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Nutritional regulation of metabolic hormones implicated in the postnatal programming of obesity – the case of leptin and ghrelin

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Presenté par

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Scientific communications

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Abbreviations

List of abbreviations	
aMSH	alpha-melanocyte stimulating hormone
A/DAG ratio	acyl / des-acyl ghrelin ratio
ACTH	Adrenocorticotrophic hormone
AgRP	agouti-related protein
Akt	protein kinase B
AMPK	AMP-activated protein kinase
ARH	arcuate nucleus of the hypothalamus
ATP	Adénosine triphosphate
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BMI	body mass index
BMP	bone morphogenetic protein
BrdU	Bromodeoxyuridine
Ca2+	calcium ion
CART	cocaine-and-amphetamine regulated transcript
CCK	choleocystokinin
CNS	central nervous system
CPT1	carnitine palmitoyltransferase 1
CRF / CRH	corticotrophin releasing factor / hormone
CSF	cerebrospinal fluid
Db	leptin receptor gene
DMH	dorsomedial nucleus of the hypothalamus
E	embryonic day
erbB2	receptor tyrosine-protein kinase erbB2
ERK	extracellular signal-regulated kinase
FBG	fasting blood glucose
FFA	free fatty acids
FTO	fat mass and obesity-associated protein
G	gestational day
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHSR	growth hormone secretagogue receptor
GLP-1	glucagon-like peptide 1
GnRH	gonadotropin-releasing hormone
GOAT	ghrelin-O-acyltransferase
HDL	high-density lipoprotein
HFD	high-fat diet
HPA	hypothalamic-pituitary-adrenal
HPT	hypothalamic-pituitary-thyroid
ICV	intracerebroventricular
IGF-1	insulin-like growth factor 1
IL	interleukin
IP	intraperitoneal

IR	**Immunoreactive**
IRS1	**insulin receptor substrate 1**
IUGR	**intra-uterine growth restriction**
JAK	**janus kinase**
Kcal	**kilocalories**
KO	**knock-out (genetics)**
LDL	**low-density lipoprotein**
LGA	**large for gestational age**
LL	**large litter**
MCH	**melanin-concentrating hormone**
MCR	**melanocortin receptor**
ME	**median eminence**
MetS	**metabolic syndrome**
mRNA	**messenger ribonucleic acid**
NL	**normal litter**
NPY	**neuropeptide Y**
Ob-R	**leptin receptor**
Ob	**leptin gene**
P	**postnatal day**
p	**phosphorylated**
PI3K	**phosphoinositide kinase 3**
PKC	**protein kinase C**
PLC	**phospholipase C**
POMC	**proopiomelanocortin**
PP	**pancreatic polypeptide**
PTP1B	**protein-tyrosine phosphatase 1b**
PVH	**paraventricular nucleus**
PWS	**prader-willi syndrome**
PYY	**peptide YY**
ROS	**reactive oxygen species**
SL	**small litter**
SOCS3	**suppressor of cytokine signaling 3**
SRP	**stresscopin-related peptide**
STAT3	**signal transducer and activator of transcription 3**
STZ	**streptozotocin**
T3 / T4	**triiodothyronine / thyroxine**
TCPTP	**tyrosine-protein phosphatase non-receptor type 2**
TNF-a	**tumor necrosis factor alpha**
TRH	**thyrotropin-releasing hormone**
TSH	**thyroid-stimulating hormone**
UCP	**uncoupling protein**
V3	**third ventricle**
VEGF	**vascular-endothelial growth factor**
VMH	**ventromedial nucleus of the hypothalamus**

Abstract

Abstract

It is increasingly accepted that the nutritional and hormonal environment during the perinatal development of an organism can have profound and lasting effects on its metabolic health. In particular, it has been recognized that accelerated growth during critical periods of development, whether in the context of maternal obesity and fetal overnutrition or fetal undernutrition followed by rapid catch-up growth, can increase the risk of obesity and associated metabolic diseases such as diabetes and cardiovascular disease.

The hypothalamus has a crucial role in the regulation of metabolic functions such as energy and glucose homeostasis and anatomic processes, and perturbed neonatal development of the neuronal circuits involved in these regulatory processes can reproduce many of the metabolic defects also associated with perinatal overnutrition. It was recently demonstrated that certain nutritionally regulated hormones such as leptin and ghrelin are involved in the neonatal development of the hypothalamus, and that disturbances in their postnatal signalling can cause persistent adverse metabolic effects.

