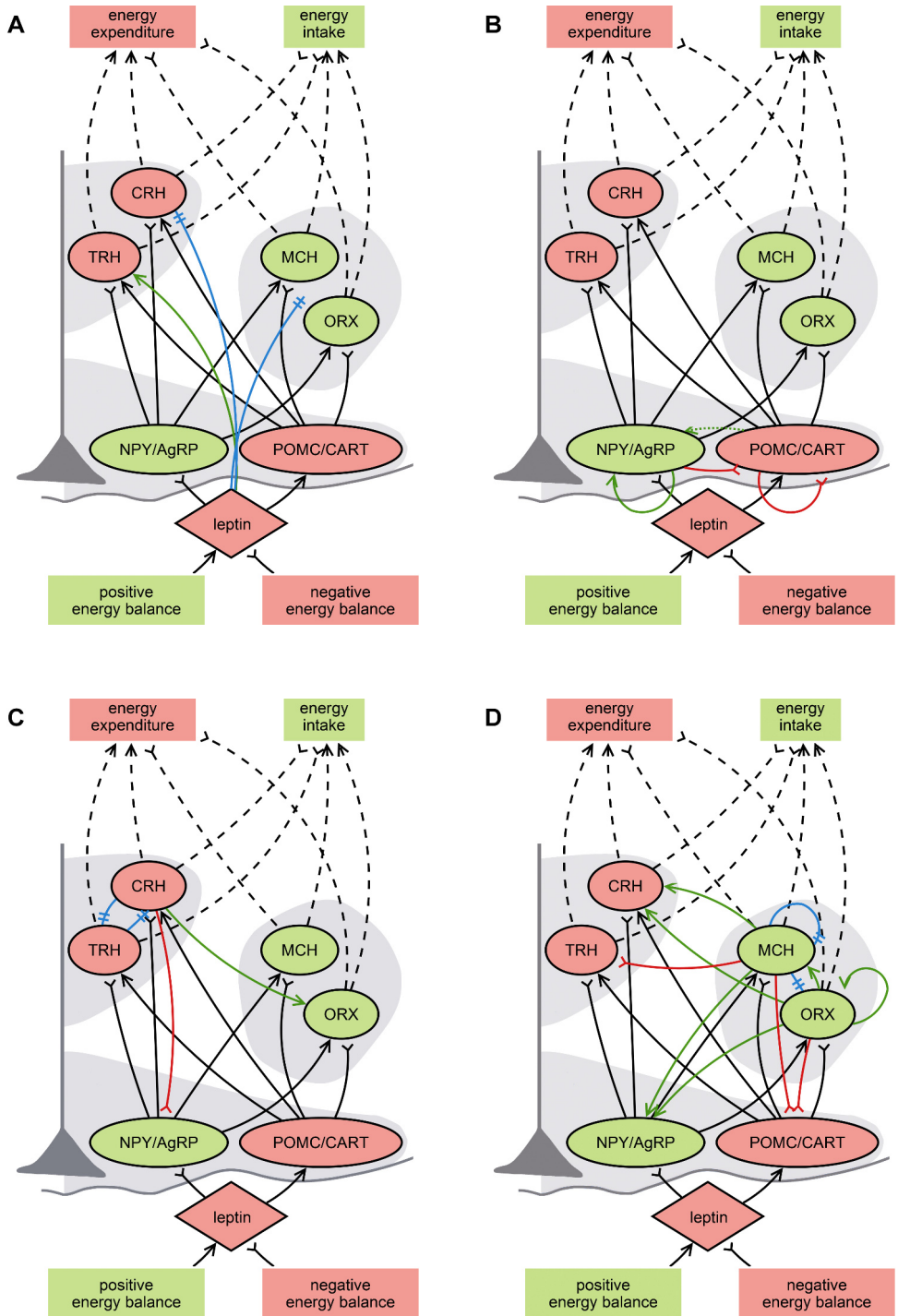
Figure 4. Still simplified diagrams of the hypothalamic regulation of energy balance. Besides the main pathway (see Figure 3), these schematics show additional connections from leptin (**A**), the ARC (**B**), the PVN (**C**), and the LHA (**D**). Note that in A, the connection to the LHA does not contact MCH or ORX neurons directly. Green arrow, stimulating effect; red inverted arrow, inhibiting effect; blue crossed line, unspecified effect; solid line, direct connection; dashed line, indirect connection; dotted line, probable connection; activity of green (red) nodes leads to more positive (negative) energy balance. See the text for details.

Ontogeny

In order to programme a certain system or function, an environmental stimulus must occur during a period in development when the system or function is still plastic. Therefore, we will now turn to the development of the hypothalamic peptides that are known to be involved in the regulation of energy balance, their ontogeny. In rodents, the energy balance-regulating system is structurally and functionally immature at the start of postnatal life. The basic anatomy of the rat hypothalamus is established prenatally, with its nuclei expressing specific neuropeptides being recognisable before birth [395], but the connections between the hypothalamus and its input- and output systems [516, 517], and those within the hypothalamus itself [213, 214], develop mostly after birth, in the first few postnatal weeks. This rapid postnatal development is also reflected in overall brain growth: in neonatal rats, total brain weight increases by a factor five between birth and weaning [587].

Developing rat pups go through some major transitions. Whereas the foetus receives mainly glucose, lactate, and amino acids via the placenta, at birth the source of energy changes to high-fat mother's milk [169]. Only a few weeks later, the pups are weaned and make a more gradual transition to the high-carbohydrate, low-fat adult diet [20, 169]. At the same time, the pups have to make the transition from obtaining all energy and fluids from the dam by suckling, to the two separate processes of feeding and drinking [592]. As will be described below, different mechanisms appear to regulate these different types of ingestive behaviour.

Rat pups as young as one day old already regulate their milk intake according to how deprived they are [107, 259]. The only cue that suckling rats have been shown to use to regulate their milk intake is the distension caused by gastro-intestinal fill [375, 483]. This response is mediated primarily by the vagus nerve [373, 375] and hence by the brainstem rather than by the hypothalamus. Other signals that influence food intake in adult rats, such as the nutritional value of the stomach contents, serum leptin levels, and manipulations of levels of glucose and free fatty acids, were not found to affect intake in suckling rats [374, 483, 502]. It appears that the regulation of energy balance in the suckling pup is limited to optimising energy intake for growth, and intake is only restricted by a full stomach to prevent gross overeating. Thus, there only seems to be short-term regulation of intake in suckling pups, no long-term regulation [228]. Furthermore, this system regulating milk intake appears to be fully functional at birth.

Thermoregulation and the regulation of adult forms of ingestion then develop in the early postnatal period. From day 1 on, pups can already regulate their temperature by

moving towards or away from a heat source [321], whereas mechanisms for thermogenesis develop over the first two weeks of life [374]. Under certain conditions, suckling pups can be induced to ingest fluids from soaked towels lining the floor and this 'independent ingestion' has been used as a model to study the development of adult feeding behaviour [228]. Using this model of independent ingestion, it has been reported that in the first 10 days of life, gastric distension is the only cue that terminates intake. From then on, the nutritive value of the gastric content starts to play a role [reviewed in 228, 516]. Around the same age, pups start to adjust their intake according to their level of fatty acids [reviewed in 592], whereas a similar response to glucose levels does not appear until the age of 4 to 5 weeks [228, 259]. Another major development event is the differentiation between feeding and drinking; young pups simply increase their intake when they are dehydrated, and only from around the age of 20 days they will reduce their milk intake when dehydrated, a phenomenon called dehydration anorexia [reviewed in 228, 516].

The development of the regulation of energy balance is accompanied by changes in mRNA and protein levels of the reviewed peptides. The ontogeny of these peptides is summarised below and depicted in **Figure 5**.
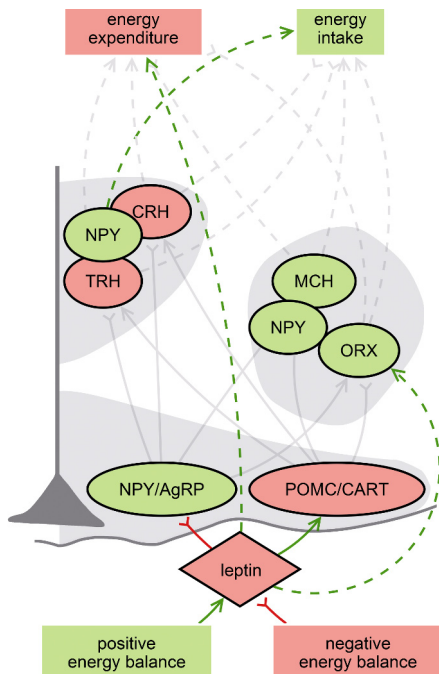
Figure 5. A simplified diagram of the hypothalamic regulation of energy balance in juvenile life. Energy balance does not appear to be tightly regulated in the neonatal period. The few connections that have been reported to be in place are summarised in this figure. The adult connections are not yet present and/or active and are shown in light grey. Green arrow, stimulating effect; red inverted arrow, inhibiting effect; solid line, direct connection; dashed line, indirect connection; activity of green (red) nodes leads to more positive (negative) energy balance. See the text for details.

Leptin

In rats, leptin can first be detected in foetal plasma on day 19 of gestation [304]. In nearly full-term foetuses (day 20–21), leptin levels strongly resembled those of the pregnant dams [247, 304]. Leptin mRNA is already expressed by rat adipose tissue at birth, and its

expression and serum levels are immediately regulated by the nutritional status of the neonatal pup [131]. In addition, Ob-Rb, the leptin receptor, has been shown to be expressed in the foetal brain as early as day 14 of gestation [40, 89].

During the lactation period, leptin undergoes some major changes. A first, relatively small increase in serum leptin levels can be detected in rat pups of 1 to 2 days old [247, 564], followed by a second and larger peak around days 7 to 12 [425, 509, 564] (although some investigators have reported constantly high serum leptin levels throughout the first two postnatal weeks [132], or a persistently low level after the initial small peak [247]). Interestingly, this leptin surge is unrelated to changes in body weight and fat content in the neonatal period [6, 564]. The high leptin levels do coincide with elevated leptin mRNA in neonatal adipose tissue, suggesting that the peak originates from the pups' own leptin production [675]. Both the neonatal pituitary, which has high leptin expression during this period [425], and the dam's milk [90] may contribute to the leptin surge. In concert with the changes in leptin levels in the neonatal period, hypothalamic levels of Ob-Rb and its mRNA rise significantly between birth and weaning [426, 564].

Leptin's functionality in the regulation of energy balance appears to be partial in the neonatal period. In rats as young as one week old, leptin injections are found to reduce gain in body weight and especially fat mass, without an effect on milk intake [339, 502, 503, 574]. Instead, these effects seem to be the result of an increase in energy expenditure [57, 574]. Leptin is already effective in increasing POMC and decreasing NPY mRNA in the ARC of rats in this neonatal period [503], and a robust positive relation between leptin levels and fat mass was reported on day 10 [679]. The exact timing of the development of this system seems to differ between mice and rats. In mice, serum leptin levels were not altered after milk deprivation on day 8 [6], energy expenditure was not yet increased by leptin injections on day 9 [418], and daily leptin injections in the second week of postnatal life were not found to affect hypothalamic neuropeptide expression [4].

During this period of partial functionality in energy balance regulation, leptin has a neurotrophic role. In the absence of leptin, general brain development and that of the hypothalamic circuitry specifically are impaired [3, 66]. Leptin shares this property with insulin, which is also implicated in brain development. The neurotrophic actions of insulin include stimulation of neurite outgrowth, protein synthesis, and neuronal survival [243, 261, 535]. Leptin's neurotrophic effects may actually persist until adulthood, as leptin administration in *ob/ob* mice significantly alters the synaptic input on both NPY and POMC neurons in the ARC [488]. Furthermore, the lining of the third ventricle has been shown to contain neural progenitor cells that can be induced by neurotrophic factors to proliferate and differentiate into functional hypothalamic neurons [324, 670]. This residual plasticity of the hypothalamic circuitry in adulthood provides an additional route by which environmental signals (including leptin) can regulate energy balance [182].

ARC peptides

The four reviewed peptides that are expressed by the ARC: NPY, AgRP, POMC, and CART, are already expressed in the prenatal rat brain [40]. However, ARC projections to other hypothalamic nuclei only develop during the early postnatal period [65]. During this period, there are also dynamic changes in the levels of the peptides and their gene expression.

Orexigenic ARC peptides · NPY peptide is detected in the rat foetal midbrain as early as day 13 or 14 of gestation [179, 297, 666]. NPY mRNA levels rise during gestation to reach near adult levels around birth [348, 558]. Like leptin, NPY gene expression is elevated during the lactation period, with a peak around day 16 [449, 558]. At the same time, NPY mRNA is transiently expressed in hypothalamic areas that do not produce NPY in adulthood. Suckling rat pups express NPY mRNA in the dorsomedial nucleus, PVN, LHA, and PFA, albeit at lower levels than in the ARC [212, 558]. Alongside the developmental changes in NPY mRNA, NPY peptide levels show a rapid postnatal rise and in the ARC reach adult levels by the time of weaning [9, 357]. Immunohistochemistry studies have shown that the number of cell bodies containing NPY peptide rises gradually until birth, with declining numbers afterwards [179, 666]. After day 10, NPY cell bodies can only be visualised when axonal transport is chemically blocked by colchicine administration; a finding that is consistent with the simultaneous increase in NPY-immunoreactive fibres throughout the hypothalamus [179, 297, 666]. In a more recent study, by staining for NPY and AgRP peptide simultaneously, the origin of these postnatally developing fibres was proven to be the ARC [211]. Indeed, the developmental pattern of AgRP resembles that of NPY, with increasing expression during the first postnatal weeks and a peak around day 16 [211, 447].

In the neonatal period, NPY and AgRP already appear to have some functionality. Maternal deprivation has been shown to increase expression in the ARC already on day 2 (NPY) and at least from day 11 (AgRP) [212, 335]. Furthermore, NPY injections into the PVN increased intake of water and milk as early as day 2; on day 15 the pups showed a preferential increase in milk intake [88]. As has already been mentioned, intrahypothalamic fibres in the neonatal rat are still incomplete and NPY is expressed in several hypothalamic nuclei. Therefore, NPY may exert most of its actions locally at the site of expression, rather than after being axonally transported from the ARC to other hypothalamic regions.

Anorexigenic ARC peptides · POMC mRNA is first detected in the midbrain on day 13 of gestation [251]. During the lactation period, hypothalamic POMC expression is either stable [14] or increases towards weaning [4]. ARC POMC expression then increases significantly between weaning and young adulthood [313, 652]. Hypothalamic POMC peptide has been detected as early as day 12 of gestation [314, 545], with α-MSH, the cleaved product, only appearing between day 15 and day 19.5 of gestation [115, 314, 423]. Postnatally, POMC and α-MSH protein in the ARC go through a rapid increase, to peak around day 21 to 28 [314, 423]. About early CART ontogeny, there is only limited information. One study in mice has

reported low levels of hypothalamic mRNA on postnatal day 5, with near adult levels on days 10 and 22 [4]. However, the developmental patterns reported by this study for NPY, AgRP, and POMC were different from those found in most other studies.

In contrast to NPY, α-MSH does not seem to have much functionality early in life. In one-week-old rat pups, many PVN neurons are responsive to NPY, whereas only a few show a response after administration of an α-MSH agonist [410]. At the age of 4 to 5 weeks, however, the number of PVN neurons responsive to NPY has decreased, whereas the number of neurons responsive to MTII has increased dramatically [410]. This phenomenon may ensure a high intake in neonatal life, by minimising anorexigenic signalling in early life.

PVN & LHA peptides

Less detailed information is available about the development of the peptides of interest in the PVN and the LHA: CRH, TRH, MCH, and ORX. Gene expression is detected in the foetal rat brain for all four peptides [26, 71, 81, 209, 575]. The peptide is generally also detected in the hypothalamus before birth [71, 187, 452, 575]. Neonatally, there is a gradual increase in expression and protein levels of most peptides, and adult levels are generally reached around the time of weaning [81, 104, 122, 148, 209, 575, 599, 616, 671], although TRH peptide levels may keep on rising between weaning and young adulthood [187].

Functional tests were reported for ORX. In the neonatal period, leptin administration was shown to increase ORX mRNA in the LHA [671], where the normal effect in adults would be inhibition of expression [370]. Interestingly, the neonatal leptin administration that increased ORX expression did not affect body weight and blood glucose levels, whereas 24 hours of milk deprivation did reduce body weight and blood glucose levels, but did not affect ORX expression levels [671]. Therefore, the neonatal leptin effect on ORX mRNA was interpreted to reflect a developmental role, rather than an effect on energy balance regulation [671].

Ageing

After the major developmental events in juvenile life, in adulthood there is little change in the hypothalamic system that regulates energy balance. Only as rats reach old age, some more changes can be noted in the expression and protein levels of these peptides. From middle-age on (around one year in rats), the regulation of energy balance starts to deteriorate.

At ages ranging from 12 to 26 months, hypothalamic expression and peptide levels for NPY, AgRP, POMC, and CRH have mostly been reported to be lower than in young rats (2 to 4 months) [198, 215, 300, 334, 662], although unchanged levels for NPY, POMC, and CRH were also reported at these ages [300, 334, 662]. Interestingly, ARC CART mRNA was shown to increase between 4, 13, and 25 months of age [662].

Likewise, aged rats show changes in functionality of these peptides. Fasting-induced upregulation of NPY and AgRP and downregulation of POMC and CART were reduced in

middle-aged and old rats [215, 300, 662], and with NPY, this effect was shown to occur as early as 40 weeks of age [285]. PVN CRH expression showed a fasting-induced downregulation at 72 weeks, but not at 9 weeks of age [300]. The orexigenic effects of injections of NPY and ORX-A were also shown to subside with increasing age [8, 595], and in the case of ORX-A, this coincided with a reduced number of hypothalamic receptors [595].

The aforementioned changes also seem to be accompanied by changes in eating behaviour. Food intake per kilogram body weight has been found to be lower in aged animals [300, 595], although others report an unchanged food intake even at the age of 33 months [662]. By middle-age, rats already show a reduction in refeeding response after food deprivation [215, 300].

Development in humans and rats

If we want to extrapolate any of our data and conclusions to the human situation, it is important to consider the respective timing of the ontogeny of the relevant systems in humans and rats. At birth, humans are further in their development, and many developmental events that occur in the early postnatal period in rats, take place in the third trimester of human pregnancy [99, 135, 213, 405]. NPY immunoreactivity is first detected in the human ARC at about 21 weeks of gestation [333], and in non-human primates, NPY/AgRP projections to the PVN increase dramatically during the third trimester of gestation [206] and seem to be nearly complete by birth [213, 214]. Therefore, caution is needed when extrapolating findings from one species to another.

Energy balance programming

As has been shown in the previous section, a large part of the development of the energy balance-regulating system occurs in the perinatal period in both man and rat, although the exact timing of developmental events differs between the two species. With the knowledge of the previous section, one can imagine that the perinatal period with its rapid development may be a critical period, and that during this critical time window, the organism is vulnerable to environmental influences. One can also imagine that different timing of an external stimulus, relative to the stage of development of the organism, can produce different outcomes. Also, different types of stimulus (e.g. undernutrition *vs.* overnutrition, global *vs.* specific nutrients, maternal *vs.* foetal/neonatal) may produce different outcomes. Therefore, in this section, we will discuss developmental programming of energy balance according to the type and timing of the stimulus.

Indicators of developmental programming of energy balance

To identify programming of energy balance, different approaches have been taken. There are basically three types of outcome that can be measured in order to investigate this phenomenon. An indirect way of looking at energy balance is to measure body dimensions

and body composition. Because positive energy balance results in fat deposition and allows growth, these measurements can give an indication of enduring positive or negative energy balance in the (recent) past. Relevant parameters are body weight, body length, body mass index (BMI), fat mass and lean mass, and whether or not there is complete catch-up growth. These parameters are most apparent, and in humans often are the first indication that energy balance may be disturbed. Another way of investigating energy balance programming is to examine components of energy balance directly. Energy intake, resting energy expenditure, and activity-related energy expenditure together determine energy balance. These parameters may be somewhat less explicit in everyday life, but they can be studied relatively easy, also in the human situation. The third approach to investigate energy balance programming is to study the peptides and hormones that are responsible for the regulation of energy balance. Properties like gene expression, peptide levels, epigenetic modifications, and functional changes can be studied. As these measurements require invasive techniques, this approach is less suitable for use in the human situation.

Naturally, a combination of the three approaches will generate the most complete description of the phenomenon of developmental programming of energy balance. Our understanding of the phenomenon has much advanced in recent years; many studies and review papers have been published on this subject since the start of the present project [e.g. 68, 204, 257, 406, 455, 578, 598]. Now, various influences of the perinatal environment on energy balance parameters will be discussed; first briefly for the human situation and then in different rat models.

Programming of energy balance in humans

Epidemiological evidence suggests that the early environment can have a profound influence on energy balance. With these studies it must be kept in mind, however, that in the human situation, the underlying cause of low birth weight or restricted foetal growth varies and is often unknown [181, 664]. In addition, there are many confounding factors (e.g. the living conditions) that may obscure the real effects of the early environmental influence.

Body dimensions & body composition

Although higher adult body weight and BMI have repeatedly been reported with increasing birth weight [112, 113, 298, 400, 508], the notion that low birth weight and impaired foetal growth may also programme increased adiposity is gaining recognition. Over the last decade or so, researchers have increasingly investigated effects on more refined indicators of obesity, such as body composition (lean *vs.* fat mass) and fat distribution (e.g. waist-to-hip ratio, skinfold ratios). These studies have shown that the positive relationship between birth weight and adult BMI results mostly from a positive relationship with lean mass, but not with fat mass [189, 298, 364, 560, 639, 644, 674]. Moreover, low birth weight has now been shown to be associated with a higher fat percentage in later life [241, 271, 281, 674], and with a detrimental distribution of fat (i.e. more central, abdominal, and

visceral) [31, 155, 269, 270, 280, 344, 350, 369, 392, 600, 614, 639]. The fact that these studies were performed in diverse populations (from different European countries {Belgium, Finland, France, the Netherlands, Spain, and the United Kingdom}, the United States of America {non-Hispanic white, non-Hispanic black, and Mexican-American}, Brazil, Guatemala, and Jamaica), with different ages (from young children to old age), and in both sexes, underlines the robustness of these associations.

In recent years, the significance of the rapid postnatal catch-up that often follows perinatal undernutrition, rather than that of a low birth weight per se, has been stressed. Several studies have shown that rapid early growth (with the definition of early ranging from the first week of postnatal life to about three years) increases the risk for later adiposity and obesity [143, 456, 458, 459, 579, 644].

Energy: intake & expenditure

Relatively few studies have directly assessed energy balance parameters in low-birth weight subjects. For energy expenditure, only some neonatal data are available. These suggest that infants that are born small for gestational age have higher energy expenditure than both premature appropriate-for-gestational-age very-low-birth weight infants [62, 78, 91, 95] and at-term appropriate-for-gestational-age infants [121, 278]. Energy intake was generally similar to that of premature infants of the same body weight [62, 91, 95]. One study reported a higher intake per kilogram body weight in SGA infants, compared to appropriate- and large-for-gestational-age infants of the same postnatal age [464]. However, this difference may largely be due to simple body-weight differences between the groups, because relative energy requirements diminish as body size increases (also see Chapter 2). In a more long-term study, a sample of prepubertal SGA children that did not catch up had a food intake below the recommended energy intake for their age [63].

Peptides and hormones

In humans, measurements of the third category (that of the peptides and hormones that are involved in the regulation of energy balance) have largely been limited to the circulating hormones. Serum leptin levels have been investigated most thoroughly.

In neonates, several studies have found positive correlations of leptin with birth weight, birth length, and BMI [205, 282, 393, 402, 457]. As the strongest correlation was usually found with BMI, these associations most likely reflect the deficit in fat deposition in low-birth weight infants. However, a programming effect is suggested by the fact that subjects that were born with a low birth weight were found to have high leptin levels with respect to their BMI at several different ages (ranging from one year to adulthood) [283, 399, 484]. Another report that suggests programming of leptin levels, studied the influence of early nutrition in preterm infants [559]. It was shown that adolescents that had received preterm formula had more leptin per kilogram fat mass than adolescents that had received a control diet in infancy [559].

Besides altered leptin levels, a few studies have shown increased ghrelin levels in SGA infants at birth [157, 454], but not at the age of one year [275]. There is also some evidence (in neonates and children) that the HPT axis may be disturbed in SGA subjects [390, 505].

Evidence for developmental programming

Summarising, there is quite some evidence that the early nutritional environment can have permanent effect on the body dimensions of humans. Although direct measurements of energy balance and its regulation are still scarce, disturbances have been found, some of which seem to persist into adult life. As these kinds of measurements are more invasive, and some can only be performed postmortem, they are obviously not employed in humans on a large scale. That is why different animal models were designed to study these effects more closely.

Programming of energy balance in animal models

The use of experimental animal models has some substantial advantages over studies in humans. In contrast to the human situation, with animal models for perinatal restriction of growth and nutrition, the exact cause of the observed symptoms is known, and the degree of control over the subsequent environment is far greater. In addition, animal models permit the use of more invasive methods than in humans.

Experimental animal models for developmental programming have been designed in various species, including primates, sheep, guinea pigs, and rats [47, 408, 465]. In this review, we will focus on studies in the rat, although a few studies in mice are also included. In rats, both pre and postnatal manipulations of nutrition have been used to induce developmental programming of energy balance, including ligation of the uterine arteries, maternal protein- or global undernutrition, and manipulations of litter size [47, 408, 465]. These different models produce different phenotypes. Here, we will first describe effects on the body dimensions and body composition of the major models that have been used in rodents. Then, the effects on energy balance and its regulation will be discussed.

Body dimensions & body composition

Nutritional manipulation of the dam · Prenatal manipulations of foetal nutrition, via the diet of the pregnant dam, exert long-term effects on the body dimensions of the offspring, with or without an immediate effect on birth weight of the pups. Two major types of this kind of manipulation are maternal low-protein diets and global maternal food restriction to different degrees (ranging from 30% to 70% of control intake).

Whether a maternal low-protein diet actually reduces birth weight of the pups, appears to depend on the exact composition of the diet and other details in the methodology, because some studies (mostly using a low-protein Hope Farms diet) report lower birth weights [50, 383, 552, 604], whereas others (mostly using the 'Southampton diet') have

reported normal birth weight after maternal low-protein diet during gestation [7, 347, 403, 510]. After a maternal low-protein diet, body weight either stays reduced or normalises to control levels, with the outcome apparently independent of birth weight and the experimental diet used during pregnancy [7, 38, 39, 50, 347, 383, 510, 552, 604, 676]. Two studies have reported rapid catch-up growth with increased body weight [403, 466]. Adult body composition after a maternal low-protein diet has mostly been reported to be normal [38, 39, 510, 676], although some of these studies did report an altered fat percentage in either males or females. One study found increased leptin and triglyceride levels in males, but not females, with otherwise normal body weight and fat mass [676]. This suggests that, although the body composition may be normal, its regulation can still be disturbed in these animals.

Maternal food restriction usually reduces birth weight of the resulting pups [49, 129, 256, 286, 477, 602, 632, 634, 675], except when the food restriction is limited to the first two weeks of pregnancy or in some cases when intake is only mildly restricted to 70% of control intake [15, 291, 292, 322]. After maternal food restriction, rats show either complete or incomplete catch-up growth [15, 256, 286, 341, 477] so that in rats with a low birth weight, adult body weight was either reduced, normal, or elevated compared to that of controls [129, 602, 633, 634]. Several studies have found normal body composition after prenatal maternal food restriction [129, 176, 256, 274, 286, 291, 292, 322, 360, 675]. However, increased and decreased adiposity have also been reported. Within studies, these different outcomes can be attributed to sex differences, different effects at different ages, strain differences, and timing of the food restriction [15, 129, 176, 292, 322, 360]. Between studies, the method of determining body composition (e.g. BMI, weight of different fat pads, total lipid determination by carcass analysis, dual-energy X-ray absorptiometry) and the severity of the food restriction may explain a large part of the variation in outcome. One group that uses severe maternal food restriction (to 30% of control levels) has consistently found a persistent lower body weight, combined with increased fat mass and leptin levels in both males and females [341, 602, 632-634]. Leptin levels usually reflected body composition [129, 274, 286, 360, 602, 632-634, 675], although in one study increased leptin levels appeared to precede the increased fat percentage [129]. In summary, although studies using the 'Vickers model' present a constant exception, most studies have found normal body composition after prenatal maternal food restriction.

Because a considerable part of the developmental events that occur *in utero* in humans, take place after birth in rats, postnatal manipulations are also frequently used as a model. When the same maternal dietary manipulations that are used prenatally are either started or continued in the lactation period, the reductions in body weight are generally longer lasting and less catch-up growth is reported [49, 50, 127, 129, 216, 256, 286, 383, 466, 467, 469, 470, 507, 552, 580, 601, 676]. Concomitantly, an obese phenotype is observed less frequently than with strictly prenatal manipulations [127, 129, 216, 256, 286, 360, 552, 676]. There may be less catch-up growth after these postnatal manipulations because the

condition is too severe to recover from (especially when prenatal and postnatal malnutrition are combined), or at weaning the animals may have reached the end of the time-window in which complete catch-up is possible. Alternatively, the fact that these animals do not seem to be 'programmed for obesity' may reflect a different type of programming than with exclusive prenatal maternal dietary manipulation.

Nutritional manipulation of the offspring · <u>Uterine artery ligation in the pregnant dam</u> reduces the blood flow to the foetuses [653] and is frequently used as a model for placental insufficiency, the most common cause of low birth weight in westernised countries [245]. To approach the human IUGR situation as closely as possibly, often only pups that are growth restricted according to similar criteria as those used in humans are selected for studies [see 440]. This obviously results in a birth weight that is reduced by definition [e.g. 150, 386, 450, 539, 597]. Nevertheless, studies that did not use pup selection have also reported a lower birth weight in rats born after uterine artery ligation [e.g. 254, 325, 597, 653]. The long-term effects on body weight seem to be dependent on the exact timing of the ligation. When performed on day 17 of gestation, the weight deficit is usually persistent [92, 150, 265, 451, 541], whereas after ligation on day 19 of gestation, complete catch-up growth has been reported [450, 525, 638]. Some studies also found a return to normal body weight after ligation on day 16 or 17 [585, 597]. Newborn pups that were growth restricted by uterine artery ligation were shown to have a fat percentage that was either reduced or comparable to that of control pups [254, 386]. Juveniles and adults that do not completely catch up in body weight have been shown to have normal BMI, fat percentage, and serum leptin levels [150, 152]. The ones that do catch up to control body weight also have normal leptin levels when young (at an age when their body weight is still reduced) [450, 506]. Rats that stay at the same body weight as control rats after catch-up have elevated leptin levels and increased fat mass in adulthood [450]. The group that reported overweight in adulthood, found normal or increased fat mass at the age that body weights were similar to those of controls [557, 638] and increased fat mass afterwards [551, 557]. In summary, when there is complete (or even overcomplete) catch-up in body weight, the animals' body composition is disturbed and shifted towards a more obese phenotype. If the catch-up growth stays limited, however, body composition remains normal. It seems likely that the capacity for true growth of organs and other lean tissue is curbed by the early growth restriction, and if there is catch-up beyond a certain point, any additional 'growth' is in fat only.

A method to manipulate early postnatal nutrition that targets the offspring directly (rather than indirectly via the diet of the dam) is to manually adjust the number of pups nursed in a litter [311, 312]. In this way, both neonatal under- and overnutrition can be achieved. By definition, birth weight is not affected by these manipulations, because they take place after birth. Shortly after redistribution into litters of different sizes, differences in body weight become apparent. Rats that are raised in a <u>small litter</u> of only 2 to 5 pups receive more milk, resulting in a higher growth rate and body weight before weaning [19,

120, 175, 238, 345, 371, 433, 493, 494, 536, 537]. Although a few studies report normalisation of body weight [34, 238, 345, 431, 624], this elevated body weight is generally found to persist into adulthood and middle-age [19, 34, 64, 117-120, 158, 242, 323, 367, 371, 372, 417, 433, 461, 462, 493, 495, 649, 651]. The opposite is true for rats that are raised in a large litter of 14 to 24 pups, which have less milk available per pup. These rats grow much slower during the lactation period and have a significantly lower body weight [19, 92, 150, 175, 238, 258, 345, 371, 433, 494]. Again, some studies report normalisation [34, 345, 658], but most researchers find that body weight is persistently reduced [19, 34, 150, 158, 238, 258, 265, 323, 345, 371, 433, 461, 462, 649, 659]. Already during the lactation period, the two models show marked effects on body composition: overfed small-litter pups have an increased fat percentage and leptin levels, whereas these are both decreased in underfed large-litter pups [175, 345, 371, 494, 536, 537]. Thus, a disproportionate part of the added growth in small-litter pups can be ascribed to adipose tissue. After weaning, when all animals are transferred to a normal feeding regime, body composition remains disturbed. In most small- and large-litter rats with persistent changes in body weight, fat percentage and leptin levels also remain altered into adulthood and middle-age [19, 64, 150, 152, 158, 238, 242, 323, 371, 372, 417, 431, 461, 495, 649, 651]. One study even reported an increased fat percentage in small-litter rats at an age when their body weight was no longer elevated [431]. Apart from a few exceptions, the effects of neonatal litter manipulations are long-lasting and also rather consistent between studies. Neonatal overfeeding by raising rats in small litters causes an immediate rise in growth velocity, with persistent higher body weight and fat mass in adulthood, resulting in an 'obese' phenotype. Neonatal underfeeding by raising rats in large litters, on the other hand, acutely reduces growth rate and causes a permanently lower body weight and fat mass, resulting in a leaner phenotype.

Response to a dietary challenge · This section has demonstrated that diverse manipulations of perinatal nutrition can bring forth different phenotypes. Even seemingly comparable manipulations have been shown to generate different long-term effects on body dimensions and body composition. What's more, some of these manipulations have been shown to alter the animals' susceptibility to 'diet-induced obesity' (which is induced by feeding a hypercaloric diet, usually a high-fat diet). Again, there is considerable variation in the reports on this effect. A maternal low-protein diet either did not affect [153] or increased [467] the susceptibility to diet-induced obesity when the manipulation was prenatal. When the manipulation was restricted to the lactation period, less obesity was observed on a highly palatable diet [467]. Several studies have reported a higher susceptibility to diet-induced obesity after prenatal maternal food restriction [274, 291-293, 341, 631, 633, 675], but unchanged obesity has also been reported [291-293, 629, 632, 634]. Here, there seems to be a difference in susceptibility between the sexes, although this sex difference may be strain-dependent: Jones reported increased diet-induced obesity in Sprague-Dawley males but not females, whereas Vickers found higher susceptibility in

Wistar females but not males. Also in rats that were neonatally overfed or underfed by raising them in <u>small or large litters</u>, conflicting results have been reported. In rats with persistent differences in body weight, some studies found no difference between the two models in their susceptibility to diet-induced obesity [158, 238]. One study, however, reported that diet-induced obesity was augmented in small-litter rats and diminished in large-litter rats [461]. From these data, we can conclude that the effects of a dietary challenge are mostly consistent with the general phenotype. More diet-induced obesity is observed in those models that under baseline conditions showed more catch-up growth and increased adiposity.

Energy: intake & expenditure

In the abovementioned rodent models, energy intake and energy expenditure have been studied using a range of different parameters. Expenditure-related parameters include resting- and total energy expenditure, (locomotor) activity, body temperature, and measurements of thyroid function and cellular metabolism. For energy intake, the variety is more in how the data are represented. Daily food intake is either given per animal (raw data), per kilogram body weight (or some other approximation of body size), or adjusted for body size in a statistical test. The results of these different representations are not always easily compared. Especially when intake is divided by body size, the results can be distorted. Because energy requirements per kilogram body weight fall with increasing body size, this calculation systematically underestimates energy utilisation by larger individuals [e.g. 606, and see Chapter 2]. Therefore, such studies are excluded from this review; only studies that report raw food intake data or intake adjusted for body size in a statistical test are included.

Nutritional manipulation of the dam · One study that induced prenatal underfeeding by a <u>maternal low-protein diet</u> reported normal food intake in the adult offspring [676]. The same study found reduced food intake when the underfeeding was (continued) during the lactation period. This was confirmed by others [383, 601], although some have also reported normal levels of food intake in these (prenatally and) postnatally malnourished rats [127, 470]. These data suggest a subtle decrease in food intake after protein malnutrition in the lactation period, whereas prenatal-only malnutrition probably does not affect energy intake. On the expenditure side, in rats with postnatal exposure increased thyroid function (pointing to increased basal metabolism) was found [469, 507], and normal-to-low activity levels have been reported after prenatal exposure [39]. Taken together, these studies suggest that in prenatally malnourished animals normal levels of intake and reduced activity may lead to positive energy balance, whereas in postnatally malnourished animals a negative balance may result from their lower food intake and increased basal metabolism.

After <u>maternal food restriction</u>, food intake was usually found to be similar to that of control animals. However, when body size is taken into account, the effects on energy intake differ according to the timing of the malnutrition: prenatally or postnatally. When pups were

exposed to the maternal diet postnatally, they often had reduced body size combined with normal food intake [129, 286, 470, 601], which results in an elevated relative energy intake. With prenatal-only maternal food restriction, both body size and food intake were usually normal [15, 176, 286, 291, 292, 675], leading to a normal relative food intake. In a few cases, both body size and food intake were elevated [129, 291, 292], which also may point to a fairly normal relative energy intake. Measurements of energy expenditure were mostly performed in prenatally underfed rats; in postnatally underfed rats, one study reported a normal thyroid function [469]. Using the 'Vickers model' of prenatal maternal undernutrition, female rats (that have a low body weight and high fat mass) were found to have reduced activity levels in adulthood [630, 631]. Other studies using prenatal undernutrition have reported normal levels of activity [292] and normal body temperature and resting energy expenditure [675]. These data are suggestive of normal total energy expenditure, which together with an unaltered food intake points to a normal energy balance for these rats that are prenatally exposed to maternal undernutrition.

Nutritional manipulation of the offspring · Food intake was not widely studied after underlined: uterine artery ligation: one study reported decreased food intake [450], whereas another found an unaltered intake per kilogram body weight [638]. In both studies, the experimental animals had similar body weight as controls (which nullifies the interpretational problems of the 'per-kilogram representation'). In both juvenile and adult rats, cellular metabolism was reduced [481, 551, 556], whereas locomotor activity has been reported to be normal [539, 597]. Taken together, a reduced or normal food intake, lower basal metabolic rate, and probably normal activity-related energy expenditure, suggest that energy balance may be either approximately normal (intake and expenditure both reduced) or more positive than in control animals (normal intake with reduced expenditure), respectively.

In virtually all small-litter rats that were heavier than controls, food intake was reported to be elevated throughout life [34, 64, 117-119, 367, 417, 462, 493, 495, 651, but see 372]. Only in young rats that would later lose their overweight, unchanged food intake was found [345, 431]. Fewer studies have reported on the expenditure side of the balance. Rats raised in small litters were found to have a higher body temperature and resting expenditure [433], and in young animals, elevated total energy expenditure was reported [651]. The latter study found that the elevation in energy expenditure was appropriate for the larger body size of the small-litter rats. Since both energy intake and -expenditure are increased in these animals, the overall effect on energy balance depends on the relative sizes of the effects on intake and expenditure. These are difficult to compare between studies. Large-litter rats, on the other hand, were generally reported to have lower energy intake and -expenditure than controls [34, 433, 462]. Again, the fact that these measurements were taken in separate studies complicates interpretations about the overall effect on energy balance in these animals.

The foregoing paragraphs have shown that different models of perinatal malnutrition can have different effects on adult energy balance. They have also shown that, although there is a lot of information about the effects of these manipulations on components of the energy balance, the exact information needed to assess a directional change in energy balance is not always available. Further, in the interpretation of these studies, it is vital to distinguish absolute measurements from adjusted data. Comparisons should only be made between data that are expressed in the same dimensions.

Response to leptin administration · A related parameter that marks the transition to the subject of the next paragraph is the anorexigenic effect of leptin. Peripheral leptin administration acutely reduces food intake in control animals, but not in rats subjected to prenatal or postnatal maternal food restriction or a postnatal maternal low-protein diet [130, 341, 470]. In small-litter rats, central injections of leptin are effective, in contrast to peripheral injections [372]. This suggests that this leptin resistance may be due to impaired leptin transport, rather than an altered hypothalamic response [372].

Hypothalamic regulation

It has been known since quite some time that perinatal malnutrition can have profound effects on brain development [e.g. 427]. Nevertheless, studies investigating programming effects on the hypothalamic peptides that regulate energy balance are relatively scarce (compared to the other two categories of measurements discussed above). Most of these have studied relatively short-term effects.

Nutritional manipulation of the dam · Weanling rats subjected to a maternal low-protein diet during gestation and lactation were shown to have a reduced number of NPY immunoreactive cells in the ARC [492]. This was combined with an increase of the concentration of NPY protein in the PVN and LHA and a tendency for an increased concentration in the ARC, whereas the NPY content of other hypothalamic nuclei was unaltered [496]. This is suggestive of an increased orexigenic drive in these animals, provided that the PVN and LHA are fully responsive to NPY. In view of the slightly hypophagic phenotype of these animals (see above), the responsiveness of these areas (or other regions further downstream) is probably reduced. Rats that were only exposed to a low-protein diet prenatally did not show any changes in ARC gene expression of Ob-Rb, NPY, AgRP, POMC, and CART at weaning [108]. In contrast, weanling pups that were subjected to the diet postnatally had increased expression of Ob-Rb, NPY, and AgRP, and decreased expression of the anorexigenic POMC and CART [108], again suggesting an increased orexigenic drive.

Prenatal maternal food restriction has been shown to drastically increase hypothalamic mRNA levels of Ob-Rb at birth, an effect that reversed by weaning to levels below normal [130]. In adulthood, hypothalamic Ob-Rb expression had normalised [274], but Ob-Ra expression was lower than in control animals [675], which points to a reduction in leptin

transport. The latter is supported by a normal reaction to central injections of leptin, with a reduced reaction to peripheral leptin [675]. Adult hypothalamic expression of the ARC peptide AgRP was reduced, whereas that of NPY and POMC was normal [274]. Despite this, the PVN in these adult animals did receive a larger number of NPY- and CART terminals [675]. This was not reflected in a change in PVN CRH expression [284], although the PVN in juvenile rats did show increased neuronal activity and CRH mRNA levels [477, 478]. When the maternal diet was continued postnatally, juvenile pups showed very low serum levels of leptin [128]. This was accompanied by reduced POMC expression and axons, but surprisingly, hypothalamic NPY expression and its protein levels in the PVN were normal [128]. There does not seem to be a predominant direction in which energy balance regulation is shifted, which is in line with the variation in the general phenotype described above for these animals.

Nutritional manipulation of the offspring · In rats that were prenatally growth restricted by uterine artery ligation, NPY mRNA and protein were both increased at weaning, whereas CRH levels were unaffected [506]. In young adulthood, the number of ARC cells expressing NPY mRNA was normal, but the levels of expression were reduced [265]. This suggests an increased orexigenic drive in the juvenile animals, which is in accordance with the complete catch-up growth reported for these animals (see above). Lower NPY expression in the adult rats is concurrent with the incomplete catch-up growth that these animals display (see above).