However, little is known about the causal link between neonatal endocrine disturbances and long-term metabolic outcomes in the context of postnatal overnutrition. The purpose of this thesis was thus to assess how the postnatal ghrelin and leptin systems are affected by postnatal overnutrition, using a divergent litter size model of overnutrition, and further to investigate the causal contribution of such endocrine alterations in the pathogenesis of obesity and metabolic disease in postnatally overnourished mice.

During the course of my thesis, we have found that postnatal overnutrition causes numerous alterations in the postnatal ghrelin system, including impaired secretion and synthesis of ghrelin, and reduced central sensitivity to ghrelin related to impaired transport of ghrelin from the periphery into the hypothalamus. The second finding may explain our observation that neonatal ghrelin treatment is relatively ineffective in altering the metabolic programming of postnatally overfed mice.

In the second part of my thesis, based on previous observations that postnatally overfed mice exhibit hyperleptinemia during neonatal development, we investigated the effects of neonatal leptin antagonism on long-term metabolic outcomes in overfed pups, and discovered that this temporally restricted partial leptin blockade was sufficient to completely normalize the fat mass and insulin sensitivity of the overfed mice in adulthood, suggesting that neonatal hyperleptinemia plays a crucial role in the adverse metabolic programming observed in postnatally overfed rodents.

Taken together these data provide the first detailed investigations on how the neonatal secretion pattern of leptin and ghrelin is altered by postnatal overnutrition, and shows that in addition to major alterations at the peripheral level, neonatally overfed mice also display impaired central responsiveness to these hormones during postnatal development. Whereas the precise contribution of the neonatal defects in ghrelin signalling to the adverse metabolic programming in overfed pups remains to be determined, we demonstrate that hyperleptinemia is a crucial factor, and that blocking excessive leptin signalling during this critical period of development can substantially alter the adult phenotype of postnatally overfed mice.

Resumé

Resumé

Il est désormais clairement établi que la vélocité de croissance dans les premiers mois de la vie influence le risque d'apparition de maladies métaboliques, telles que l'obésité à l'âge adulte. L'un des mécanismes sous-jacents à cette programmation néonatale pourrait mettre en jeu des perturbations de l'environnement hormonal périnatal pendant des périodes critiques du développement. Cette hypothèse repose sur des travaux réalisés au laboratoire montrant que pendant le développement postnatal, des hormones telles que la ghréline et la leptine peuvent influencer le développement des circuits neuronaux impliqués dans la régulation de la prise alimentaire à l'âge adulte.

Dans ce contexte, l'objectif général de ce travail de thèse a été de définir les médiateurs hormonaux par lesquels l'environnement nutritionnel périnatal peut influencer le développement de maladies métaboliques à l'âge adulte. Une attention particulière a été portée à la contribution de la ghréline et de la leptine.

Pour induire une surnutrition postnatale, nous avons utilisé un modèle murin de surnutrition postnatale induit par la réduction de la taille de portées. Au troisième jour de vie post-natale, les portées ont été réduites à 3 petits (SL) pour induire une suralimentation et les portées contrôles ont conservé 7 petits. Comparées aux souris NL, les souris SL présentent un gain de poids rapide pendant la lactation, et présentent un surpoids à l'âge adulte même sous régime de nourriture standard. A l'âge adulte, les souris SL ont une adiposité et une glycémie à jeun plus élevée. Sous régime « obésogène » les souris SL présentent également une prise de poids et de graisse plus importante que les souris NL.

La première partie de ces travaux a été dédiée à l'étude de la nutrition périnatale sur le développement du système ghrélinergique. Les souris SL présentent des taux réduits de ghréline total et active pendant la troisième semaine de vie post-natale. Ces diminutions des taux de ghréline sont associées à une diminution de l'expression de ghreline dans l'estomac. La normalisation de la ghrélinémie ne parvient pas à restaurer un phénotype métabolique normal chez les souris SL ce qui suggère que les souriceaux SL présenteraient une résistance a la ghréline. En accord avec cette hypothèse, les

souriceaux SL présentent une atténuation de la réponse centrale suite à l'injection périphérique de ghréline. Les mécanismes sous-jacents à cette résistance à la ghréline semblent mettre en jeu un défaut de transport de l'hormone via les tanycytes de l'éminence médiane.