Weaning small-litter rats were shown to have reduced Ob-Rb expression [371], which is in agreement with the high serum leptin levels found in these rats (see above). ARC NPY, AgRP, and CART mRNA levels were all increased, but this orexigenic signal did not seem to reach the PVN and LHA as expression of TRH, MCH, and ORX were unaltered [371]. This was also suggested by the fact that NPY peptide levels in both the ARC and the PVN were normal [494]. In young adulthood, expression of the ARC peptides NPY, AgRP, and CART is normal, as well as CRH and TRH expression in the PVN and MCH and ORX expression in the LHA [64, 372]. At this age, leptin transport across the blood-brain barrier appears to be impaired [372], which indicates a state of leptin resistance. This leptin resistance seems to develop only after weaning, since weanling small-litter rats are still responsive to peripheral injections of leptin [537]. Taken together, these studies suggest that the 'obese' phenotype of adult small-litter rats (see above) may be at least partly attributable to central leptin resistance caused by high neonatal leptin levels and the resulting hyperproductivity of the ARC. Additionally, studies by Davidowa and colleagues suggest that in these rats, neurons of several hypothalamic nuclei have an altered response to many of the orexigenic and anorexigenic signals [reviewed in 213, 214].

Interestingly, juvenile large-litter rats also show a shift towards orexigenic signalling. In the ARC, AgRP and NPY expression and NPY peptide levels were shown to be increased, whereas CART expression was unchanged [371, 494]. This resulted in elevated PVN NPY

peptide levels [494], but did not affect its expression of TRH, nor MCH and ORX expression in the LHA [371]. Unlike small-litter rats, juvenile rats raised in large litters had normal hypothalamic Ob-Rb expression [371]. Instead, some of the short forms of Ob-R were expressed at increased levels [371]. One study suggested that ARC NPY mRNA was no longer elevated in young adulthood [265], although there still seemed to be a small tendency towards higher expression levels. These results generally appear to be in agreement with the phenotype described above. The acute effects of juvenile food restriction seem to be mostly orexigenic, although apparently not enough to achieve full catch-up growth. Information on the long-term effects of this model is still largely missing.

Although the studies described here have all used nutritional manipulations, perinatal non-nutritional manipulations have also been shown to programme hypothalamic (an)orexigenic signalling. One example is neonatal stress, which has been shown to have long-term effects on levels of POMC, CRH, ORX, and ORX receptors [166, 316].

The neonatal role of leptin · As has been mentioned in a previous section, leptin is not fully functional in the regulation of energy balance during the neonatal period. Instead, it seems to play a more developmental role. It is responsible for the proper development of intrahypothalamic connections [66, 69] that occurs during the early postnatal period [65]. Even general brain development seems to depend on leptin, as the brains of leptin-deficient mice show a variety of abnormalities that can be rescued by juvenile leptin treatment [3]. In recent years, several researchers have hypothesised that altered neonatal leptin levels may play a key role in developmental programming [68, 109, 257, 376, 407, 467, 511, 628]. This hypothesis is supported by several recent studies that manipulated perinatal leptin levels.

Interestingly, the direction of the reported effects differed between these studies. Some researchers have found a beneficial effect of perinatal leptin administration on adult body adiposity [446, 486]. Moreover, one study reported the absence of an anorexigenic reaction to peripheral leptin in adulthood when leptin action was blocked neonatally [18]. In contrast, others have reported increased fat mass, leptin levels, and/or food intake [124, 368, 605, 632] and leptin resistance [368, 605, 675] in adult rodents subjected to perinatal leptin administration. Similarly, different effects of perinatal leptin on susceptibility to diet-induced obesity were reported. Some studies found perinatal leptin to be protective against diet-induced obesity [18, 582], whereas others reported increased weight gain on a high-fat diet after neonatal leptin injections [632, 675].

Based on some of the positive effects mentioned above, several groups have investigated whether perinatal leptin administration might rescue the obesity-prone phenotype of rats that were programmed by perinatal nutritional manipulations. Their results have been mixed. Rats that were malnourished by a maternal low-protein diet throughout gestation and lactation had lower body weight in adulthood, similar leptin levels, and similar susceptibility to diet-induced obesity as controls [580]. When the low-protein dams were infused with leptin during the perinatal period, weight gain on the high-fat diet

was abolished [580]. The effect of perinatal leptin on control rats was not investigated in this study, which hampers the interpretation of the results. Notably, the body weight of saline-treated low-protein pups appears to reach normal control levels on the high-fat diet. Another group has investigated the effects of neonatal leptin injections on the obese phenotype of rats subjected to prenatal maternal food restriction. These underfed rats have a higher baseline adiposity (at least the males) and both sexes are more susceptible to diet-induced obesity than control rats [631, 632]. When prenatal undernutrition was followed by neonatal leptin injections, baseline adiposity was reduced in males with no effect on diet-induced obesity [632], whereas in females, neonatal leptin reduced the effects of the high-fat diet to that found in controls, without an effect on baseline adiposity [631]. Notably, neonatal leptin injections aggravated diet-induced obesity in control males, but not in control females. A third group attempted to rescue the obese phenotype of weanling rats raised in small litters by using neonatal leptin injections. In female small-litter rats, leptin injections reduced the fat percentage to that of control females raised in normal litters [537]. In male rats, however, the fat percentage at weaning was not altered by leptin injections in small-litter animals, whereas it was significantly reduced by leptin in normal-litter males [536]. So, neonatal leptin rescued the obese phenotype in weanling female small-litter rats, but not in males.

Summarising, perinatal leptin supplementation can have beneficial or detrimental effects on energy balance and body composition in both normal and programmed rats. The outcome is probably determined by the exact timing and levels of leptin, as well as the phenotypic background of the animal. Therefore, we recommend extreme caution when investigating the option of providing infants with supplemental leptin as a proposed obesity-protective agent [581]. An additional concern is the reduction in skeletal growth that was reported in some of the studies, resulting in reduced body length [446, 632], which is usually undesirable in the human situation.

Summary

In this section, we have seen ample evidence for developmental programming of energy balance and its hypothalamic regulation in experimental animals. Studies have investigated outcomes in all three categories of measurements of energy balance (i.e. body size and -composition, energy intake and -expenditure, and hypothalamic neuropeptides). Persistent changes have been found in all of these parameters in various rodent models of perinatal malnutrition. Nevertheless, the precise effects that have been reported differed considerably between the models and also between individual studies. Many of these apparent discrepancies can be explained by (small) differences between studies in the timing, nature, and severity of the manipulation or other subtle variations in their methods, such as the genetic background, sex, and age of the experimental animals. All these possible obscuring factors obligate the researcher to take extreme care when combining results between studies to reach a final conclusion.

Because of its distinct role during development, leptin has been hypothesised to play a major role in programming of energy balance regulation. Evidence for this hypothesis has been published in recent years, in parallel with the execution of our own studies. Of course, leptin cannot be the sole factor responsible for programming. Examples of other proposed candidates are insulin, glucocorticoids, growth hormone, and thyroid hormone [123, 180, 491].

Concluding remarks

In this introductory chapter, we have presented the concept of developmental programming. It explains how changes in the environment during a critical time window in early development can permanently alter the phenotype of an organism. In this manner, animals can be 'fine-tuned' to their expected future environment. Although there has been controversy on this subject, it is now generally believed that energy balance and its regulation can also be programmed. Before reviewing the literature on this topic, an overview of the hypothalamic regulation of energy balance was given.

In the last section, we have discussed a substantial number of studies that have investigated developmental programming of energy balance in different species, using different techniques, and from different angles. From these studies, we can conclude that early nutrition can truly programme energy balance and its regulation in both humans and animals. The direction of the programming effects that were reported appears to be variable and dependent on the environment; both the perinatal and the adult environment. One thing that becomes apparent from the discussed animal studies is that developmental programming of energy balance does not necessarily entail detrimental changes: in some cases the programmed changes were favourable, such as reductions in fat mass. In contrast, mostly adverse effects on adult body composition were reported in humans. This striking disparity, between metabolic effects with different directions in animal models on one hand and consistent detrimental effects in humans on the other, may result from (interaction with) the obesogenic environment that most humans (unlike most experimental animals) encounter in later life.

With the inconsistencies between animal studies, a comprehensive picture of the impact of perinatal nutrition on energy balance in later life has thus far remained elusive. If we intend to extrapolate conclusions between studies, and from animal models to the human situation, it is vital to identify the exact circumstances leading to each outcome and to standardise the variable methodology which researchers have used to investigate this subject. Therefore, in the present thesis we aim to study developmental programming of a range of energy balance-related parameters in a single animal model. This chapter has provided us with the necessary knowledge to further investigate developmental programming of energy balance.

Objectives, design, and outline of the thesis

Outline (Floor Remmers)

2 | OBJECTIVES, DESIGN, AND OUTLINE OF THE THESIS

In this chapter, the research aims are defined, followed by an overview of the design and techniques we used to answer these questions. The chapter concludes with a short description of the topics covered in each chapter.

Research objectives

We have investigated energy balance and its hypothalamic regulation after early postnatal food restriction in the rat. As was described in the previous chapter, the perinatal nutritional environment can permanently alter adult metabolic function. Additionally, in rodents an important part of development of the hypothalamus has been shown to occur in the neonatal period. Based on the literature, we hypothesise that in rats, early postnatal food restriction may permanently alter energy balance and its regulation by the hypothalamus. To test this hypothesis, we studied both acute and long-term effects of the experimental manipulation of early nutritional status. In this way, we aimed to determine whether developmental programming of energy balance does take place and to identify early changes that might play a causal role in energy balance programming.

To characterise the effects on energy balance, the three types of parameters that were discussed in the previous chapter were measured at several different moments in life: body composition, energy balance, and hypothalamic regulation. Body weight, body length, body mass index (BMI), and serum leptin levels were used to describe the general phenotype of the experimental rats and to approximate body composition. Measurements of food intake and resting energy expenditure provided direct information on energy balance. In order to investigate energy balance regulation, gene expression of hypothalamic neuropeptides was determined, providing an indication of the production rate of these peptides. In this manner, we obtained a comprehensive overview of the effects of early postnatal food restriction on energy balance and its regulation.

Design and techniques

Experimental animal model

In order to investigate developmental programming of energy balance we have chosen to use an animal model of early postnatal food restriction: raising rats in large litters.

Model choice

There are a number of reasons why rats are preferred over humans as the study object. The causes of perinatal undernutrition in humans vary significantly and are often unknown. Human life is further characterised by ample individual variation in early and later

environment. These different types of variation all act as confounding factors in the analysis of the effect under study: that of perinatal undernutrition. The use of an animal model provides control over and standardisation in the induction of perinatal undernutrition as well as the subjects' genetic background and environmental conditions. In small mammals, the energy balance-regulating system bears a good similarity to that of humans. In addition, their lifespan is considerably shorter than that of humans, making long-term studies more feasible. The choice for the rat as our model organism (rather than mouse, guinea pig, or hamster for example) was prompted by its frequent use in earlier programming studies. This allows for some careful extrapolation of results between studies.

In the rat, we decided to perform the manipulation of nutrition postnatally. The rationale for this was the timing of hypothalamic development: although the basic hypothalamic anatomy is already established at birth [395], much of its connectivity and functionality arise during the lactation period [65, 213, 214, and see Chapter 1]. This makes the early postnatal period a good candidate to be a critical window for programming of the hypothalamic regulation of energy balance. As we have seen in the previous chapter, manipulation of litter size is a reliable model to manipulate neonatal nutrition and to programme adult body size in rats. These manipulations have produced a more consistent phenotype than manipulations through the maternal diet (see Chapter 1) and therefore seem to be a more robust model. In order to model perinatal malnutrition, we have used the large-litter model.

Because we studied all parameters in animals subjected to a single model in one large experiment, all animals encountered very similar circumstances. Therefore, the obtained results can truly be combined to reach a conclusion about developmental programming of energy balance and its regulation in this model in the rat.

Early postnatal food restriction by raising rats in large litters

Early postnatal food restriction was induced by enlarging litter size. After a normal gestation, litters were adjusted the day after birth to either 10 or 20 pups for control and food-restricted (FR) litters, respectively. In the large FR litters, less milk is available per pup than in control litters, which results in undernutrition and reduced growth [175]. After weaning on day 25, all animals received *ad libitum* food.

This manipulation of neonatal nutrition is believed to programme the adult phenotype because, in contrast to food restriction in later life, it results in persistently lower body weight [262, 650]. In later life, food restriction only induces reversible growth restriction with complete catch-up [262, 650]. Our model of early postnatal food restriction is essentially a model of hyperparity [374]: a situation in which there are more pups than available nipples (of which the rat has twelve). In this situation, most dams will divide the litter into sublitters and nurse them alternately [374]. 'Side effects' of raising rats in large litters may be that the dam spends less time with the litter [210], with less maternal care per individual pup [134], but this does not result in a reduction in the number of milk ejections [524].

Previous studies in our laboratory using this model already showed a persistent reduction in body weight [150, 263] and reduced adiposity [150, 152]. In addition, several changes were found that point to altered hypothalamic function: impaired reproductive function [149, 151, 622] and changes in the growth hormone axis [258, 264, 266]. These latter findings suggest that another hypothalamic function, regulation of energy balance, may also be programmed by early postnatal food restriction.

Methodological considerations

In order to study the differences between the two groups, we have to ensure that the group difference is the only difference between them. Several steps were taken to avoid additional differences. Neonatal male and female pups were assigned to either a control litter or an FR litter by computer-generated random numbers. Because this randomly distributed the pups of a biological litter between foster dams, any genetic- and prenatal environmental differences between the animals were distributed equally. Additionally, by distributing males and females separately, we ensured that all experimental litters had a sex ratio of 1:1. At weaning, all pups were socially housed with other animals from their own experimental group. This was done to create a similar environment for all rats. Animals were killed at four different ages. At the first time-point, which was before weaning, a number of FR and control litters were randomly chosen for sacrifice. At the end of the lactation period, the remaining animals were allocated to one of three subsets for sacrifice at the other three time-points, according to their group, sex, foster litter, and body weight. In this manner, these characteristics were equally distributed between the different age groups. Finally, all animals were killed in the first half of the light phase of the day (FR rats and controls were alternated) and females were killed on the day of pro-oestrus. This was done to avoid variation of the circadian rhythm and of the oestrous cycle interfering with the FR effect. Hypothalamic gene expression of the neuropeptides of interest discussed in the previous chapter has been shown to vary considerably with the time of day [56, 104, 380, 584, 593, 641, 669]. Overall, the first half of the light phase appears to be the most stable moment of the day. In addition, serum leptin levels peak at night and are relatively stable in the first half of the light phase [59, 401, 641, 669]. Any remaining circadian variation in these peptides was equally distributed between the groups because FR and control rats were killed in an alternating manner. In females, gene expression of some of the relevant neuropeptides, as well as serum leptin levels were shown to vary with the phase of the oestrous cycle. For most peptides, the morning of pro-oestrus is relatively stable, followed by an increase in the (late) afternoon [35, 60, 61, 388, 475, 555, 613].

Regular measurements

In order to monitor the phenotype of the animals, some easily measurable parameters were taken on a regular basis. These included body weight, body length, tail length, total length, BMI, and food intake. Weight and length together determine the size of the animals,

which allows their growth to be assessed. In the literature, body weight is often given as an overall indication of the size of rats. On the other hand, body length and tail length give an indication of skeletal growth. Further, BMI is calculated as the ratio of body weight to body length squared. This describes body weight relative to length and is often used as an estimate of obesity. However, it is important to keep in mind that a larger BMI not only points to more fat mass, but at the same time can also be indicative of more fat-free mass [185, 343]. BMI has recently been validated as a proxy for obesity in the rat [448].

Daily food intake was used to calculate energy intake (conversion from grams to kilojoules). When interpreting energy intake data, it is important to consider the body size and body composition of the subject. Energy use is body-size dependent: large animals have higher energy requirements than small animals. However, large animals are more energy-efficient than small animals and therefore have lower requirements per kilogram body weight [e.g. 538, 648]. This is why correction for differences in body size by simply dividing energy intake (or -expenditure) by body weight or another proxy produces misleading results: this method systematically underestimates energy utilisation by larger individuals. Instead, it is recommended to use analysis of covariance to adjust energy-related data for body composition [17, 497, 606]. The metabolic activity of adipose tissue is relatively low, and therefore energy use is best predicted by fat-free mass [441, 497, 606]. In this thesis, energy intake (and -expenditure, see next section) data are adjusted using analysis of covariance. Preferably, BMI or an estimate of fat-free mass was used as a proxy for body composition; if these were not available, body weight was used. In addition, the raw data (per animal) are always reported.

Indirect calorimetry

Indirect calorimetry uses respiratory gas exchange measurements to calculate energy expenditure. This method is based on the fact that living organisms use oxidation to obtain energy from their body stores (e.g. glucose and lipids stored in glycogen and adipose tissue). In oxidation, the body uses oxygen to transform the (stored) nutrients' carbon-hydrogen bonds to energy, carbon dioxide, and water. Therefore, energy expenditure (or production) can be calculated from oxygen uptake and carbon dioxide release [e.g. 154, 168, 643]. A metabolic monitor is designed to frequently sample oxygen and carbon dioxide levels in passing air. When connected to a cage fitted with a perspex top (instead of the normal wire top), it allows for accurate measurements with minimal disturbance of the rats.

In this thesis, we have measured resting energy expenditure during the light phase of the day, when rats are naturally inactive. To remove the highly variable component of activity-related energy expenditure, bouts of activity (identified by elevated rates of oxygen consumption and carbon dioxide production) were excluded: only stretches of stable measurements were used for calculations, see **Figure 1**. As with energy intake, it is important to consider body size and -composition when interpreting data on energy

expenditure. Therefore, results were expressed both per animal and after adjustment for a proxy of body composition.
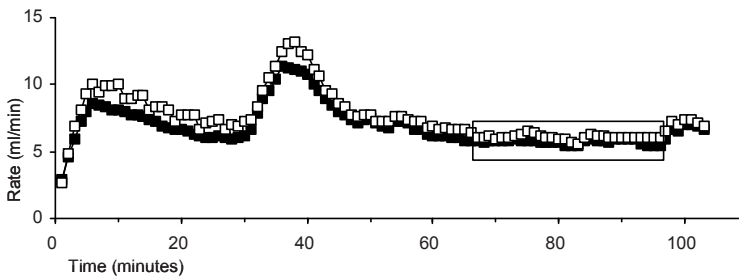

Figure 1. A representative example of an indirect calorimetry measurement. Resting energy expenditure is calculated from oxygen consumption rate (□) and carbon dioxide production rate (■) when both are stable for at least 20 minutes (boxed area).

Radioimmunoassay

The radioimmunoassay (RIA) is a method that is used to quantify the amount of substances (e.g. hormones) in body fluids or tissue extracts. It is based on the principle of competitive binding [reviewed in 44]. In this thesis, a RIA kit was used to determine leptin peptide concentrations. This kit uses ^{125}I-labelled rat leptin to compete with leptin in the serum sample for binding with a leptin antibody, so that the sample's leptin concentration can be derived from the amount of radioactivity bound to the antibody [see 346]. RIAs described in this thesis were kindly performed by the Endocrine Laboratory of the department of Clinical Chemistry at the VU University Medical Center.

Quantitative polymerase chain reaction

Real-time, or quantitative, reverse transcription polymerase chain reaction (qRT-PCR) can be used to quantify the amount of a specific mRNA in a tissue sample. It is based on 'normal' PCR, which uses DNA polymerase, a natural enzyme, to exponentially amplify a specific DNA fragment [e.g. 620]. For quantitative analysis of a PCR, a fluorescent dye is used to monitor the amplification process [e.g. 82, 655]. This dye has a high affinity for double-stranded DNA and produces a fluorescent signal only when bound. By recording fluorescence levels at the end of each cycle, template amplification can be monitored in real time during the PCR. To use mRNA as the template sequence, the PCR is preceded by reverse transcription, which synthesises a strand of complementary DNA (or cDNA) from single-stranded mRNA.

This technique has been used before to assess changes in hypothalamic neuropeptide expression in a status of negative energy balance [126]. In the present thesis, gene expression of hypothalamic neuropeptides was investigated using qRT-PCR on mRNA isolated from punches that were microdissected from the arcuate nucleus (ARC),

paraventricular nucleus (PVN), and lateral hypothalamic area (LHA), see **Figure 2**. The department of Neuroscience and Pharmacology of the Rudolf Magnus Institute of Neuroscience at the University Medical Center Utrecht generously made their facilities available for this study.
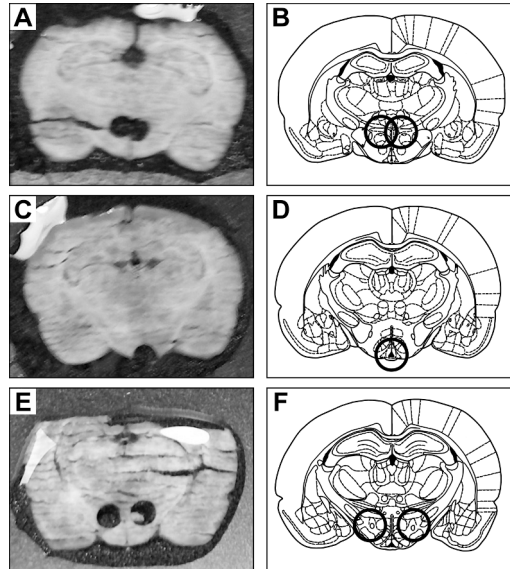

Figure 2. Microdissection on rat brain slices. Representative sections with the PVN (**A**), ARC (**C**), and LHA (**E**) punched out and the corresponding diagrams (**B**, bregma −2.12 mm; **D**, bregma −2.56 mm; **F**, bregma −3.14 mm) from the rat brain atlas [472], adapted with permission.

In situ hybridisation histochemistry

This technique visualises mRNA molecules in the tissue where they are expressed. Once the mRNA is visible under a microscope, its image can be captured and subsequently analysed for quantification. The principle behind *in situ* hybridisation is the formation of hybrids between a specific mRNA sequence in the investigated tissue and a manufactured RNA strand that contains a visible label and is complementary to the original sequence [e.g. 656]. In the study described in this thesis, the probe was labelled with fluorescein. This dye emits green light of around 520 nm when illuminated by light with a wavelength of 495 nm. Photomicrographs captured by a camera attached to a fluorescence microscope can be analysed using imaging software.

In this thesis, *in situ* hybridisations were performed for neuropeptide Y and pro-opiomelanocortin, both expressed in the hypothalamic ARC, see **Figure 3**. The department of Anatomy and Neurosciences at the VU University Medical Center generously made their facilities available for this study.
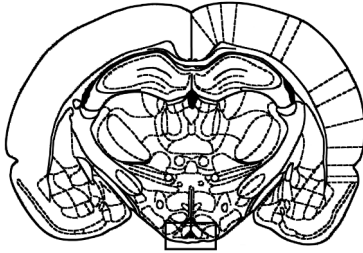
Figure 3. Representation of a rat brain slice with the area of interest (boxed area). Adapted from the brain atlas [472], with permission.

Thesis outline

This thesis reports studies on the short- and long-term effects of early postnatal food restriction on energy balance in the rat, and its hypothalamic regulation. To investigate this, rats were raised in large litters and their development and outcomes were compared to those of rats raised in normal litters.

In **Chapter 1**, we introduced the concept of developmental programming, explained what is known about hypothalamic energy balance regulation, and reviewed the existing literature on developmental programming of energy balance and its regulation. The present chapter has introduced the objectives of the studies described in this thesis, and the experimental methods with which we aim to reach these objectives. The experimental part of the thesis starts in **Chapter 3** with a general description of the phenotype of the animals utilised in this thesis, as a basis for the subsequent studies. The study uses BMI and serum leptin levels to estimate effects of neonatal food restriction on body composition. In **Chapter 4**, energy intake and energy expenditure of adult male rats are determined; the latter via indirect calorimetry. This study examines long-term effects of early postnatal food restriction on energy balance itself. **Chapter 5** investigates both acute and long-term effects of early postnatal food restriction on the regulation of energy balance; gene expression of several relevant hypothalamic peptides is measured using quantitative PCR. In **Chapter 6**, *in situ* hybridisation is used to determine gene expression of two major regulating peptides in the hypothalamic ARC of young adult males. Finally, **Chapter 7** discusses the results and methodology of the preceding chapters. There, the outcomes of the separate studies in this thesis are united and regarded in the light of existing literature and the hypothesis and questions brought forward in the present chapter. The chapter concludes with suggestions for future experiments to further elucidate energy balance programming.

Neonatal food restriction permanently alters rat body dimensions and energy intake

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Mariann Fodor †
Henriette A. Delemarre-van de Waal

Growth pattern (Floor Remmers)

3 | NEONATAL FOOD RESTRICTION PERMANENTLY ALTERS RAT BODY DIMENSIONS AND ENERGY INTAKE

Neonatal food restriction in rats, by means of increased litter size, has been used as a model for developmental programming by several investigators. However, the results reported have been inconsistent and difficult to compare between studies. In the present study, we aim to characterize the effects of this model throughout life in both sexes of one particular strain. On the second day of life, Wistar rat pups were randomly assigned to a litter of 10 (control) or 20 (food-restricted, FR). All litters had an equal number of males and females, and pups were weaned on day 25. Body dimensions and food intake were measured regularly until the age of one year. Serum leptin levels were determined in four subsets of different ages. Food restriction acutely reduced growth in all body dimensions and serum leptin levels. Despite catch-up after weaning, all these parameters remained reduced throughout life. Male and female FR rats had a significantly reduced absolute energy intake throughout life. Male FR rats had significantly higher energy intake adjusted for body weight immediately after weaning. During catch-up growth, both FR males and females showed significantly enhanced feed efficiency. These results suggest that neonatal food restriction programmed both male and female Wistar rats to remain small and lean in adult life, with a lower food intake. Low neonatal leptin levels may play a mechanistic role in this process.

Introduction

The finding that in humans a low birth weight is associated with an increased risk of developing hypertension, cardiovascular disease, high adiposity, and diabetes in adult life, has led to the concept of 'developmental programming' or 'developmental origins of health and disease' [203, 227]. This concept implies that differences in the environment during the plastic period of development can permanently alter organ function in later life [227]. It is proposed that the perinatal level of nutrition can lead to adaptations of the organism to optimise survival in an environment with similar food availability. Only when there is a mismatch between the perinatal environment (and hence the expected later environment) on the one hand and the actual later environment on the other hand, developmental programming has these adverse effects in adult life [203].

Different animal species including primates, sheep, guinea pigs, and rats have been employed to study developmental programming, using both prenatal and postnatal manipulations of nutrition, including ligation of the uterine arteries, maternal protein- or global undernutrition, and manipulations of litter size [47, 408, 465]. These different approaches have yielded variable outcomes. Using rats raised in large litters, we and others have previously reported on the effects of neonatal food restriction. Even restricted to this one model, conflicting outcomes have been reported. After neonatal food restriction, body size was either permanently reduced [34, 150, 233, 234, 238, 262, 263, 345, 371, 433, 462, 649, 650, 659] or normalized to control levels [345, 371, 658, 659]. Adiposity was reported to be reduced [152, 175, 371, 649] or unchanged [345] in juvenile life, whereas in adulthood

adiposity either normalized [150, 345] or stayed reduced in large-litter rats [150, 233, 234, 238, 371, 649]. Energy intake of neonatally food-restricted (FR) rats was found to be either reduced [34, 462] or unchanged [345]. Serum levels of leptin, a hormone that is secreted by adipocytes in proportion to the body fat content [389] and regulates food intake and energy expenditure via the hypothalamus [544], until now have only been examined in relatively young large-litter animals [152, 371, 494].

In the present study, we aim to characterize the effects of neonatal food restriction by the large-litter model throughout life in both sexes. Body dimensions, food intake, and serum leptin levels were measured at different ages and the relationship between these parameters was investigated.

Methods

Experimental animals

Primiparous timed-pregnant Wistar rats (Harlan, Horst, the Netherlands) arrived on day 14 or 15 of gestation and were housed individually under controlled lighting (12h light, 12h dark) and temperature (21.5 ± 0.5 °C). Animals had unlimited access to standard rat chow (Ssniff R/M-H; Bio Services, Uden, the Netherlands; 12.8 MJ/kg metabolisable energy) and tap water at all times. Pups were born spontaneously on day 22 or 23 of gestation. The first morning after birth was designated as postnatal day 1. On day 2, male and female pups were allocated to either a control litter with 10 pups or an FR litter with 20 pups using computer-generated random numbers. In FR litters, less milk is available per pup than in control litters, resulting in undernutrition [175]. Male-to-female ratio was 1:1 in all litters. On day 25, the pups were weaned and socially housed; two (males) or four (females) animals of the same experimental group per cage. Note that rat pups start taking solid food from day 16 [20], and therefore the period of food restriction did not necessarily last until weaning. At three time-points in the study, subsets of animals were sacrificed for further study (75 on day 10, 64 on day 25, and 64 around day 77). Therefore, the number of animals used in the present study declines with progressing age, see **Table 1**. All procedures were approved by the Animal Experimentation Ethics Committee of the Vrije Universiteit and the VU University Medical Center in Amsterdam, the Netherlands.

Body dimensions

Body weight was measured regularly throughout life. Body- and tail length were measured regularly from day 2 until day 70 in manually restrained animals from the tip of the nose to the anus, and from the anus to the tip of the tail, respectively. At the age of 1 year, body and tail length were measured under pentobarbital or O_2/CO_2 anaesthesia before sacrifice. Total length was calculated as the sum of body length and tail length. Body mass index (BMI), a simple, non-invasive way to approximate body composition in rats [448], was calculated as the ratio of body weight (g) to body length (cm) squared.

Table 1. The numbers of animals (or, in the case of energy intake: numbers of cages) used in the different parts of the study

		males		females		all
		control	FR	control	FR	animals
body dimensions	days 2–10	67	73	69	68	277
	days 15–21	48	54	49	51	202
	days 28–70	32	38	33	35	138
	days 84–380	16	22	17	19	74
energy intake	days 28–70	16	18	8	8	50
	days 84–350	8	8	4	4	24
serum leptin	day 10	10	10	10	8	38
	day 25	8	8	8	8	32
	day 77	8	8	8	8	32
	day 380	9	12	8	10	39

Food intake

Food intake of the lactating dams was determined daily from day 5 until day 16. In this way, disturbance of the newborn litter [315] and measuring chow intake from the pups after day 16 [20] were avoided. After weaning, food intake per cage was measured weekly until day 70 and thereafter every other week until day 350.

The food provided during the measurement was weighed both at the beginning and at the end of a 24-hour period. Mean daily energy intake was calculated from daily food intake per cage, the energy density of the diet provided (12.8 MJ/kg), and the number of animals per cage (i.e. one for the lactating dams, two for males, four for females). Daily energy intake was linearly interpolated to days without data-points. Total energy intake was then computed as the sum of daily energy intake for all days from the first measurement after weaning (day 28) until the last food intake measurement (day 350). Feed efficiency, or the amount of weight gained per MJ of energy intake, was calculated from the weight gain and the total energy intake during a period.

Serum collection and leptin radioimmunoassay

Serum leptin levels were measured in a subset of the animals that were sacrificed at the ages of 10 and 25 days (suckling and weanling rats), and around 77 and 380 days (young adult and middle-aged rats; 38, 32, 32, and 39 rats respectively, see **Table 1** for numbers per group). To avoid effects of the oestrous cycle on serum leptin concentrations [388], adult females (day 77 and 380) were sacrificed on the day of pro-oestrus. To that end, their cycle was monitored during the last 2 weeks before sacrifice by daily vaginal smears which were stained with Giemsa stain. At each time-point, all animals were sacrificed between the first 1.5 and 4.5 hours of the light phase to avoid circadian variations in leptin levels [401]. Rats were euthanised by CO_2 inhalation, followed by decapitation. Since CO_2 inhalation is not indicated until after day 10 [443], at this age animals were decapitated without prior CO_2 inhalation. Upon decapitation, trunk blood was collected, stored at 4 °C for a maximum of 4 hours, and then centrifuged at 3000 rpm for 15 minutes. Serum was stored at −80 °C until

leptin levels were determined using a rat leptin radioimmunoassay (Linco Research, St. Charles, MO, USA). A sample volume of 50 µl was tested in duplicate. Within each age group, all samples were run in the same assay. The coefficients of intra- and inter-assay variation were both 5% and the detection limit was 0.5 ng/ml. Values below this limit were substituted by the value of 0.4 ng/ml.

Data analysis

The results were analysed using SPSS for Windows, version 12. Differences were considered significant at $P<0.05$. All data were checked for normality and are expressed either as mean ± SD or, in the case of adjusted means, mean ± SEM. The number of animals in each analysis is shown in **Table 1**.

One-way ANOVA with group as independent variable was used to investigate litter size, energy intake data of the dams, and energy intake and feed efficiency of the male and female pups. Repeated measures ANOVA was used to analyse energy intake of the dams and body dimensions of the pups (body weight, body-, tail-, and total length, and BMI). Body dimensions, growth rates, and leptin levels of the pups were then analysed using a multilevel approach [610]. The mixed model procedure was used with the foster dams as subjects. Group and sex of the pups, plus their interaction only if this was significant, were covariates in this analysis. In case of a significant interaction, the group effect was assessed for males and females separately. In addition, serum leptin levels and energy intake of the pups were tested with body weight or BMI as an extra covariate, in order to analyse the parameters independently of these covariates. Correlations were determined using Pearson's bivariate correlation analysis.

To avoid any unwanted effects of the dams, the redistribution of the pups over dams nurturing control and FR litters (hereafter: control and FR dams) on the second day of life was done randomly. Possible interfering interactions of the biological dams with the group effect were tested in univariate ANOVA. Furthermore, a multilevel approach was used to test and adjust for possible effects of the foster dams on the pups' body dimensions and serum leptin levels.

Results

Details of delivery and redistribution

In order to have 14 control litters and 8 FR litters as planned, 33 dams were used. The dams gave birth to 379 pups, with an average litter size at birth of 11.5 pups. After the redistribution of the pups on day 2, 14 control dams and 8 FR dams raised 140 and 160 pups, respectively. Of these 300 pups, 19 FR and 4 control animals died early (mostly before weaning), leaving 277 animals (67 control males, 73 FR males, 69 control females, and 68 FR females) in the study, see **Table 1**. On day 10, the number of pups in a control litter was 9 or 10, whereas that of FR litters ranged between 18 and 20. On day 25, all control litters had 10

pups, and FR litters had 17 to 19 pups. The original litter size of the foster dams was similar between control dams (11.8 ± 1.6) and FR dams (12.3 ± 2.1; *P*>0.550, see the **supplemental Tables** for df and *F*-values of all analyses) and also between control pups (12.2 ± 2.4) and FR pups (11.8 ± 2.4; *P*=0.190). Of the 277 pups in the study, 86% were cross-fostered, whereas 14% (18 controls and 21 FR pups) remained with the same dam after the random redistribution on day 2.

Maternal energy intake during lactation

Daily energy intake of all the dams increased by over 100% between day 5 (478 ± 92 kJ/day) and day 16 (977 ± 70 kJ/day). Mean total energy intake of lactating dams was determined for 10 control and 6 FR dams. Fostering the increased number of pups did not increase total energy intake in FR dams (9.9 ± 0.7 MJ) above that of control dams (9.2 ± 0.9 MJ; *P*=0.125). In a repeated measures ANOVA for day 5 until 16, mean daily intake of FR dams (821 ± 27 kJ; mean ± SEM) also did not differ significantly from that of control dams (767 ± 21 kJ; *P*=0.140).

Body dimensions of FR rats

In repeated measures ANOVA, body weight, body length, tail length, total length, and BMI were lower in FR animals than in controls for days 4–10, days 4–21, days 4–70, and days 4–380 (*P*<0.001 in all tests). Body dimensions of the pups throughout the experiment were investigated using multilevel analyses. Body weight throughout the experiment is shown in **Figure 1A**.

On day 2, before the groups were assigned, there were no significant differences between FR and control rats for body weight, body length, tail length, total length, and BMI (*P*=0.549–0.922). From day 4 until the end of the experiment around day 380, body weight, body length, and BMI were significantly smaller in FR animals than in controls (*P*<0.001–0.047), except BMI in females on days 42 and 56. For tail length and total length, the difference between the groups became significant from day 10 and 7, respectively (*P*<0.001–0.012).

The body dimensions of FR rats relative to those of control rats are shown in **Table 2**. In FR rats, all relative body dimensions decreased during lactation, followed by a rapid but incomplete catch-up growth after weaning, when all animals had unlimited access to food. This pattern is shown in **Figure 1B** for relative body weight.
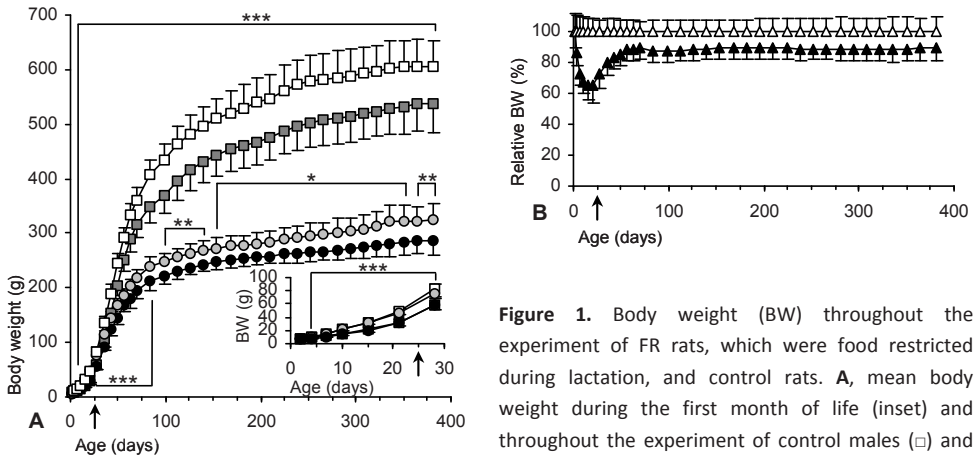
Figure 1. Body weight (BW) throughout the experiment of FR rats, which were food restricted during lactation, and control rats. **A**, mean body weight during the first month of life (inset) and throughout the experiment of control males (□) and females (●) and FR male (■) and female (●) rats. **B**, body weight expressed as a percentage of control (△) body weight for FR animals (▲). Values are mean ± SD. Weaning is indicated by an arrow. * $P<0.05$, ** $P<0.01$,*** $P<0.001$ for FR *vs.* control for both sexes (inset), males (upper), or females (lower).

Table 2. Characteristics of FR growth relative to control growth: minimal and final percentages of body weight, body-, tail-, and total length, and BMI of FR animals relative to those of control animals and periods when growth velocity was significantly lower and higher in FR animals than in controls

	FR relative to controls			growth velocity of FR rats	
	lowest %	on day	final %	lower between days	higher between days
BW	64.5	21	88.7	2–15 ($P<0.001–0.021$[a])	21–56 ($P\leq0.001–0.010$)
BL	86.2	21	96.5	2–7 ($P\leq0.001$)	21–49 ($P=0.006–0.045$[b])
tailL	84.9	21	97.1	7–10 ($P=0.026$)	28–49 ($P=0.002–0.036$)
totL	85.7	21	96.8	2–10 ($P<0.001–0.019$)	21–49 ($P<0.001–0.028$)
BMI	84.1	15	95.4	2–4 ($P<0.001$)	21–28 ($P<0.001$)

BW, body weight; BL, body length; tailL, tail length; totL, total length.
[a] $P=0.052$ for males between days 7 and 10; [b] $P=0.064$ between days 28 and 35.

Growth velocity was expressed as the percentage of gain per day, rather than the absolute gain per day, to adjust for the smaller body size of FR animals. Further, growth velocity was always computed over a period of time and is displayed in **Figure 2** (body weight, only for females) at the end of that period, i.e. the mean growth rate for days 2–4 is displayed at day 4. Patterns of growth velocity are shown in **Table 2**. Growth rates of all body dimensions were significantly lower in FR rats for some time during the lactation period, and elevated for some time after weaning. The different body dimensions showed different patterns: BMI had the shortest period of decreased and increased growth rate, whereas body weight showed the longest periods of altered growth velocity.

In summary, neonatal food restriction acutely reduced gain in all body dimensions during the lactation period, so that weanling FR animals weighed only 64% of controls. After weaning, FR rats had an increased growth rate and showed incomplete catch-up growth.
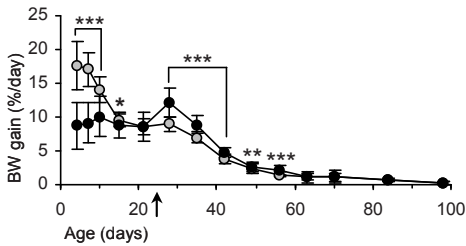
Figure 2. Body weight (BW) gain per day expressed as a percentage of body weight in female control (⬤) and FR (●) animals. Growth velocity patterns in male control and FR rats were similar to those in females and are not shown. Values are mean ± SD. Weaning is indicated by an arrow. * *P*<0.05, ** *P*<0.01,*** *P*<0.001.

Sex differences in effect on body dimensions

In the multilevel analyses, a significant interaction between group and sex indicates differential effects of neonatal food restriction in the sexes. A significant interaction was found for body weight from weaning until the age of about ten months (data not shown, see the **supplemental Tables** for *P*-values). At these ages, the group effects found in males were stronger than those found in females. Before and after this period, effects were similar for both sexes. For body-, tail-, and total length, significant interactions were found only around weaning. Stronger group effects were again found in males. On days 42 and 56, BMI showed a significant interaction for group and sex, with females showing no effect of group on BMI at these ages. Note however, that early postnatal food restriction did affect BMI in females before, between, and after these time-points. Growth rates of the body dimensions showed very few significant interactions between group and sex. One instance was the body-weight growth rate for days 7–10, which was significantly reduced in FR females (*P*<0.001), but just failed to reach significance in males (*P*=0.052).

As a by-product of the analysis to investigate differences in body dimensions between FR and control rats, some sex differences in these parameters were found. Body weight and length were higher in males during most of the lactation period, and the difference grew larger after weaning. During the lactation period, females had a higher BMI, but afterwards BMI became higher in males. Growth rates also differed between the sexes, especially after weaning. Males grew significantly faster than females in body weight from weaning until about 8 months. Body length grew faster in males from weaning until 9 weeks, whereas BMI increased faster in males from 7 weeks until the end of the experiment.