La seconde partie de mon travail de thèse a consisté à étudier l'importance de la leptine dans la programmation nutritionnelle. Etant connu que les souris SL présentent des taux anormalement élevés de leptine pendant la deuxième semaine de vie postnatale, nous avons émis l'hypothèse que le blocage partiel de la leptine chez les souris SL pourrait avoir des effets bénéfiques sur le métabolisme de ces souris. Les animaux injectés néonatalement avec l'antagoniste de la leptine ne présentent pas de différences de poids par rapport aux animaux contrôles. En revanche, l'injection de l'antagoniste de la leptine chez les souriceaux SL induit une amélioration de leur masse grasse et une normalisation de leur glycémie. Cette amélioration du phénotype métabolique des souris SL est associée à un rétablissement de la sensibilité centrale à l'hormone leptine.

Ainsi, les travaux réalisés au cours de ce doctorat confortent l'importance de l'environnement hormonal périnatal, et en particulier de la ghéline et de la leptine, dans la programmation nutritionnelle.

Introduction

1. The epidemic of metabolic disease

1.1 Definition of obesity and the metabolic syndrome

1.1.1 Obesity

Obesity is defined as an accumulation of body fat that substantially exceeds the range at which the organism would function optimally. It is most commonly classified using the body mass index (BMI), which is a measure of relative weight derived by dividing an individuals weight in kilograms by his height in meters squared. The resulting values are typically grouped into four main categories:

Underweight:	**BMI < 18,5**
Normal weight:	**BMI 18,5 – 25**
Overweight:	**BMI 25 – 30**
Obese:	**BMI > 30**

Although the BMI gives only a rough estimate of adiposity and is subject to confounding factors like variations in lean mass, it is nonetheless considered to correlate reasonably well with adiposity on a population level (Romero-Corral et al., 2008), whereas other measures such as waist circumference and waist-to-hip ratio may be more appropriate on the individual level.

1.1.2 The Metabolic Syndrome

First described by Gerald Reaven (Reaven, 1988), the Metabolic Syndrome (MetS), is a disorder of energy metabolism that is characterized by the co-existence in the same individual of a number of medical signs that are each separately considered as risk factors for several overt diseases. The incomplete and still developing understanding of this disorder has given rise to numerous definitions, but it is typically diagnosed as the presence of at least 3 of the following 5 signs:

Abdominal obesity:	Defined as a waist circumference > 102 cm for men and > 88 cm for women
Elevated fasting glucose	Fasting Blood Glucose (FBG) > 5,6 mM
Hypertension	Blood pressure > 140/90 mmHg
Elevated triglycerides	Triglycerides > 1,7 mM
Reduced HDL	High-Density-Lipoprotein (HDL) < 40-50mg/dL

Abdominal obesity, along with any 2 of the remaining 4 symptoms would generally be classified as MetS. While MetS by itself is usually asymptomatic, it substantially elevates the risk for other overt health problems such as Type 2 Diabetes and cardiovascular disease (Ford, 2005; Wilson et al., 2005; Mottillo et al., 2010). Epidemiologically, obesity, and in particular abdominal obesity, is strongly associated with all other components of MetS to the point where obesity is commonly considered to be the main driver of MetS (Despres, 2006).

1.2 Trends in the prevalence of obesity/MetS

After remaining at a relatively stable level throughout the years 1960-1980, the past three decades has seen a more marked increase in the prevalence of obesity as measured by BMI. While the United States has experienced the most drastic shift, with the prevalence of obese adults more than tripling between the years 1980 and 2008 (Ogden and Carroll, 2010), a similar pattern can be observed in most western countries. In France for instance, the prevalence of adults with BMI > 30 increased from 8,5 to 15% just between 1997 and 2012 (Le Goff et al).

To account for this staggering increase in the prevalence of obesity and related comorbidities, many putative causes have been put forward. Most frequently mentioned are increased food intake due to greater and easier access to fast foods, as well as a decrease in physical activity due to motorized transports, growing prevalence of sedentary "desk jobs" and increasing leisure time spent with sedentary activities such as watching television and playing computer games.

Due to the proliferation of different diagnostic criteria, the prevalence and trends in MetS is more complicated to assess. In the US, the prevalence of the central component of MetS – abdominal obesity – more than tripled from 12,7 to 38,3% in men and from 19,4 to 59.9% in women (Okosun et al., 2004). The age-adjusted prevalence of MetS increased from 24,1 to 27% between the two measurement periods 1988-1994 and 1999-2000 (Ford et al., 2004), it is considered to currently affect between 25-30% of the population of US and Europe (Grundy, 2008).