Energy intake and feed efficiency of FR rats

Energy intake was determined per cage, with animals raised by different dams housed in each cage. Therefore, the multilevel approach (which has the foster dam as subject) could not be applied, and ANOVA was used for analysis. Because the number of cages in which energy intake was measured differed largely between males and females, food intake was analysed separately for both sexes. In both male and female FR rats, total energy intake between day 28 and 350 was lower (*P*=0.001 for both sexes), see **Figure 3A**. In male FR rats, mean daily energy intake was reduced at all ages except on days 294 and 308 (*P*<0.001 for days 28–49, 98, 224; *P*<0.010 for days 56–84, 154–182, 210, 238; *P*<0.050 for days 112–140,

196, 252–280, 322–350), see **Figure 3B**. On days 294 and 308, FR males tended to eat less than control males ($P \leq 0.100$). In FR females, mean daily energy intake was lower on days 28, 56 ($P<0.005$), 63, 182, 210, 238, 280 ($P<0.050$), 322, and 336 ($P<0.010$) and tended to be lower on days 35, 42, 49, 70, 196, 224, and 252 ($P<0.100$).
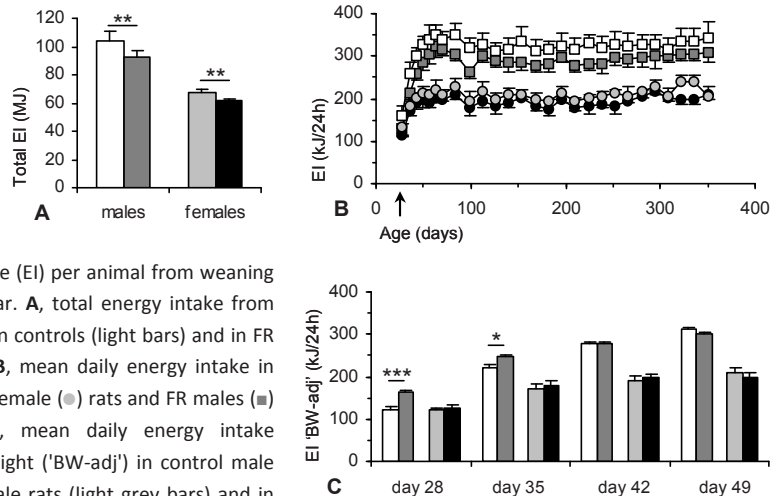

Figure 3. Energy intake (EI) per animal from weaning until the age of a year. **A**, total energy intake from day 28 until day 350 in controls (light bars) and in FR animals (dark bars). **B**, mean daily energy intake in control male (□) and female (●) rats and FR males (■) and females (●). **C**, mean daily energy intake adjusted for body weight ('BW-adj') in control male (white bars) and female rats (light grey bars) and in FR males (dark grey bars) and females (black bars) in the first four weeks after weaning. Values are mean ± SD (A and B) or mean + SEM (C). In B, weaning is indicated by an arrow. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ in A and C (for P-values in B, please refer to the text).

Energy intake was also analysed adjusted for body weight and BMI, when these parameters reached significance as a covariate. After adjustment for body weight (i.e. including body weight as a covariate), FR males consumed more energy than control males on day 28 ($P<0.001$) and day 35 ($P=0.029$), see **Figure 3C**, but not on days 42–210, 238–294, and 322–350 ($P=0.096–0.993$). On day 224, FR males consumed less energy after adjustment for body weight ($P=0.007$). Energy intake adjusted for body weight was similar in FR and control females ($P>0.245$ on days 28, 63, and 70). In FR males, energy intake adjusted for BMI was significantly lower on day 35 ($P=0.001$; 219 ± 5.9 kJ/day *vs.* 252 ± 6.3 kJ/day in controls). In females, intake adjusted for BMI was similar in FR and control animals ($P>0.150$ on days 63 and 70).

The efficiency with which the animals converted energy from food into body mass was computed from body weight and energy intake, and therefore was analysed separately for males and females in univariate ANOVA. FR rats showed increased feed efficiency from weaning until young adulthood, with a prolonged effect in females, see **Figure 4**.

In summary, early postnatal food restriction reduced absolute food intake throughout life, but food intake irrespective of body size was only altered in the immediate postweaning period. Between weaning and young adulthood, FR rats showed elevated feed efficiency.
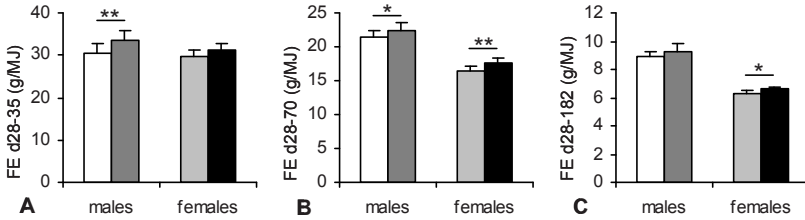
Figure 4. Feed efficiency (FE) of control (light bars) and FR (dark bars) animals from day (d) 28 until day 35 (**A**), until day 70 (**B**), and until day 182 (**C**). Values are mean + SD. * *P*<0.05, ** *P*<0.01.

Serum leptin levels of FR rats

Serum leptin levels were determined at four different ages. The results were analysed separately for each age. From the 141 animals in the leptin radioimmunoassays, one weanling (day 25) FR male was excluded because of methodological problems. On day 10, 10 (6 males and 4 females) of the 18 FR rats had leptin levels below 0.5 ng/ml.

There was no significant interaction between group and sex for leptin levels. In FR rats, serum leptin levels were significantly decreased at 10, 25 and around 380 days (*P*=0.019, 0.022, and 0.044, respectively), and showed a strong trend towards decrease around 77 days (*P*=0.052) in multilevel analyses. In univariate ANOVA (which was permitted, because there was no significant influence of the foster dams in the multilevel analyses), the group effect was significant at all ages, see **Figure 5**. Relative to controls, leptin levels were 20% on day 10, 62% on day 25, 78% around day 77, and 80% around day 380. Leptin was positively associated with body weight and BMI at all ages, although on day 25 the correlation with BMI failed to reach significance, see **Table 3**.
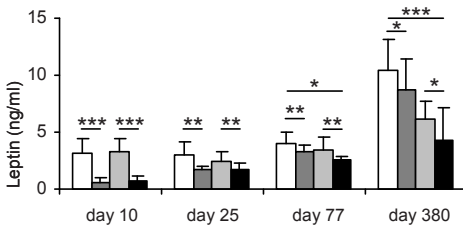

Figure 5. Serum leptin levels in suckling, weanling, young adult, and middle-aged control male (white bars) and female (light grey bars) rats and FR males (dark grey bars) and females (black bars). Values are mean + SD. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 in univariate ANOVA.

Table 3. Correlations of serum leptin levels with body weight and BMI

	leptin & BW	leptin & BMI
day 10	R=0.804, *P*<0.001	R=0.539, *P*<0.001
day 25	R=0.460, *P*=0.009	R=0.331, *P*=0.074
day 77 (sex)	R=0.566, *P*=0.001	R=0.358, *P*=0.048
day 380 (sex)	R=0.651, *P*<0.001	R=0.741, *P*<0.001

BW, body weight. Around day 77 and 380 correlations were adjusted for sex using partial correlations.

As extra covariates, body weight (*P*=0.032 on day 77, *P*<0.001 on day 380) and BMI (*P*<0.001 on day 380) had a significant effect on serum leptin levels. After adjustment for

these body dimensions, leptin levels were similar for FR and control animals (P=0.169–0.926).

In adult rats (days 77 and 380), leptin levels were significantly higher in males than in females. Around day 380, serum leptin adjusted for body weight or BMI was higher in females than in males (P=0.042–0.046).

In summary, leptin levels were extremely low in suckling FR rats. Although catch-up was already apparent prior to weaning, similar levels to control rats were never attained.

Interfering effects of biological and foster dams

In univariate ANOVA, biological dam only had a significant interaction with the group effect on day 10 for body weight in males (P=0.048), on day 10 and 15 for tail length (P=0.036 and P=0.014, respectively), and on day 15 for total length (P=0.016).

The effect of foster dam was determined in the multilevel approach that was used to analyse body dimensions, growth rates, and leptin levels. Foster dam explained a significant amount of the variation in the body dimension parameters until around weaning (P=0.003–0.050). After day 35, no significant influence of the foster dams was found. In addition, variation in serum leptin was not explained by effects of the foster dams.

A possible effect of the dams could not be determined for energy intake and feed efficiency, since these measurements were performed per cage, and animals with different biological and foster dams were housed together.

Discussion

Many developmental events that take place in the third trimester of pregnancy in humans, occur in the early postnatal period in rats [99, 135]. Since programming is proposed to arise during periods of intense development [203, 227], the rat may be vulnerable to programming effects in early postnatal life. In this study, neonatal food restriction was induced by raising rat pups in large litters.

Description of the FR phenotype

Dams raising large litters had a similar energy intake as control dams. A higher intake in FR dams could have been expected if they were attempting to produce more milk. Since data on the body composition of the dams were not obtained, we cannot exclude the possibility that the FR dams were increasing milk production from their own energy reserves. However, from other studies it is known that although milk production may increase with increasing litter size, the amount of milk available per pup actually decreases [175, 424, 524]. Therefore, in the FR litters less energy and nutrients were assumed to be available per pup.

Neonatal food restriction led to an acute reduction of growth in all body dimensions. After weaning, this was followed by a distinct period of incomplete catch-up growth, resulting in animals that remained lighter (body weight), shorter (length), and thinner (BMI)

than control animals throughout the experiment. In contrast, food restriction later in life is known to induce reversible growth restriction with complete catch-up [262, 650]. Therefore, in this study, early postnatal food restriction by means of raising rats in large litters was believed to program adult body dimensions.

FR rats ate less than control animals throughout the experiment. Analysis of energy intake irrespective of body size (by using body weight or BMI as a covariate) abolished most of the differences in energy intake between FR and control animals, indicating that energy intake of FR rats was appropriate for their smaller size. Only in recently weaned males, adjustment for body weight revealed a higher energy intake in FR males, whereas adjustment for BMI pointed towards a lower energy intake. Since at this age, energy intake correlated more strongly with body weight (R=0.877) than with BMI (R=0.583), we believe that body weight is a more appropriate body dimension to use for the adjustment. Therefore, FR males had a higher energy intake shortly after weaning, which may have permitted their partial catch-up growth. In addition, feed efficiency was enhanced during the period of catch-up growth in both sexes.

Early postnatal food restriction acutely reduced serum leptin levels. Despite some recovery, leptin levels were still significantly reduced in adulthood. In adulthood, leptin levels in FR rats were appropriate for their smaller body size, suggesting that the lower leptin levels may have been caused predominantly by lower fat mass. The fact that leptin levels relative to those of controls were stable between day 77 and day 380 suggests that body fat levels of FR animals relative to those of controls were stable throughout adulthood.

In summary, in the present study, raising rats in large litters induced an acute reduction in growth of all body dimensions and low leptin levels. After weaning, an increased feed efficiency was probably responsible for the incomplete catch-up growth, resulting in adult rats that were still both smaller and thinner, with a lower food intake that was appropriate for their smaller body size. Soft tissue, and especially adipose tissue, appeared to be more vulnerable than bone tissue, as evidenced by the asymmetrical growth reduction.

Differential effects in FR males and females

In this study, animals of both sexes were studied. Male and female values differed widely for the parameters measured in this study, especially in adulthood. Males were both larger and heavier than females and, consistent with the literature, had higher leptin levels [346, 564], with the latter sex difference reversing after correction for body size [487]. Still, most of the effects of neonatal food restriction seemed to be comparable between the sexes. In cases where both sexes were analysed in a single test, the interaction between group and sex signifies differential effects of food restriction in males and females. Neonatal food restriction affected both sexes throughout the experiment, but nevertheless there were some differential effects of food restriction on body dimensions and energy use in males and females.

For body weight, at most time-points after weaning, the effect of neonatal food restriction was stronger in males than in females. Length was only differentially affected around the time of weaning. This may be caused by the different timing of onset of puberty in the sexes. BMI was similarly affected in males and females, except on two time-points, when there was no significant effect in females. Because BMI was significantly affected at all other time-points, this may have been due to inaccuracy in the measurement. Growth rate was generally affected similarly in males and females, as were serum leptin levels.

The effect of neonatal food restriction on energy intake was more significant in males than in females. However, as males and females were tested in separate analyses, these effects cannot be compared directly. At most time-points however, food intake in FR rats was reduced by a similar percentage in males and females (data not shown). Moreover, for females fewer cages were available for measurement, leading to a lower power in the analysis, which may also explain the higher *P*-values in the female tests. Feed efficiency, like food intake, was analysed separately for males and females, and therefore, no direct analysis of differential food-restriction effects in males and females could be performed. Nonetheless, feed efficiency was increased in FR females for much longer than in FR males. Because in adulthood the feed efficiency becomes progressively lower, perhaps the efficiency of FR females was no longer increased in adulthood. Its contribution to the total feed efficiency may have been overshadowed by the high efficiency in juvenile life.

In summary, body weight appeared to be less affected in females, whereas other body dimensions, and growth in all body dimensions, were similarly affected in both sexes. Differential effects on food intake and feed efficiency could not be determined from these data.

Several other studies have also found permanent programming of body weight in both sexes, although others have reported complete catch-up growth in females but not males. An explanation for these discrepancies may lie in the strain of rats used: Wistar or Sprague-Dawley rats were used in studies that reported persistent effects in females [150, 238, 263, 433, the present study], whereas the others used Long-Evans or other black-hooded rats [345, 658, 659]. Alternatively, if females are truly less affected, the differences may be harder to detect in smaller studies.

Large litters and other models that manipulate perinatal nutrition

In contrast to the varying effects in females mentioned above, neonatal food restriction generally affects body weight permanently in males [34, 150, 233, 234, 263, 345, 371, 433, 462, 649, 650, 659]. Body length has been reported to be reduced either persistently [262, 433, 649, the present study] or only transiently [371, 658, 659]. These conflicting results cannot be explained by the different strains, sexes, number of animals, litter sizes, age of placing in litters, age at weaning, or the age at measurement used in the different studies. Perhaps other subtle methodological differences can explain this discrepancy. Adiposity (either fat mass measured directly, or a proxy like BMI) was reduced in juvenile large-litter

rats in most studies [152, 175, 371, 494, 649, the present study]. One that reported unchanged adiposity used few animals in each group and only a small difference in size between the normal and large litters [345]. In adult large-litter rats, adiposity was generally still reduced [150, 238, 371, 649, the present study]. However, one study showed a significant effect in males, but not in females (although FR females seemed to tend towards lower adiposity) [150] and in another study there were no significant differences, but large-litter rats tended towards a higher fat percentage than their small-litter counterparts [345]. This latter study also found no change in adiposity in juvenile rats. It also reported unchanged food intake, whereas other studies showed lower absolute food intake in large-litter rats [34, 462, the present study]. Lastly, feed efficiency has been reported to be unchanged in large-litter rats [345]. Another study, one that used small-litter rats, reported increased feed efficiency in rats that were growing faster than controls [417], similar to the present results. Summarizing, some of the differences in outcome of studies using the large-litter model may be explained by subtle differences in the methods used, such as the rat strain or sex used.

Apart from raising rats in large litters, perinatal nutrition can also be manipulated by other methods, such as maternal protein- or global undernutrition during gestation or lactation. Using these models, persistent changes have been reported in parameters like body composition and energy intake. In general, prenatal malnutrition induced transiently reduced body dimensions, with increased adiposity and higher food intake in adulthood [129, 676]. On the other hand, postnatal malnutrition seems to lead to either reduced or normal energy intake and body size and -composition [129, 676, the present study]. Of course, the exact phenotype varies with the model used [e.g. 47, 408, 465]. For example, the model used by Vickers (maternal food restriction to 30% during gestation) produced animals with a decreased body size, but with increased adiposity and food intake (per g body weight) and seems to induce more severe effects on males than on females [629-632].

Possible mechanisms of developmental programming

The persistent changes caused by manipulations of perinatal nutrition, both in the present study and in previous reports, raise the question of the mechanism by which early undernutrition can cause programming.

The catabolic effects of leptin, which serve to reduce food intake and increase energy expenditure when energy reserves are high [544], only emerge during the first postnatal weeks [502, 574]. Instead of a role in regulating energy balance, in the neonate leptin seems to play a more developmental role. In its absence, hypothalamic pathways that are involved in the regulation of energy balance are permanently disrupted, a phenotype that can be rescued by neonatal administration of leptin [66]. Therefore, disturbed neonatal leptin levels have been suggested to play a mechanistic role in developmental programming [67, 257, 407]. The present data do not contradict this hypothesis. Leptin levels in the present study were very low on day 10, during undernutrition, and were more severely affected at that

time than any of the other parameters investigated. It should be kept in mind however, that the present data do not allow us to discern whether leptin levels were low throughout the lactation period or whether the timing or peak of the neonatal surge of leptin [509, 564] may be affected in these large-litter rats. Moreover, it is important to recognize that evidently this discussion must remain speculative, since we did not explicitly investigate the effects of leptin administration.

Other factors have also been suggested to play a mechanistic role in developmental programming. Especially, other hormones such as insulin, glucocorticoids, growth hormone, and thyroid hormone have been put forward as alternative candidates [123, 180, 491]. Until we are able to manipulate the levels of one of these hormones without affecting any of the others, it will not be possible to dissect their individual contributions.

Technical considerations

In the set-up used in the present study, the effects of neonatal food restriction could have been influenced by the effects of two dams; the biological and foster dam. To avoid unwanted effects of the biological dams, pups were randomly cross-fostered on day 2 of life. To adjust for possible effects of the foster dams, a multilevel statistical approach was employed. Assessment of the influence of both dams revealed some significant interactions with the experimental group, but only in the period before and around weaning. Notably, the reported group effects were unaffected after statistical adjustment for the dams. Therefore, we believe that variability between the dams did not interfere greatly with the effects induced by neonatal food restriction itself.

When evaluating the effects of this model, it is important to realize that food restriction may not be the only consequence of raising rats in large litters. It is possible that the dams and littermates behaved differently towards individual pups in these litters of different sizes. This may include altered nursing behaviour of the dams or altered huddling behaviour of the pups, for example, which might lead to altered energy use. These possible additional changes are inherent in the large-litter model however, and do not compromise the results. Nevertheless, it is good to bear this in mind.

Both BMI and serum leptin are proxies for fat mass. But although a low BMI is indicative of a low fat mass [448], BMI also correlates with fat-free mass [343]. Circulating leptin is not only derived from adipose tissue, but is also transferred through the milk from the dam to her pups [90]. Nevertheless, leptin levels are directly related to the amount of body fat under normal circumstances [346], in neonatal rats [679], and also in our previously studied FR animals [152]. In addition, in the present study, there was a good correlation of leptin with body weight and BMI at all ages studied. Therefore, although BMI and leptin are no perfect indicators of body fat, we are confident that the low BMI and leptin levels found in this study point to less body fat in FR animals. However, it is important to bear in mind that they are only proxies for the actual adiposity in these animals.

Conclusions and implications

In the present study, raising rats in large litters produced acute growth restriction in all body dimensions, followed by incomplete catch-up growth in both males and females. During the catch-up phase, FR rats had an increased feed efficiency.

This enhanced efficiency of energy use might result from a lower investment in reproduction [149, 151], lower basal metabolism and body temperature [433], or activity levels, for example. Regardless of the mechanisms behind it, it would be interesting to investigate whether this increased feed efficiency does increase the susceptibility to a high-fat diet during the catch-up phase. If that were the case, the apparent discrepancy between FR rats and the developmental-programming hypothesis would be solved. For it is generally found that perinatal undernutrition is associated with an increased risk for adiposity and metabolic problems [203, 227], instead of a reduced risk as found in the present study. Then, a mismatch between the poor early environment (large litter) and the rich later environment (e.g. high-fat diet) might induce increased adiposity. In recent years, the importance of this mismatch, rather than early undernutrition per se, has been stressed [203, 287].

When hypothesizing that neonatal leptin levels may have played a role in programming the adult body dimensions and food intake of the FR rats in the present study, it is interesting to consider recent studies that administered leptin neonatally. Several studies have investigated the effects of prenatal undernutrition, neonatal leptin administration, and a high-fat diet in combination. Neonatal leptin administration per se has produced either increased [124, 632], normal [605, 631, 675], or decreased [486] adiposity in adulthood. The obesity induced by a high-fat diet was also differentially influenced by neonatal leptin administration in different situations: neonatal leptin aggravated the diet-induced obesity in male rats and mice [632, 675], but not in male rats that were prenatally underfed [632] and female control rats [631], whereas it protected from diet-induced obesity in female rats that were prenatally underfed [631] and in male rats when it was administered orally instead of subcutaneously [486]. The subtle differences between these animal models that may cause these different effects remain to be elucidated, but the timing of the neonatal leptin surge [509, 564] may be an interesting candidate. As indicated by Yura *et al.* in one of these studies [675], an early leptin surge is associated with adult obesity and possibly with accelerated neonatal growth (small litter). Since the large-litter FR rats in the present study showed reduced adult adiposity, one might speculate that the neonatal leptin surge may be delayed or decreased in these animals.

The present study provides a complete overview of both short- and long-term effects of raising rats in large litters on body dimensions and energy intake. Together with other models, the large-litter model provides the opportunity to investigate the specific conditions under which perinatal malnutrition leads to deleterious long-term effects, and how these might eventually be prevented.
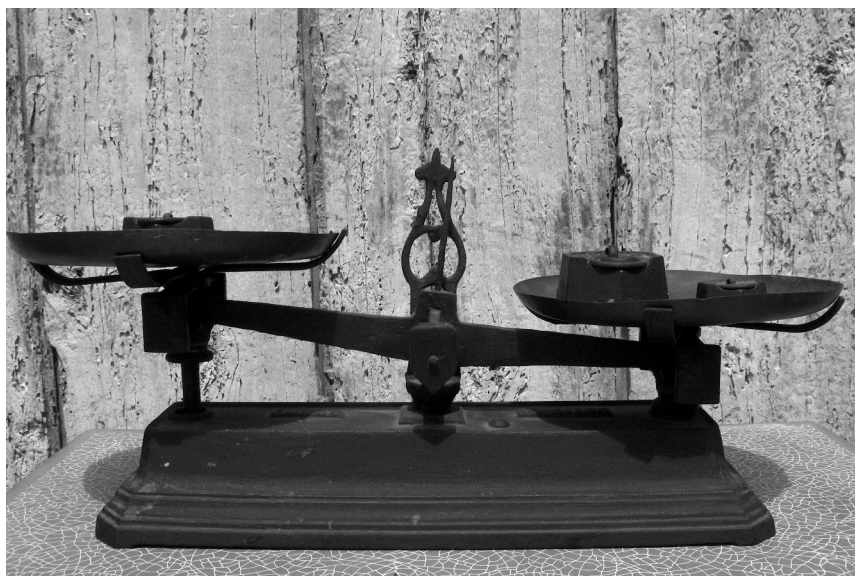
Acknowledgements

Supplemental data

The supplemental Tables with this chapter can be found from page 181.

4

Energy intake and resting energy expenditure in adult male rats after early postnatal food restriction

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Balance (Floor Remmers)

4 | ENERGY INTAKE AND RESTING ENERGY EXPENDITURE IN ADULT MALE RATS AFTER EARLY POSTNATAL FOOD RESTRICTION

Both in man and in animal models, changes in food intake and body composition in later life have been reported after alterations in perinatal nutrition. Therefore, we hypothesised that early postnatal undernutrition in the rat induces permanent changes in energy balance. Food restriction during lactation was achieved by enlarging litter size to twenty pups, whereas control animals were raised in litters containing ten pups. Energy intake and resting energy expenditure were determined in adult males. Early postnatal food restriction resulted in acute growth restriction followed by incomplete catch-up in body weight, body length, and body mass index (BMI). At the age of 12 months, middle-aged food-restricted (FR) males had significantly lower absolute resting energy expenditure (200 *vs.* 216 kJ/24 h, *P*=0.009), absolute energy intake (281 *vs.* 310 kJ/24 h, *P*=0.001) and energy intake adjusted for BMI (284 *vs.* 305 kJ/24 h, *P*=0.016) than controls, whereas resting energy expenditure adjusted for BMI did not differ significantly between the groups (204 *vs.* 211 kJ/24 h, *P*=0.156). The amount of energy remaining for other functions was lower in FR males (80 *vs.* 94 kJ/24 h, *P*=0.044). Comparable data were obtained at the age of 6 months. These results indicate that in rats energy balance can be programmed by early nutrition. A low early postnatal food intake appears to programme these animals for a low energy intake and to remain slender in adult life.

Introduction

Epidemiological studies that linked low birth weight with later disease [227] have led to the theory of developmental programming [203]. The early environment, encountered during a sensitive period, is believed to influence the development and hence the function of the organism permanently [231]. Humans that are born small for gestational age represent an example of developmental programming. These individuals are thought to be adapted to a poor environment, and when confronted with a rich, western environment they have an increased risk of insulin resistance, hypertension, obesity, and cardiovascular disease (collectively called the metabolic syndrome) in adult life [203, 227]. Other examples of developmental programming are various animal models that manipulate the perinatal nutritional environment [47, 255, 465]. Studies using these models have shown that, depending on the exact nature and timing of the manipulation, programming can act in different directions. For instance, different effects on energy balance have been reported after perinatal malnutrition [406].

In different rat models, adult food intake [34, 129, 462, 676] and body fat [129, 150, 158, 629, 676] were either increased, decreased, or unchanged. In man, programming of energy balance has also been reported. Although obesity rates have been reported to be lower after perinatal malnutrition [508], there are now several studies that associate low birth weight with a more central distribution of fat [155, 614] and a lower lean body mass [189].

An important part of the regulation of energy balance takes place in the hypothalamus. Whereas in man, a substantial part of the development of the hypothalamus and the brain is

completed *in utero*, in rats much of this development occurs postnatally [65, 135, 211, 332]. Therefore, we have used early postnatal food restriction in rats to study developmental programming of energy balance. We have previously shown that raising rats in large litters reduced body weight into adulthood [150, 263] and decreased the fat percentage in adult males [150]. These animals also showed disruptions in several processes that are regulated by the hypothalamus; a delayed onset of puberty [149], impaired testicular function [622], and changes in the growth hormone axis [258].

If the hypothalamus is affected in these animals, then its regulation of energy homeostasis may also be affected, which could ultimately lead to permanently altered energy balance. Changes in energy balance might contribute to the phenotype of these animals. Therefore, the aim of the present study was to elucidate whether early postnatal food restriction alters energy intake and resting energy expenditure (REE) in adult and middle-aged male rats.

Methods

Experimental animals

Primiparous timed-pregnant Wistar rats (Harlan, Horst, the Netherlands) arrived on day 14 or 15 of gestation and were housed individually under controlled lighting (12h light, 12h dark) and temperature (21.5 ± 0.5 °C). Animals had unlimited access to tap water and standard rat chow (Ssniff R/M-H; Bio Services, Uden, the Netherlands; 12.8 kJ/g metabolisable energy, 19.0% protein, 3.3% fat, 36.5% starch, 4.7% sugar, and 4.9% crude fibre), unless mentioned otherwise. Pups were born spontaneously on day 22 or 23 of gestation. From day 20 of gestation, the presence of pups was checked daily in the morning and the first day of life was designated postnatal day 1. On day 2, male and female pups were allocated to either a control litter of ten pups or a food-restricted (FR) litter of twenty pups using computer-generated random numbers. Male-to-female ratio was 1:1 in all fostered litters. In large FR litters, less milk has been shown to be available per pup than in control litters, resulting in undernutrition [175]. On day 25, the pups were weaned and males were housed two per cage, paired with another animal of the same experimental group. Subsets of animals were killed at different ages for another study. A subset of 39 of the male animals in the experiment survived until the age of one year and was used in the present study (16 controls and 23 FR animals). All procedures were approved by the Animal Experimentation Ethics Committee of the Vrije Universiteit and the VU University Medical Center in Amsterdam, the Netherlands.

Body dimensions

Body weight was measured regularly throughout life. At the age of 12 months (day 380), body length was measured from the tip of the nose to the anus under pentobarbital or O_2/CO_2 anaesthesia before the animals were killed for further study. Body mass index (BMI)

was calculated as the ratio of body weight (g) to body length (cm) squared. In a previous study, we showed that at the age of 6 months control and FR males had a fat-free mass (FFM) of, respectively, 76% and 81% of their body weight [150]. Therefore, an estimate of FFM at 6 months was calculated as 0.76 x body weight in controls and 0.81 x body weight in FR males.

Food intake

Individual food intake was determined in 16 adult and middle-aged control animals and 23 FR animals at the ages of 6 and 12 months. The animals were housed individually at least one day before the measurement to become accustomed to the testing cage. The food provided was weighed at the beginning and the end of a 24-hour period. Individual energy intake was calculated from 24-hour food intake and the energy density of the diet (12.8 kJ/g).

Indirect calorimetry

REE was determined by means of indirect calorimetry in the same 16 control and 23 FR animals at the ages of 6 and 12 months. The animals were housed individually at least one day before the measurement to become accustomed to the testing cage. All measurements were carried out during the light, inactive phase of the day. During the measurements, no food and water were available. A metabolic monitor (Deltatrac II MBM-200; Datex-Ohmeda, Helsinki, Finland), adapted to fit the animal cages, was used to measure resting oxygen consumption rate (V_{O2}) and carbon dioxide production rate (V_{CO2}) every minute. The lower limit for reliable measurements was 5 ml/min for both V_{O2} and V_{CO2}, restricting us to the measurement of adult males; neither females nor younger animals reached this limit of reliability. Before each measurement, the metabolic monitor was calibrated with a gas mixture of 95% O_2 and 5% CO_2. Mean V_{O2} and V_{CO2} values from stable measurements with a duration of at least 20 minutes and a coefficient of variation $\leq$ 5% were used for calculations. REE was calculated using the modified Weir formula [643]: REE (kJ/24h) = 4.184 x {5.50 x V_{O2} (ml/min) + 1.76 x V_{CO2} (ml/min)}, without adjustment for urinary nitrogen excretion [154]. To avoid possible effects of circadian rhythm on energy expenditure interfering with the group effects, control and FR animals were measured in an alternating manner. After the two energy balance measurements were completed, the animals were socially housed with the same individual as before.

Data analysis

The results were analysed using Statistical Product and Service Solutions software for Windows, version 12 (SPSS Inc., Chicago, IL, USA). All data were checked for normality and are expressed as means ± SEM (except in Figures 1 and 2, where means are shown with SD for better visibility). After exclusion of animals with missing values or a variation > 5% in the

indirect calorimetry, data were analysed for 14 control and 22 adult FR males at 6 months and for 16 control and 21 middle-aged FR males at 12 months. All outcome measures were initially analysed by means of one-way ANOVA. To confirm that the food restriction in the FR litters was distributed evenly over the pups within a litter, differences in variance of preweaning body weight between the groups were tested using Levene's test for homogeneity of variances. Potential confounding effects of biological and foster dams were tested in univariate ANOVA. Foster dam nested within group and the interaction between biological dam and group had no long-lasting significant effect and were omitted in further analyses. Energy utilisation is known to correlate with body size, and more specifically with FFM, and it has been recommended to adjust for FFM in an ANOVA when comparing energy utilisation between subjects with different body compositions [17, 606]. In a previous study we have shown that at the age of 6 months FR males indeed have a different body composition than controls [150], confirming the need for adjustment. At 6 months, we estimated FFM by means of the values found in this previous study. At 12 months, BMI was available as another estimate of body composition. Therefore, energy balance data were tested in a univariate ANOVA with estimated FFM (eFFM) as a covariate at 6 months and BMI at 12 months, as recommended [17, 606]. If these covariates did not have a significant effect, they were omitted from the analysis.

Results

The 39 animals used in the present study were born from 21 of the 33 dams in the complete experiment and on day 2 were fostered to six different dams for each group. The original litter size of the foster dams nurturing FR pups (12.3 ± 1.0 pups) was not different from that of the foster dams that nurtured control pups (11.7 ± 0.8 pups, $P>0.600$). Nor did the original litter size of FR pups (12.0 ± 0.5 pups) differ from that of control pups (12.2 ± 0.5 pups, $P>0.800$). Of the 39 pups in this study, 85% were cross-fostered, whereas 15% (3 control and 3 FR animals) remained with the same dam after the random redistribution on day 2.

Early postnatal food restriction resulted in a persistent reduction in body weight, body length, and BMI. Mean body weights of control and FR rats are shown in **Figure 1**. Body weight on day 2 (before the redistribution into control and FR litters) was 7.7 ± 0.13 g. Body weight was lower in FR rats from day 4 until day 380 ($P<0.001$). Relative to control values, body weight of FR animals decreased during lactation to 60% at weaning. After weaning, relative body weight of FR rats increased to 86% on day 70 and then stabilised so that on day 380 FR animals weighed 89% of control weight (**Figure 2**). During the lactation period, the variance in body weight did not differ significantly between the groups ($P>0.200$), although on day 21 there was a trend towards larger variance in the FR group ($P=0.093$). Body dimensions of FR and control rats at 6 and 12 months are shown in **Table 1**. At both 6 and 12 months, body weight was lower in FR males ($P<0.001$). At 6 months, eFFM, which was

computed as 76% of body weight in controls and 81% of body weight in FR rats, was lower in FR animals (*P*=0.029). At 12 months, body length (*P*<0.001) and BMI (*P*=0.024) were lower in FR animals than in controls.

At 6 months, we could not obtain measurements with a variation below 5% for three animals (two controls and one FR rat), despite repeated attempts. These animals were excluded from all analyses at this time-point. At 12 months, two FR animals had to be excluded; one had to be killed prematurely, one had missing body length data at the time of killing.
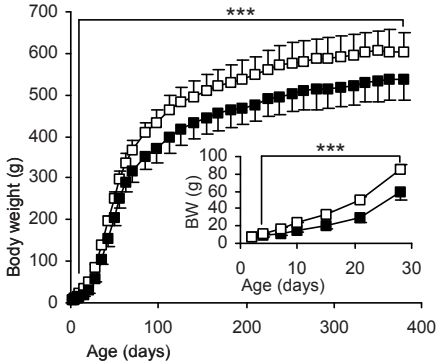

Figure 1. Body weight (BW) throughout the experiment and during the first month of life (inset) of male FR rats (■, *n*=23), which were food restricted during lactation, and male control rats (□, *n*=16). Values are expressed as mean ± SD (where the error bars are not visible, they are within the symbol). *** *P*<0.001 for FR *vs.* control from day 4 until 380.
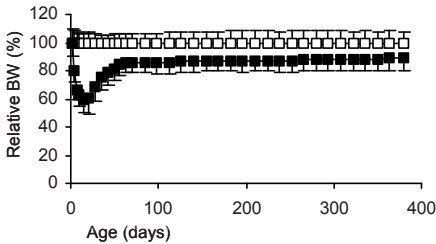

Figure 2. Body weight (BW) expressed as a percentage of control body weight for FR animals (■, *n*=23) and control animals (□, *n*=16). Values are expressed as mean ± SD.

Table 1. Body dimensions of control and FR males at the ages of 6 and 12 months

	6 months					12 months				
	control (*n*=14)		FR (*n*=22)			control (*n*=16)		FR (*n*=21)		
	mean	SEM	mean	SEM	*P*	mean	SEM	mean	SEM	*P*
BW (g)	526.4	12.5	461.1	8.7	***	604.2	11.9	538.4	11.4	***
eFFM (g)	400.0	9.5	373.5	7.1	*	n.d.		n.d.		
BL (cm)	n.d.		n.d.			27.1	0.2	26.1	0.1	***
BMI (g/cm²)	n.d.		n.d.			0.82	0.01	0.79	0.01	*

BL, body length; BW, body weight; n.d., no data. * *P*<0.05, *** *P*<0.001 for FR *vs.* control at the same age.

Energy intake

At both 6 and 12 months, FR animals consumed a significantly smaller absolute amount of food than control animals (**Table 2**). Energy intake correlated with estimated body composition at both 6 months (eFFM, R=0.699, *P*<0.001) and 12 months (BMI, R=0.540, *P*=0.001). Energy intake was adjusted for eFFM at 6 months and for BMI at 12 months to account for differences in body composition between the groups. Adjusted energy intake at 6 months (**Figure 3A**) was lower in FR males (285.0 ± 4.2 kJ/24h) than in control males (303.4 ± 5.3 kJ/24h, *P*=0.012). At 12 months, adjusted energy intake (**Figure 3B**) was also lower in FR rats (284.4 ± 5.1 kJ/24h) than in controls (304.9 ± 5.9 kJ/24h, *P*=0.016).
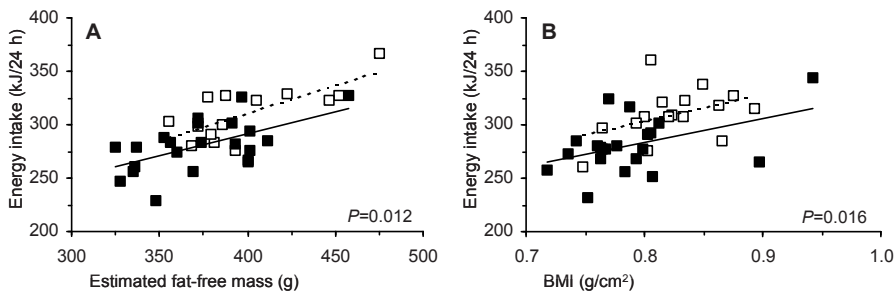

Figure 3. Relationship between estimated body composition and energy intake at 6 months (**A**) and 12 months (**B**) in control (□, dashed line, *n*=14 in A, *n*=16 in B) and FR rats (■, solid line, *n*=22 in A, *n*=21 in B). The given *P*-values indicate a significant effect of experimental group on energy intake when body composition (eFFM or BMI) was included as a covariate.

Resting energy expenditure

Mean values for V_{O2} and V_{CO2} were 6.5 ± 0.1 and 6.0 ± 0.1 ml/min at 6 months and 6.9 ± 0.1 and 6.5 ± 0.1 ml/min at 12 months, respectively.

At both 6 and 12 months, FR animals had a significantly lower absolute REE than control animals (**Table 2**). REE correlated with estimated body composition at both 6 months (eFFM, R=0.870, *P*<0.001) and 12 months (BMI, R=0.680, *P*<0.001). At 6 months, energy expenditure adjusted for estimated body composition (**Figure 4A**) was not significantly different between FR males (192.0 ± 2.2 kJ/24h) and controls (198.1 ± 2.8 kJ/24h, *P*=0.099), nor did adjusted energy expenditure at 12 months (**Figure 4B**) differ significantly between FR rats (204.1 ± 3.2 kJ/24h) and controls (211.3 ± 3.6 kJ/24h, *P*=0.156).
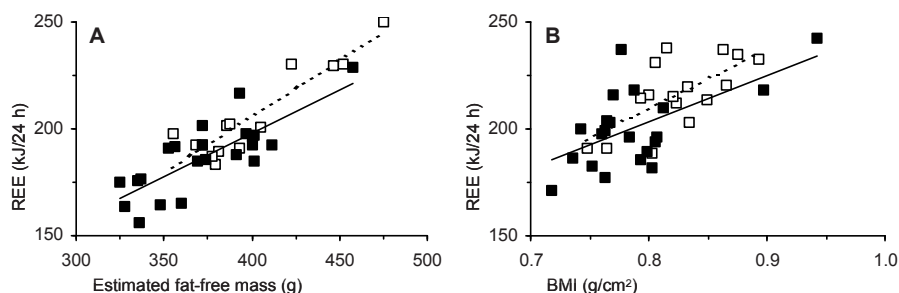
Figure 4. Relationship between estimated body composition and REE at 6 months (**A**) and 12 months (**B**) in control (□, dashed line, *n*=14 in A, *n*=16 in B) and FR rats (■, solid line, *n*=22 in A, *n*=21 in B).

Energy intake minus resting energy expenditure

When energy balance is neutral, energy intake equals total energy expenditure (TEE), so the difference between energy intake and REE represents the amount of energy available for other functions such as locomotor activity. This parameter did not correlate with BMI or eFFM (*P*>0.480). Energy intake minus REE was lower in FR rats than in control rats at both 6 (*P*=0.038) and 12 months (*P*=0.044; **Table 2**).

Table 2. Energy intake (EI), REE, and EI minus REE of control and FR males at the ages of 6 and 12 months

	6 months					12 months				
	control (*n*=14)		FR (*n*=22)			control (*n*=16)		FR (*n*=21)		
	mean	SEM	mean	SEM	*P*	mean	SEM	mean	SEM	*P*
EI (kJ/24h)	310.9	6.7	280.3	5.1	**	309.8	23.8	280.7	25.4	**
REE (kJ/24h)	205.6	5.5	187.2	3.6	**	216.2	4.1	200.3	4.0	**
EI − REE (kJ/24h)	105.2	4.1	93.1	3.6	*	93.6	4.5	80.4	4.3	*

* *P*<0.05, ** *P*<0.01 for FR *vs.* control at the same age.

Discussion

In the present study, early postnatal food restriction of male rats resulted in an acute reduction in growth, followed by incomplete catch-up growth, and permanently altered energy balance. At both 6 and 12 months, FR rats consumed and expended less energy than controls. After subtraction of REE from energy intake, FR animals had less energy available for other functions. When estimated adult body composition was taken into account, energy intake was lower after early postnatal food restriction, whereas energy expended in rest was similar to that of controls.

Programming of energy balance

In the present study, male FR rats remained lighter than control males until the end of the experiment at the age of 12 months. This suggests that early postnatal food restriction

can programme later size. This is in contrast to food restriction later in life, which has been shown to induce reversible growth restriction with complete catch-up [262, 650]. BMI was also reduced in FR animals. Although BMI is not a direct measure for fat mass, it is strongly correlated with the percentage of body fat in both man [328] and rats [448]. Therefore, the present results suggest that at least in rats early postnatal food restriction can programme a low level of adult adiposity. This may be through a reduced energy intake, but from the present data it is not possible to discern cause and effect in the relationship between BMI and food intake. Although REE was reduced in the FR animals, it seemed appropriate for the altered body composition. Therefore, programming of REE does not seem to have taken place. In the case of neutral energy balance, energy intake equals TEE. Since REE includes basal metabolic rate (BMR), the thermic effect of food, and energy expended for growth, the difference between TEE and REE represents activity-related energy expenditure (AEE) [645]. Energy intake minus REE, or AEE, was reduced in FR males. Therefore, these animals may either be less active or expend less energy during their activity. In adult rats, the energy expended for growth is negligible. If during the development of these animals, energy intake was also reduced without a change in BMR, there may have been less energy available for growth. This may explain, at least in part, the permanent reduction in body weight, body length, and BMI in the animals in the present study.

Early and late effects

When analysing data on late effects of early insults, it is important to separate the effects of the early insult from those of events later in life [384]. Therefore, in the present study both unadjusted data and data adjusted for estimated adult body composition were presented. Energy intake and REE were both reduced in FR animals when early size (i.e. control or FR) was the sole independent variable. Adding estimated adult body composition as a covariate removed the effect on REE, but not that on energy intake. This suggests that later events may have been more important in determining REE than early postnatal food restriction, but that the food restriction was the most important determinant of energy intake in these animals. Here it should be noted that the adjustment for fat free mass as advised [17, 606] is essential for this result. When adjusted for the less recommended crude body weight instead of the metabolically active FFM, energy intake was not significantly different between the groups (data not shown). This emphasizes the importance of choosing the appropriate parameter for adjustment of energy balance data. The difference between energy intake and REE, or AEE, was independent of adult size and therefore the differences between the groups were most probably due to the early postnatal undernutrition in the FR group.

If the differences between the groups are to be attributed to true programming, the effects must be permanent. Therefore, the animals were tested in adulthood. Animals were retested when middle-aged at the age of one year to verify whether the effects were truly

permanent. Since similar results were obtained at both ages studied, we are rather confident that permanent programming really occurred.