1.3 Childhood obesity

1.3.1 Trends in prevalence

Perhaps more troubling than the surge in obesity in adults, is the even more striking increased prevalence of obesity in children. Determining rates of obesity in children is more complicated due to their continuous growth, but is typically defined as the deviation in BMI from the age and sex-specific norm, so that children with a BMI in the 85th percentile for their age group would be considered overweight and a BMI in the 95th percentile considered as obese. In the United States, the prevalence of obesity in children aged 6 -19 more than quadrupled from 4,4 to 18,2% between the years 1970 and 2010, while even children as young as 2-5 years old saw an increase in obesity from 4,8 % in 1972 to 12,1% in 2010 (Fryar et al., 2012). In most other European countries for which data is available, including France, a similar rising trend in the prevalence of overweight children is apparent (Lobstein et al., 2005).

1.3.2 Childhood obesity and adult disease

Childhood obesity has consistently been found to be a strong risk factor for obesity and its associated comorbidities in adulthood (Park et al. 2012; Allcock et al. 2009). Although it remains unclear whether the increased risk for such morbidities is merely due to the strong tendency for childhood obesity to persist in adulthood, there is evidence that the adverse effects of childhood obesity on health in adulthood, such as increased risk of coronary heart disease, diabetes, colorectal cancer, arthritis and gout, may be independent of whether the individual remains obese in adulthood (Must et al., 1992). Childhood obesity thus provides one important example of how an adverse metabolic state during the important periods of childhood development may impact the long-term metabolic health of the individual.

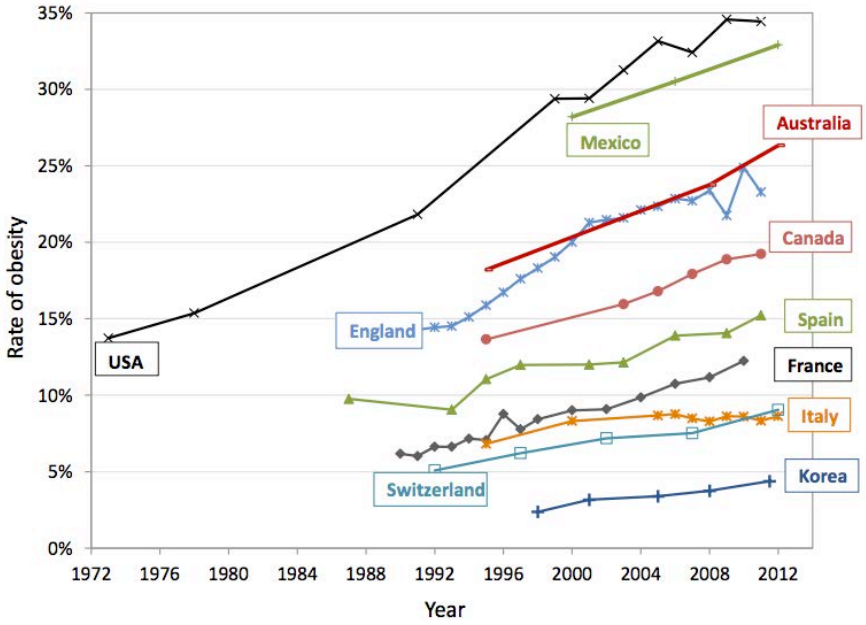

Figure 1: Trends in adult prevalence of obesity in OECD countries. Age- and gender-adjusted rates of obesity. Measured height and weight in Australia, England, Korea, Mexico and the United States, self-reported in other countries From OECD Obesity Update 2014, OECD analysis of health survey data.