Energy balance in other models

Postnatal manipulations of litter size appear to yield consistent results. Other studies using large litters have also found a permanently reduced body weight [34, 150, 158, 462, 650] and fat mass [150, 158], a lower food intake in young adulthood [34], and a far lower cumulative absolute food intake from weaning until over a year of age [462]. Studies using overfeeding in small litters have found opposite results: animals were permanently heavier than control animals [34, 64, 493], had an increased fat mass or BMI [64, 493], and a larger absolute food intake in young adulthood [34, 64, 493]. New in the present study is that food intake of male FR rats was not only significantly lower in absolute terms, but it was even reduced when their altered body composition was taken into account.

In comparison with the present observation of an appropriately reduced REE, a previous study using early postnatally overfed male small-litter rats showed increased TEE at the age of 5 weeks, but not in older animals [651].

Comparing the results of the present study with those of others that used different models of perinatal undernutrition is more complicated, however, as the direction of the changes observed appears to be highly dependent on the exact timing, type, and severity of malnutrition [47, 255, 465]. Maternal 'caloric' and protein restriction during gestation or lactation have produced an increased, reduced, or normal body weight and fat mass in adulthood [129, 255, 406, 629, 676], depending on the timing and severity of malnutrition. Moreover, 50% food restriction of the dam during gestation increased adult food intake, but the same insult during lactation did not [129], whereas a low-protein diet during lactation reduced adult food intake [676]. In general, a lower food intake has been found in models with incomplete catch-up growth, whereas a higher food intake was found after postnatal overnutrition or prenatal undernutrition followed by overcomplete catch-up. Studies using prenatal maternal malnutrition have found reductions in TEE with unaltered REE (suggesting reduced AEE) [114] and an actual reduction in activity levels [39, 630] in adult males, a consequence also suggested by the results of the present study. In contrast, activity was not reduced in our previous study in young adult males that were prenatally growth restricted by bilateral uterine artery ligation [539].

Unlike the early postnatally FR rats, most of the humans that are born small for gestational age or after intra-uterine growth restriction catch up during infancy [301]. However, it was shown that prepubertal children born small for gestational age that did not catch up had a food intake below the recommended energy intake for their age [63]. These data seem to be in accord with the reduced food intake in the early postnatally FR rats with incomplete catch-up growth in the present study. Studies in neonates have suggested that infants that are born small for gestational age have a higher energy expenditure per kilogram body weight or FFM than weight-matched controls [91, 121]. Although these data

on REE relate to acute instead of long-term effects, they do indicate that perinatal malnutrition can also affect energy expenditure in man.

The differences outlined above warn us to exert extreme caution when attempting to extrapolate outcomes of perinatal malnutrition, not only between rats and man, but also between different animal models. Seemingly comparable manipulations of prenatal or early postnatal nutrition can yield widely differing results [47, 255, 465].

Because of the different timing of birth relative to development, early postnatal food restriction in rats is probably somewhat similar to undernutrition in human foetuses during the third trimester, although the potential for catch-up growth is evidently different between the two. It could be speculated that the window of plasticity for body dimensions, adiposity, and food intake may close before the end of the lactation period in rats, whereas in man it may extend into the postnatal period.

Technical considerations

A concern when using large litters to reduce early postnatal food intake is the lack of control over the distribution of the available milk within litters. There may be competition between the pups over the milk supply and as a consequence the pups in a litter may be food restricted to different degrees [190]. The fact that the variance in body weight during the lactation period was similar between control and FR males suggested that in the present study all FR pups were food restricted roughly to the same degree.

The energy balance measurements in the present study were restricted to adult male rats. Investigating the possible effects of early postnatal food restriction on energy expenditure in females would be interesting. Unfortunately, we were unable to investigate this, because of the limitations of the metabolic monitor.

In the present study, actual measurements of FFM were not available. The variables eFFM and BMI were chosen as estimates for FFM. By extrapolating the percentage of FFM from one population to another, we introduced an uncertainty. Especially because at 6 months the population of the present study was heavier than that used in the other study [150], most probably because of the different diets the animals received. Therefore, energy intake and REE were also determined at another age, when BMI was available as a parameter of body composition. Although it is usually employed for its correlation with fat mass, BMI describes body weight relative to length, and hence does not discriminate between fat mass and FFM. It therefore also increases with increasing FFM [343]. The fact that the analyses using these different covariates produced comparable results at both ages suggests that the estimates BMI and eFFM were equally suitable approximations for FFM.

Implications

In the present study, we showed that male rats that were food restricted early postnatally remained lean with a reduced food intake in adult life. This fits in with the relatively recent idea that promoting catch-up growth in low-birth weight infants may not be

beneficial for their long-term outcome. Several studies in man as well as in animals have suggested that fast and early catch-up, sometimes through super-nutritious food, can be detrimental [456, 579, 629, 630]. On the other hand, rats with this modest phenotype may not have sufficient supplies for normal growth [258, 263] and possibly other matters such as reproduction [149, 622] and locomotor activity. In summary, the present study demonstrates that in rats early postnatal food restriction can programme energy balance in later life. The present study provides additional support for the hypothesis that early nutritional insults may have long-term metabolic consequences.

Acknowledgements

Hypothalamic neuropeptide expression of juvenile and middle-aged rats after early postnatal food restriction

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5 | HYPOTHALAMIC NEUROPEPTIDE EXPRESSION OF JUVENILE AND MIDDLE-AGED RATS AFTER EARLY POSTNATAL FOOD RESTRICTION

Rats subjected to early postnatal food restriction show persistent changes in energy balance. The hypothalamus plays a major role in the regulation of energy balance. Therefore, we hypothesised that early postnatal food restriction induces developmental programming of hypothalamic gene expression of neuropeptides involved in this regulation. In the hypothalamus of juvenile and middle-aged rats that were raised in control (10 pups) or food-restricted (FR) litters (20 pups), gene expression was investigated for neuropeptide Y (NPY), agouti-related protein (AgRP), pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) in the arcuate nucleus (ARC); corticotropin-releasing hormone and thyrotropin-releasing hormone in the paraventricular nucleus (PVN); and melanin-concentrating hormone (MCH) and orexin in the lateral hypothalamic area (LHA). Early postnatal food restriction acutely and persistently reduced body size. Juvenile FR rats had significantly reduced CART gene expression and increased MCH expression. In middle-aged FR rats, POMC and CART mRNA levels were significantly reduced. The ratio between expression of the ARC orexigenic peptides (NPY and AgRP) and anorexigenic peptides (POMC and CART) was increased in juvenile, but not in middle-aged, FR rats. These results suggest that in neonatal rats, food restriction already triggers the ARC, and to a lesser extent the LHA, but not the PVN, to increase expression of orexigenic relative to anorexigenic peptides. In addition, with enduring small body size and normalised hypothalamic gene expression, the adult FR rats appeared to have accepted this smaller body size as normal. This suggests that the body weight set-point was differently programmed in animals with early postnatal food restriction.

Introduction

Epidemiological studies linking low birth weight with disease in adult life [227] have led to the concept of developmental programming, which entails that differences in the environment during the plastic period of development can permanently alter physiology [203, 231]. Over the years, evidence has accumulated that perinatal events can permanently alter energy balance both in humans and in animal models. For example, human foetal growth restriction is associated with a more central distribution of fat and a lower lean body mass in later life [455]. In rat models of perinatal malnutrition, changes in food intake and body composition have been reported, but the direction of these changes appears to be highly dependent on the exact nature and timing of the malnutrition [406]. These changes in energy balance suggest that the regulation of energy balance may be programmed.

The peripheral hormone leptin and several nuclei of the hypothalamus play an important role in this regulation of energy balance. Leptin is secreted by adipocytes in proportion to their fat content and thereby indicates the body's energy reserves [389, 544]. In the hypothalamic arcuate nucleus (ARC), the expression and release of the orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) are inhibited by leptin, whereas those of the anorexigenic pro-opiomelanocortin (POMC, the precursor for α-melanocyte-

stimulating hormone) and cocaine- and amphetamine-regulated transcript (CART) are stimulated [e.g. 544, 668]. NPY/AgRP and POMC/CART neurons in the ARC project to the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) among others, where levels of the anorexigenic peptides corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) and the orexigenic peptides melanin-concentrating hormone (MCH) and orexin (ORX), respectively, are modified [e.g. 668].

In rats, the connections from the ARC to the PVN and LHA develop mostly in the early postnatal period [67, 211], and therefore, rats raised in large litters can be used to study developmental programming of energy balance. These rats were previously shown to have reduced growth during lactation, followed by incomplete catch-up growth after weaning [150, 263]. Young rats showed reduced leptin levels and fat percentages [152], and in adult males, the fat percentage was still reduced [150]. In the present set of animals, we have reported lower body weight, body length, body mass index (BMI), energy intake, and serum leptin levels until the age of one year [512, 513 (Chapters 3 and 4)]. In addition, a subset of adult males in this group was shown to have a resting energy expenditure that was reduced, but seemed appropriate for their smaller body dimensions [513].

The alterations in energy balance found in these large-litter rats suggest that early undernutrition induced permanent changes in the regulation of energy balance. Moreover, these animals showed disruptions in several other processes that are regulated by the hypothalamus: a delayed onset of puberty [149], impaired testicular function [622], and changes in the growth hormone axis [258]. Therefore, the hypothalamus seems a likely candidate for programming. In this study, we investigated acute and long-term programming effects of early postnatal undernutrition (in juvenile and middle-aged rats, respectively) on the mRNA expression levels of neuropeptides in the ARC, PVN, and LHA, using quantitative real-time polymerase chain reaction (PCR). In addition, the relationships between hypothalamic neuropeptide expression levels and the parameters that were reported previously [512, 513] were assessed.

Methods

Experimental animals

Primiparous timed-pregnant Wistar rats (Harlan, Horst, the Netherlands) arrived on day 14 or 15 of gestation and were housed individually under controlled lighting (12h light, 12h dark) and temperature (21.5 ± 0.5 °C). Animals had unlimited access to standard rat chow (Ssniff R/M-H; Bio Services, Uden, the Netherlands) and tap water at all times. All procedures were approved by the Animal Experimentation Ethics Committee of the Vrije Universiteit and the VU University Medical Center in Amsterdam, the Netherlands.

Pups were born spontaneously on day 22 or 23 of gestation. The first morning after birth was designated as postnatal day 1. On day 2, pups were allocated to either a control litter of 10 pups or a food-restricted (FR) litter of 20 pups using computer-generated random

numbers. In the large FR litters, less milk is available per pup than in control litters, resulting in undernutrition [175] and growth restriction [263]. Male-to-female ratio was 1:1 in all litters. On day 25, the pups were weaned and socially housed; two (males) or four (females) animals of the same experimental group per cage. A subset of 94 animals was used for this study.

Body dimensions and tissue collection

Body weight was measured regularly throughout life. Body length of manually restrained animals from the tip of the nose to the anus was measured on day 2 and on the day of killing. BMI was calculated as the ratio of body weight (g) to body length (cm) squared.

During the experiment, subsets of animals were killed at different ages. Suckling pups (males and females) were sacrificed on day 10 of life and middle-aged males and females around day 380 of life. In addition, weanling males were killed on day 25. In females, the expression levels of the neuropeptides in the ARC [60, 475], PVN [61, 613], and LHA [437, 555] are known to vary with the stage of the oestrous cycle. Therefore, adult females were killed on the day of pro-oestrus. To that end, in the last 2 weeks before sacrifice, their cycle was monitored by daily vaginal smears that were stained with Giemsa stain. In addition, since neuropeptide expression is also known to have a circadian rhythm in the ARC [380, 669], PVN [104, 641], and LHA [56, 593], all animals were killed during the first half of the light phase (between 1.5 and 5.5 hours after lights on).

Rats were euthanised by CO_2 inhalation, followed by decapitation. Since CO_2 inhalation is not indicated until after day 10 [443], at this age, animals were decapitated without prior CO_2 inhalation. After decapitation, brains were rapidly dissected, snap-frozen in dry ice-chilled 2-methylbutane, and stored at −80 °C. Trunk blood was collected, stored at 4 °C for a maximum of 4 hours, and then centrifuged at 3000 rpm for 15 minutes. Serum was stored at −80 °C until assayed. Serum leptin levels were determined previously using a commercial radioimmunoassay kit [512 (Chapter 3)].

Microdissection

Brain punches containing the ARC, PVN, or LHA were dissected as reported previously [126]. One day prior to dissection, brains were transferred from −80 °C to −20 °C. On the day of dissection, the brains were temperature-adapted in a −8 °C cryostat and mounted with TissueTek OCT (Sakura Finetek, Zoeterwoude, the Netherlands). Using a rat brain atlas [473], coronal 300-μm sections were cut, transferred to a flexible black rubber strip, and immediately covered with RNAlater reagent (Sigma-Aldrich, St. Louis, MO). Neonatal rat brains have a higher water content [290], and therefore on day 10, the brains were mounted using Shandon M-1 embedding matrix (Thermo), and cut at 150 μm instead of 300 μm. Punches were taken using a 2-mm-diameter stainless steel punch needle (Zivic Laboratories, Pittsburgh, PA), and the punches from each individual nucleus were placed into a 1.5-ml centrifuge tube containing RNAlater and stored at −20 °C until further processing. For the

ARC, one punch was taken from three successive sections starting at bregma –2.6 mm. For the PVN and LHA, two punches were taken from two successive sections starting at bregma –1.6 mm and bregma –2.8 mm, respectively. Using this technique, it is possible to excise tissue of distinct hypothalamic nuclei, with only a slight chance that small parts of adjacent nuclei are included.

RNA isolation and cDNA synthesis

RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For each sample, 300 µl TRIzol was used and the other volumes were adjusted accordingly. The RNA pellet was dissolved in 20 µl RNase-free water. RNA was stored at –80 °C, but 8 µl was immediately treated with 0.75 µl RNase-free DNase I (Roche, Mannheim, Germany) for 30 minutes at 37 °C. The DNase I was then inactivated by 5 minutes at 75 °C. Immediately, cDNA was synthesised using 1 µl SuperScript II reverse transcriptase (Invitrogen), 1 µl oligo(dT)$_{12-18}$ primers (Invitrogen), and 1 µl 10 mM dNTPs (Roche). The reaction was incubated for 60 minutes at 42 °C and then terminated by 10 minutes at 75 °C. cDNA was stored at –20 °C until further use. For each nucleus, all control and FR samples for one age and sex were processed at the same time.

Quantitative real-time PCR

Specific primers for NPY, AgRP, POMC, CART, CRH, TRH, MCH, ORX, cyclophilin, and β-actin were designed using Primer Express Software (Applied Biosystems, Foster City, CA) and produced by Isogen Life Science (Maarssen, the Netherlands). PCR efficiencies were obtained from standard curves of serial dilutions of whole hypothalamus cDNA. Characteristics of the primers are listed in **Table 1**. For each peptide, all samples were tested in a single PCR.

Table 1. Characteristics of the PCR primers

Gene of interest	Accession number	Forward primer	Reverse primer	Product size (bp)	PCR efficiency
NPY	NM_012614	441–458	484–504	64	2.29
AgRP	AF206017	126–142	172–191	66	2.03
POMC	AF510391	465–484	512–530	66	2.12
CART	NM_017110	124–144	167–185	62	1.89
CRH	NM_031019	689–708	738–755	67	1.94
TRH	NM_013046	442–461	490–506	65	1.95
MCH	M29712	74–95	119–141	68	2.08
ORX	NM_013179	3–19	47–68	66	2.03
cyclophilin	M19533	346–367	393–413	68	1.99
β-actin	NM_031144	460–478	505–526	67	2.01

bp, base pairs.

The real-time PCR approach utilised the SYBR green method in a 96-well plate format using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Reactions were performed

in a total volume of 25 µl, containing 1 µl cDNA, 0.32 µM of each primer, and 12.5 µl of SYBR Green PCR Master Mix (Applied Biosystems). The thermal profile used for amplification was 2 minutes at 50 °C, 10 minutes at 95 °C, and 40 cycles of 15 seconds at 95 °C and 60 seconds at 60 °C. At the end of the amplification phase, a melting-curve analysis was carried out to confirm the formation of a single PCR product and hence the absence of primer-dimers. Threshold cycle (Ct) values were determined automatically by the Applied Biosystems software.

Samples were excluded 1) if more than one PCR product was formed, 2) if there was no amplification (as indicated by the software and recognised in the amplification plot), or if the resulting data-point was an extreme outlier (more than three times the interquartile range) 3) for the ratio of the reference genes or 4) for the expression level of one of the genes of interest in the nucleus. If a sample failed to reach these criteria, it was excluded from the analysis for all peptides in the nucleus.

Expression levels of hypothalamic neuropeptides were first normalised to that of the reference genes cyclophilin and β-actin [409] and then either to that of control males of the same age or to that of animals of 380 days of the same group and sex.

Data analysis

The results were analysed using SPSS for Windows, version 12. All data were checked for a normal distribution and subjected to logarithmic transformation if necessary for analysis in ANOVA. The data are expressed as mean ± SEM. Effects of group and sex were analysed using univariate ANOVA, and the effect of age was tested by Bonferroni *post-hoc* tests. Pearson's bivariate correlation analysis was used to analyse correlations. *P*-values below 0.05 were considered to be statistically significant.

Because of the small number of pups for each biological and foster dam, it was not feasible to test for possible interference of effects of the dams with group effects on the body dimensions and gene expression levels of the pups. However, in a larger study [512 (Chapter 3)], the effects of the biological and foster dams on growth of control and FR rats were found to be negligible.

Results

Characteristics of the animals

The 94 animals used in this study were born from 28 of the 33 dams in the complete experiment and fostered to 13 control and 8 FR dams on day 2. The original litter size of the FR foster dams (12.3 ± 0.7 pups) was not different from that of the control foster dams (11.9 ± 0.4 pups, *P*>0.650). Nor did the original litter size of FR pups (11.7 ± 0.3 pups) differ from that of control pups (12.1 ± 0.3 pups, *P*>0.450). Of the 94 pups in this study, 83% were cross-fostered, whereas 17% (eight control and eight FR animals) remained with the same dam after the random redistribution on day 2. Other characteristics of the animals are shown in

Table 2. In previous studies, we have shown that FR rats have persistently reduced body weight, BMI, fat mass, and food intake, whereas adult resting energy expenditure was normal for the reduced body size [150, 152, 512, 513].

Table 2. Characteristics of the animals used in the experiment

day	group	sex	n	biological litters	foster litters	body weight (g)	body length (cm)	BMI (g/cm^2)	leptin (ng/ml)
2	control	males	27	18	-	7.6 ± 0.17	5.4 ± 0.06	0.27 ± 0.01	-
	FR		30	20	-	7.5 ± 0.14	5.4 ± 0.05	0.26 ± 0.003	-
	control	females	18	16	-	6.9 ± 0.20	5.1 ± 0.05	0.26 ± 0.01	-
	FR		19	15	-	7.2 ± 0.19	5.2 ± 0.06	0.27 ± 0.01	-
					P group	0.849	0.766	0.910	
					P sex	**0.005**	**0.001**	0.757	
10	control	males	10	8	4	21.0 ± 0.54	7.7 ± 0.12	0.36 ± 0.01	3.1 ± 0.43
	FR		10	9	2	14.4 ± 0.51	6.6 ± 0.11	0.33 ± 0.01	0.6 ± 0.12
	control	females	10	9	4	19.5 ± 0.40	7.3 ± 0.11	0.37 ± 0.01	3.3 ± 0.33
	FR		9	7	2	13.2 ± 0.36	6.3 ± 0.09	0.33 ± 0.01	0.7 ± 0.14
					P group	**<0.001**	**<0.001**	**0.001**	**<0.001**
					P sex	**0.008**	**0.005**	0.510	0.645
25	control	males	8	7	5	67.1 ± 1.61	12.5 ± 0.21	0.43 ± 0.01	3.1 ± 0.39
	FR		8	8	6	45.6 ± 2.60	11.1 ± 0.30	0.37 ± 0.02	1.6 ± 0.17
					P group	**<0.001**	**0.002**	**0.018**	**0.004**
380	control	males	9	8	6	610 ± 15.91	27.2 ± 0.28	0.83 ± 0.01	10.4 ± 0.89
	FR		12	10	6	548 ± 17.38	26.3 ± 0.17	0.79 ± 0.02	8.7 ± 0.77
	control	females	8	7	4	324 ± 12.13	22.4 ± 0.21	0.65 ± 0.02	6.1 ± 0.56
	FR		10	10	6	286 ± 8.75	21.7 ± 0.13	0.61 ± 0.01	4.3 ± 0.90
					P group	**0.002**	**<0.001**	**0.035**	**0.043**
					P sex	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Data are expressed as mean ± SEM. No significant interactions were found between group and sex (*P*=0.212–0.951). *P*-values are shown in bold when the group or sex effect was significant.

Body dimensions were analysed for all animals at the same age together using ANOVA with group and sex as independent variables. Provided that there is no significant interaction between group and sex, this analysis allows for the effect of group to be investigated independent of sex differences, and *vice versa*. On day 2 (before the random redistribution into control and FR litters), body weight, body length, and BMI were similar between the future FR and control animals (**Table 2**). In FR animals, body weight was significantly reduced from day 4 until the end of the experiment on day 380 (*P*<0.001), see **Figure 1**. At the time of killing, body weight, body length, BMI, and serum leptin levels were reduced in FR animals at all three ages (**Table 2**). Body weight and BMI correlated positively with leptin at all ages (*P*<0.001–0.054, data not shown). On day 10, females were smaller than males, but had a similar BMI and serum leptin levels. In adulthood, all body dimensions were significantly larger in males.