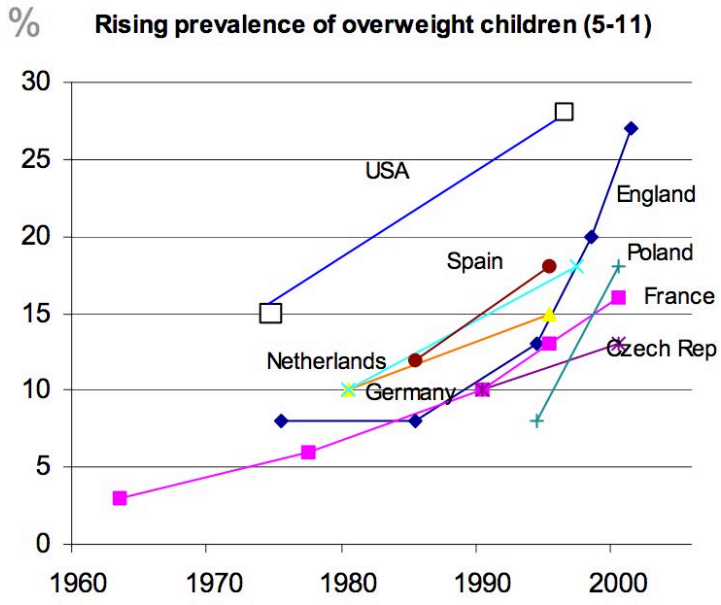

Figure 2: Prevalence of overweight and obesity among children in Europe and the US 1960-2000. From International Obesity Task Force EU Platform 2005 Briefing Paper (Lobstein et al. 2005).

2. The developmental origins of metabolic disease

2.1 The Barker hypothesis

The notion that the environment to which an individual is exposed during development may have an impact on his long-term health is known as the Barker hypothesis, or the developmental origins of disease. It proposes that adverse environmental experiences during development, such as maternal or fetal undernutrition, can act as cues for the environment that the organism is likely to experience in its adult life, and thus cause metabolic adaptations that help prepare it for better survival in this environment (Barker, 1997).

According to this theory, poor nutrition during perinatal development would cause a "thrifty phenotype" wherein the individual becomes adapted to an environment with short food supply by growing to a smaller stature, having a lower metabolic rate, and showing less behavioural activity to conserve energy (Martin and Bateson, 1999). If such individuals who experienced developmental undernutrition are later exposed to a more abundant environment, they may instead run a higher risk of developing obesity and type 2 diabetes due to the mismatch between actual and expected nutritional environment.

2.2 History and epidemiological evidence

The first clues that fetal malnutrition was linked to adult disease came from data on infant mortality in the UK in the early 20th century. The dominant cause of infant death at the time was low birth weight, and the regional pattern of infant mortality was later found to closely mirror the mortality from cardiovascular disease some 50 years later (Barker and Osmond, 1986). A link between low birth weight and increased risk of cardiovascular disease, hypertension and type 2 diabetes was later established in studies on cohorts of infants born around the same time (Hales et al., 1991; Fall et al., 1995).

2.2.1 The Dutch winter famine & the Stalingrad siege

The first evidence that directly linked fetal malnutrition to adult disease came from the cohort of infants born during the Dutch Winter Famine following the Second World War. In November 1944, the Germans enacted an embargo on food transport into the Netherlands that resulted in a brief but severe food shortage throughout the country, where the daily rations rapidly dropped below 1000 kcal/day and for a period between December 1944 and April 1945 was between 400 – 800 kcal/day, before the Netherlands were liberated in May 1945 and the food rations subsequently rose to more than 2000 kcal/day. Infants undergoing gestation during this brief period of famine were found to be significantly heavier as adults, had a higher incidence of heart disease, and diabetes, and reported poorer general health than infants that were born before or conceived after the famine (Roseboom et al., 2001).

However, these effects were found to depend on the period of gestation during which famine exposure took place. Men exposed during the first half of gestation almost doubled their risk of obesity as adults, whereas men exposed later in gestation or just after birth had a lower risk of obesity (Ravelli et al., 1976). Similarly, many of the varied adverse health effects were only found in infants exposed during early gestation (Roseboom et al., 2006). The Dutch cohort thus provided an important clue as to the existence of critical periods of development, during which a growing foetus would be particularly sensitive to environmental insults. Since early gestation exposure to famine was not even found to affect the birth weight of the foetus, these studies further suggest that the programming effects of fetal malnutrition may be independent of overall fetal growth (Schulz, 2010), providing a new dimension to the concept of foetal programming that was not evident from previous studies on the association of birth weight with adult disease.