After application of the PCR criteria (see Methods section), 75 ARC samples were analysed for neuropeptide expression, whereas the analyses for the PVN and LHA were performed on 71 and 81 samples, respectively.
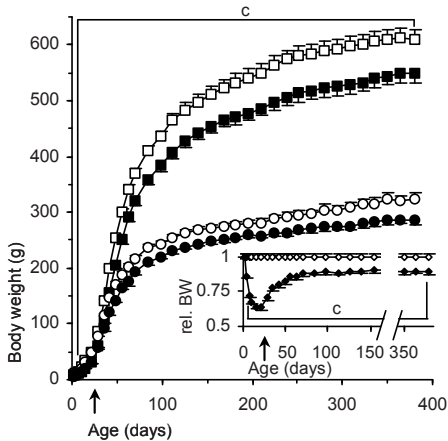

Figure 1. Mean body weight throughout the experiment of control males (□) and females (○) and FR male (■) and female (●) rats. Body weight of FR animals (◆) relative to that of controls (◇) is shown in the inset. Values are mean ± SEM. Weaning is indicated by an arrow. rel. BW, relative body weight. c, *P*<0.001 for FR *vs.* control from day 4 until 380.

ARC neuropeptide expression: effects of early postnatal food restriction

The levels of mRNA expression for the four ARC neuropeptides normalised to those of cyclophilin and β-actin are shown relative to those of control males in **Figure 2A–C**. Again, the absence of any significant interactions between group and sex allowed the simultaneous analysis of males and females.

On day 10, 25, and 380, the expression levels for NPY and AgRP mRNA did not differ significantly between control and FR rats. POMC mRNA expression was similar between the groups at the ages of 10 and 25 days, whereas its expression was significantly reduced in FR rats on day 380. CART mRNA expression levels were significantly reduced at all three ages tested. In addition, significant sex differences were found in expression levels on day 10 (NPY, AgRP, and POMC) and on day 380 (POMC and CART).
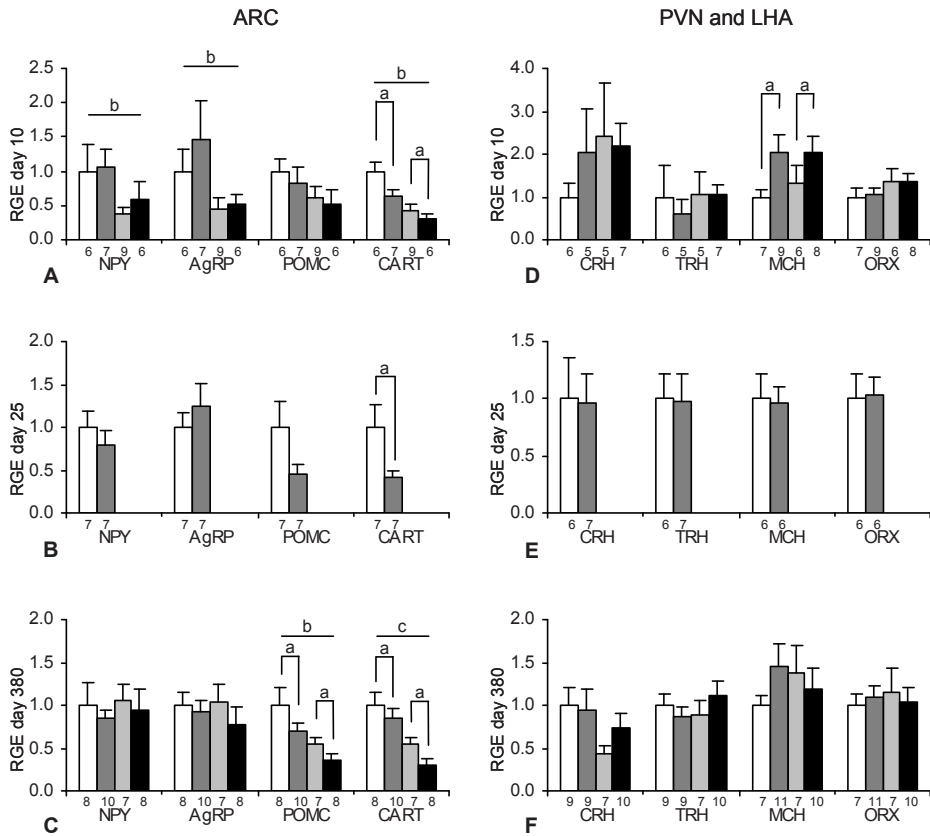
Figure 2. Neuropeptide mRNA levels relative to those of control males (white bars) in the ARC (A–C) and in the PVN and LHA (D–F) on day 10 (**A** and **D**), day 25 (**B** and **E**), and around day 380 (**C** and **F**) for FR males (dark grey bars), control females (light grey bars), and FR females (black bars). Data are expressed as mean + SEM. The number of animals in each group is indicated below the bars. No significant interactions were found between group and sex (P=0.144–0.920). RGE, relative gene expression. a, $P<0.05$ for FR $vs.$ control; b, $P<0.01$ for females $vs.$ males; c, $P<0.001$ for females $vs.$ males.

Although most of the ARC neuropeptides showed no significant changes in their expression level, in the young animals, there was a tendency in the FR group for increased expression of the orexigenic peptides (NPY and AgRP) and for decreased expression of the anorexigenic peptides (POMC and CART). Since the isolated ARC region of each animal contained both the NPY/AgRP neurons and the POMC/CART neurons, the ratio of orexigenic to anorexigenic expression (orex/anorex) in each animal was computed as the mean relative expression of NPY plus AgRP divided by the mean relative expression of POMC plus CART. This ratio was shifted significantly towards orexigenic expression in FR animals on days 10 and 25, see **Figure 3**. On day 380, there was no significant difference between the groups, but the orex/anorex ratio was significantly elevated in middle-aged females relative to males.
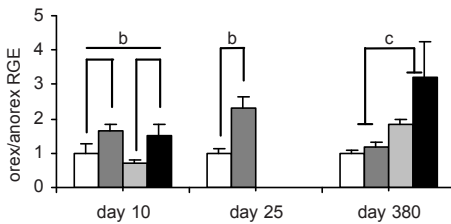
Figure 3. Mean orex/anorex mRNA expression in the ARC of FR males (dark grey bars) and females (black bars) and control female rats (light grey bars) on day 10 and 25 and around day 380 relative to that of control males (white bars) of that age. Data are expressed as mean + SEM. RGE, relative gene expression; orex/anorex, ratio of the mean mRNA expression levels of the orexigenic neuropeptides NPY and AgRP to that of the anorexigenic neuropeptides POMC and CART. b, $P<0.01$ for FR *vs.* control; c, $P<0.001$ for females *vs.* males.

PVN neuropeptide expression: effects of early postnatal food restriction

On day 10, 25, and 380, the mRNA levels of both CRH and TRH did not differ significantly between control and FR rats, see **Figure 2D–F**. No significant sex differences were found in the expression of these neuropeptides.

LHA neuropeptide expression: effects of early postnatal food restriction

On day 10, MCH mRNA levels were significantly increased in FR rats, see **Figure 2D**. This difference was not present on days 25 and 380, see **Figure 2E, F**. Expression levels of ORX were comparable between control and FR rats at all three ages tested (**Figure 2D–F**). No significant sex differences were found in the expression of these neuropeptides.

Neuropeptide expression: ontogeny

To investigate developmental changes, neuropeptide mRNA expression was computed relative to that of middle-aged animals, instead of control males at each age. This analysis was performed on males, because for males, an extra group was included on day 25. Since no significant interactions were found between age and group ($P=0.205–0.956$), control and FR males were analysed together. The absence of these interactions means that although there were group differences in expression levels, the developmental pattern was not different between the groups.

The overall effect for group (i.e. independent of age) just reached significance for CART ($P=0.043$), but not for any of the other peptides ($P=0.265–0.841$). There was a significant effect of age for all peptides except AgRP, see **Figure 4**. *Post-hoc* tests revealed significant differences between the ages. NPY mRNA levels decreased significantly between day 10 and day 380. AgRP expression showed a similar pattern, but no significant differences were found. The expression levels of the anorexigenic ARC peptides POMC and CART increased significantly between days 10 and 25 and again between day 25 and 380. Expression levels for CRH and TRH in the PVN and ORX in the LHA rose significantly from day 10 to days 25 and 380, with no further increase between weaning and middle-age. MCH in the LHA, like POMC and CART, increased between days 10 and 25 and between days 25 and 380.

Overall, the developmental patterns in females resembled those in males, with the exception that females had similar levels of NPY and AgRP mRNA at both ages (data not shown).
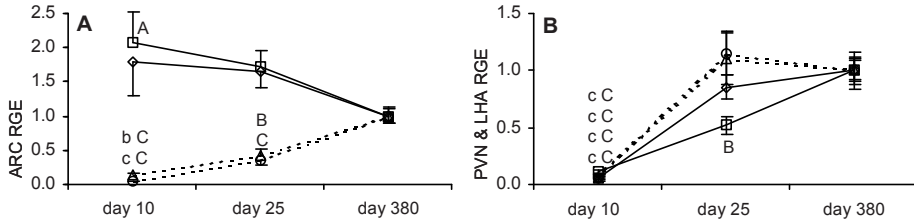

Figure 4. Expression levels in males relative to those on day 380 in the ARC (A) and in the PVN and LHA (B). **A**, relative expression levels for NPY (□), AgRP (◇), POMC (△), and CART (○); **B**, relative expression levels for CRH (△), TRH (○), MCH (□), and ORX (◇). For ease of comparison, the data-points for orexigenic neuropeptides are connected by a line and those for anorexigenic neuropeptides by a dotted line. Values are expressed as mean ± SEM. RGE, relative gene expression. a, $P<0.05$; b, $P<0.01$; c, $P<0.001$ (*vs.* day 25 in lowercase letters, *vs.* day 380 in uppercase letters), as determined by a Bonferroni *post-hoc* test.

Neuropeptide expression: correlations with other parameters

The expression levels of the orexigenic neuropeptides and those of the anorexigenic neuropeptides in each nucleus showed a strong positive correlation in all animals, see **Table 3**. Such correlations were not found for the expression levels with body weight, BMI, food intake, and leptin (except for group or sex differences).

On day 10, a decrease in BMI standard deviation score (SDS, distance from the control mean in standard deviations) between days 2 and 10 was associated with a lower orex/anorex ratio in FR animals ($R=0.643$, $P=0.018$) but not in controls ($R=0.001$, $P=0.997$), see **Figure 5A**. On day 25, there was a continuous relationship between the change in body weight SDS between days 2 and 21 and the orex/anorex ratio ($R=–0.875$, $P<0.001$), see **Figure 5B**.

Table 3. Positive correlations between the relative mRNA levels of the orexigenic and the anorexigenic neuropeptides in each age group

	10 days		25 days		380 days	
	n	correlations	*n*	correlations	*n*	correlations
NPY & AgRP	28	R=0.852, P<**0.001**	14	R=0.812, P<**0.001**	33	R=0.792, P<**0.001**
POMC & CART	28	R=0.703, P<**0.001**	14	R=0.958, P<**0.001**	33	R=0.800, P<**0.001**
CRH & TRH	23	R=0.627, P=**0.001**	13	R=0.716, P=**0.006**	35	R=0.309, P=0.071
MCH & ORX	25	R=0.358, P=0.079	12	R=0.607, P=**0.036**	36	R=0.421, P=**0.011**

P-values are shown in bold when the correlation was significant.

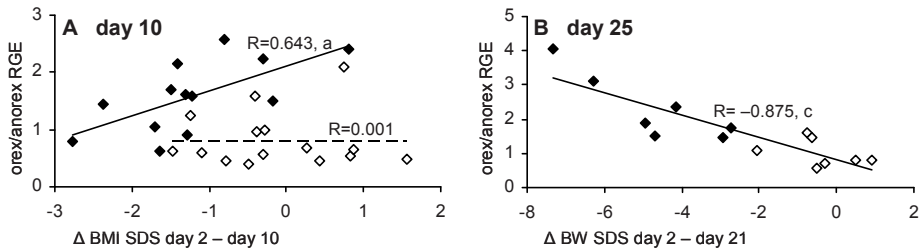**Figure 5.** Relationships between neuropeptide expression levels and growth parameters in juvenile control (◇, dashed line in A) and FR rats (◆, solid line). **A**, relationship between the change (Δ) in BMI SDS from day 2 to 10 and orex/anorex relative gene expression on day 10 is shown for control and FR rats separately; **B**, relationship between the change in body weight (BW) SDS from day 2 to 21 and orex/anorex relative gene expression on day 25 is shown for control and FR rats together. RGE, relative gene expression; orex/anorex, ratio of the mean mRNA expression levels of the orexigenic neuropeptides NPY and AgRP to that of the anorexigenic neuropeptides POMC and CART. Significance of correlation: a, *P*<0.05; c, *P*<0.001.

Discussion

In this study, raising rats in large litters transiently affected the ratio between expression of orexigenic and anorexigenic neuropeptides in the hypothalamus. In both male and female juvenile rats that were subjected to early postnatal food restriction, NPY and AgRP mRNA expression levels in the ARC had increased relative to those of POMC and CART. In addition, MCH mRNA expression in the LHA was elevated in suckling FR animals. In adulthood, despite still reduced body dimensions and lower POMC and CART expression, the ARC orex/anorex ratio had normalised.

Acute effects of early postnatal food restriction

During the lactation period, the hypothalamic circuitry that regulates energy balance in the adult rodent is not fully functional yet [67]. For example, neonatal rats do not reduce their food intake in response to leptin administration [502, 574] like adult rats do, and it is uncertain whether it alters ARC neuropeptide expression [4, 503] like in adults [544, 668]. Here we show that undernutrition does elicit an orexigenic response from the ARC already at a young age, since both the suckling and weanling FR animals reacted appropriately to the food restriction by increasing the ratio between ARC orexigenic and anorexigenic expression.

In contrast to the adult situation, where food deprivation decreases CRH and TRH expression in the PVN [276] and increases that of MCH and ORX in the LHA [46, 84, 499], early postnatal food restriction elicited little change in PVN and LHA expression. Since in rodents the projections of the ARC to the PVN and LHA are not present at birth, but rather develop during the first three weeks of postnatal life [67, 211], it is plausible that the orexigenic signal derived from the ARC of the juvenile FR rats did not reach these regions.

Long-term programming effects of early postnatal food restriction

Despite some catch-up in all body dimensions, early postnatal food restriction resulted in middle-aged animals that were persistently lighter (body weight), shorter (body length), and thinner (BMI) than control animals. Although the expression of POMC and CART was significantly reduced in FR animals at this age, the ARC orex/anorex ratio had normalised. This may suggest that the body dimensions were no longer perceived as small by the regulating system. In other words, the FR rats may have a lowered body weight set-point.

Body weight of FR rats at this age was about 10% below that of control animals. After more acute food deprivation, similar reductions in body weight have been reported to induce significant changes in hypothalamic orexigenic [84, 499, 533] and anorexigenic [276, 533] expression levels. Reductions in POMC and CART expression were similar between the present study and after acute food deprivation [533]. NPY and AgRP expression, however, instead of being elevated, rather tended to be lower in FR rats. As this resulted in an unchanged ratio between the ARC orexigenic and anorexigenic peptides, it is not surprising that the expression levels in the PVN and LHA were unchanged in the FR animals. Thus, in FR rats, POMC and CART expression appear to be normally adapted to body weight. NPY and AgRP expression, however, was lower than expected based upon body weight. If permanent changes in body weight set-point involve ARC nucleus neurons, then NPY/AgRP neurons more likely than POMC/CART neurons play a role in programming.

Possible mechanisms of body weight regulation and its programming

At all three ages tested, FR and control rats showed strong positive correlations between the expression levels of the orexigenic and anorexigenic peptides that are co-expressed within a nucleus (NPY with AgRP, POMC with CART, CRH with TRH, and MCH with ORX). This suggests that the expression of these peptides is coregulated.

On day 10, FR animals with a higher ARC orex/anorex ratio had lost fewer BMI SDS points between days 2 and 10 (and hence probably less body fat). This association was absent in control animals. Note, however, that at this age, the hypothalamic circuitry emanating from the ARC is still developing [67, 211], the orexigenic signal from the ARC did not seem to reach the PVN and LHA (present data), and FR pups have little opportunity to increase food intake during the lactation period. Therefore, this association is unlikely to be caused by increased food intake, and it must arise from another mechanism.

On day 25, and hence at the end of the food restriction period, all weanling males showed a negative correlation between the change in body weight SDS and the ARC orex/anorex ratio. A relative loss of body weight in the preceding weeks coincided with higher orexigenic expression. This may represent an attempt to increase energy reserves to permit catch-up growth at this time when food was already becoming available (because pups of different litter sizes have all been shown to start eating chow from day 16 [20]).

Our findings are largely in agreement with those of studies using other models that manipulate perinatal nutrition: a transient increase in orexigenic relative to anorexigenic

hypothalamic expression in juveniles [36, 64, 128, 335, 371, 506], with normalised expression levels in older animals [265, 372, 547]. The fact that different manipulations of perinatal nutrition produce such similar results indicates that this is an essential regulatory phenomenon. Further, it is vital to recognise that this outcome still leaves the possibility of more permanent changes in other areas involved in the regulation of energy balance, such as the brainstem or higher brain areas.

Additional findings: sex differences and ontogeny

Besides the effects of early postnatal food restriction, we also found differences in hypothalamic gene expression between the sexes and between different ages. These results generally confirmed and elaborated on previous findings.

Our male and female suckling pups showed a similar ARC orex/anorex ratio and similar expression levels of PVN and LHA peptides. This is in agreement with previous reports that found no sex differences in expression in juvenile rats for NPY, AgRP, and CRH [357, 449, 625]. In adulthood, we showed differential expression for POMC and CART, with lower levels in females, and a trend for lower levels of CRH. These data are in agreement with a previous study that showed similar NPY expression in both sexes [612], but in conflict with another study that did find a sex difference in ORX expression [289]. For CRH, conflicting data exist with either lower or higher expression in females [198, 284, 471, 546, 625], perhaps related to variation during the oestrous cycle. Most hypothalamic gene expression studies, including the present one, seem to suggest a higher orexigenic drive in adult females.

In agreement with our present data, previous authors have also reported stable or decreasing expression of NPY and AgRP over time [215, 300, 662] and mostly increasing expression of POMC and CART [313, 652, 662]. This suggests that juveniles have a stronger orexigenic drive in the ARC than adult rats. This may serve to maximise milk intake, and therefore growth, in the neonatal period [67]. Our data on increasing expression levels of PVN and LHA peptides are consistent with previous reports of rising CRH, TRH, and ORX expression during the lactation period [104, 209, 616, 671]. Conversely, unchanging levels of CRH, TRH, and MCH were also reported at this age [26, 71, 81, 599]. The overall increase in PVN and LHA expression levels with age that we found may be indicative of a tighter regulation of energy balance in the adult rat.

Summarising, the reliability of our measurements is verified by the fact that we found comparable sex differences and ontogeny to those reported previously.

Implications and conclusions

In the present study, most of the individual neuropeptides were not found to be differentially expressed in large-litter pups, although the ARC orex/anorex ratio was significantly altered. It may be that FR rats use individual strategies to promote survival and catch-up growth, resulting in either the upregulation of one or more orexigenic peptides or the downregulation of one or more anorexigenic peptides. At any rate, the differences found

in the ARC orex/anorex ratio were not solely due to the significant differences in CART mRNA expression, since omitting CART from the equation produced similar results (data not shown).

Now that the effects of early postnatal food restriction on gene expression have been described, a next step may be to investigate possible programming effects on neuropeptide protein levels and functionality. The results of a previous study which reported increased levels of NPY peptide in juvenile large-litter animals [494] suggests that protein levels may be similarly affected as gene expression. Programming of the projections between the ARC and other hypothalamic nuclei may be particularly interesting to examine. Since neonatal leptin is known to be essential for the formation of these projections [67] and leptin levels were extremely low in the suckling FR rats, the ARC projections might be expected to be permanently altered in adult FR rats due to underdevelopment in early life. In addition, studies by Davidowa and colleagues in small-litter rats suggest that hypothalamic functionality may be programmed [reviewed in 213, 214].

It has been suggested that in the case of a serious mismatch between the early and late environments, for example an abundance of highly palatable and energy-dense food in later life after a low energy intake in early life, populations may be at increased risk for obesity and other diseases [203, 227, 231, 455]. Programming of the hypothalamic regulation of energy balance may be partly responsible for these detrimental effects. In this regard, it may also be interesting to investigate the effects of highly palatable diets in adult FR rats.

Early postnatal food restriction induces persistent reductions in body size. In this study, we demonstrated that this is accompanied by a transient increase in the ratio of orexigenic to anorexigenic expression in the hypothalamic ARC. This orexigenic signal hardly affected gene expression in the PVN and LHA, which is consistent with the immature connections that were previously reported at this age. Despite still reduced body dimensions, middle-aged FR rats showed normalised levels of hypothalamic neuropeptide expression. This may indicate programming of the hypothalamic regulation of energy balance. When these results are combined with the ones that were previously obtained, a phenotype arises of juvenile rats that grow very little as a result of undernutrition, that are very thin, with a low fat mass, and that increase hypothalamic orexigenic expression, perhaps in an attempt to increase energy availability for growth [152, 512, present data]. In adulthood, these animals remain thin (low BMI) and lean (low fat percentage) despite considerable catch-up growth, with a low energy intake, but rather normal energy expenditure [150, 512, 513]. In contrast to what might be expected in such a situation, hypothalamic orexigenic expression is normal at this age (present data). In summary, the present study demonstrates that in rats, early postnatal food restriction can alter the programming of the hypothalamic regulation of energy balance. If energy balance regulation is similarly affected in perinatally malnourished humans, temporarily increased orexigenic expression may offer a partial explanation for their increased risk to develop obesity.

Acknowledgements