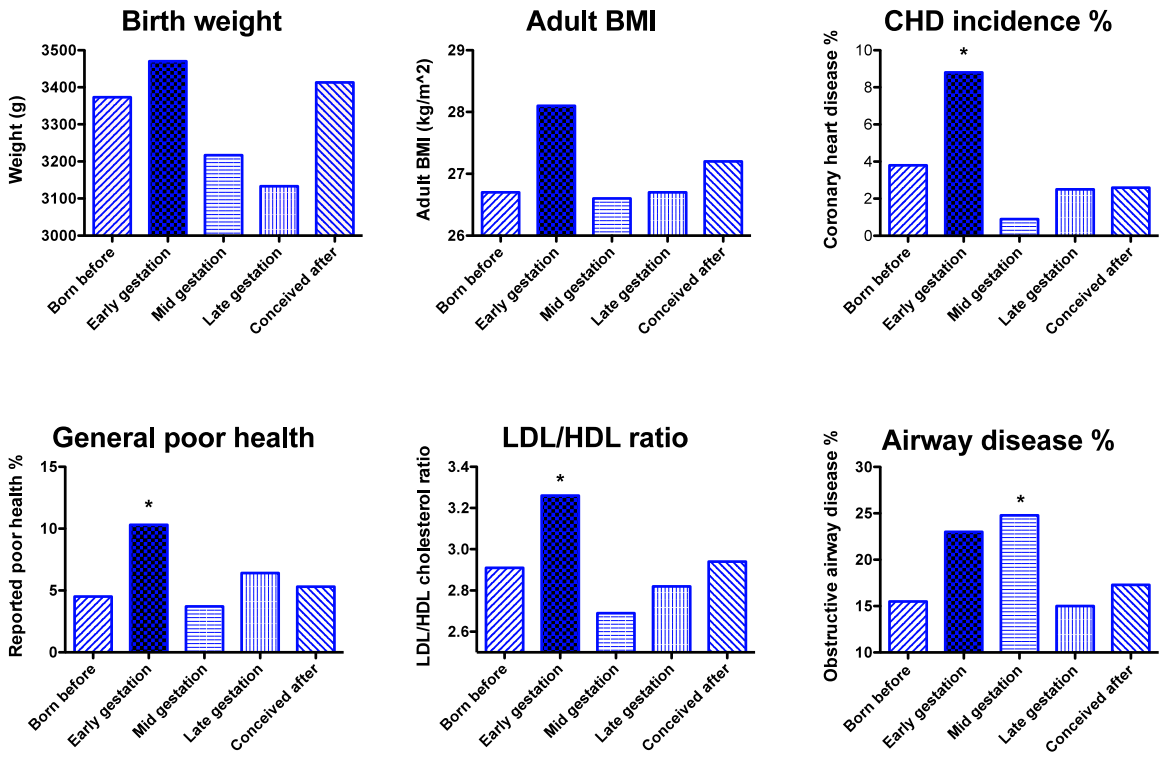

Figure 3: Long-term effects of exposure to the Dutch winter famine. One remarkable finding from this cohort was that adverse health effects of in-utero malnutrition in human foetuses seemed to be predominantly specific to exposure during early gestation, even though birth weight in this group was not different from non-exposed foetuses. * P > 0.05 compared to non-exposed infants. Adapted from Roseboom et al. 2001.

In stark contrast to the Dutch cohort, children born during the siege of Leningrad in Russia were exposed to a more prolonged famine, combined with less nutritional abundance even after the end of the siege, that prevented the catch-up growth observed in the Dutch children. In the Russian cohort, no association between exposure to famine and adult-onset obesity or metabolic disease could be found (Stanner and Yudkin, 2001), suggesting that the miss-match between perinatal and adult environments is a crucial factor in the development of metabolic disease.

Numerous epidemiological studies have since lent further support to the role of fetal malnutrition in the development of metabolic disease. To give a few examples, infant birth weight was found to be inversely associated with BMI and components of MetS in a chinese cohort (Mi et al., 2000). In the American Nurses Health Study, birth weight was inversely associated with the development of type 2 diabetes (Rich-Edwards et al., 1999), and similar results were reported in a Swedish cohort (Carlsson et al., 1999). In India, birth weight was reported to be inversely correlated with the development of cardiovascular disease (Stein et al., 1996).

While most of the epidemiological literature supports the fetal origins of disease hypothesis in one way or another, there was still controversy about the interpretation of these data due to the many possible confounding factors such as socioeconomic status and smoking, as well as methodological concerns with the statistical models used (Skogen and Overland, 2012). Hence, the effects of abnormal perinatal nutrition on disease development have more recently been extensively studied in animal models that permit a more controlled testing of the hypotheses.

2.3 Animal models of developmental programming of disease

2.3.1 Intra-uterine growth restriction

To address the question of whether fetal malnutrition is a direct causative factor in the programming of adult disease, various models have been used to induce intra-uterine growth restriction (IUGR) in animals. One of the most widely used models is global maternal food restriction during pregnancy. Restricting the maternal food supply to

30% of ad-libitum fed pregnant rats result in offspring with lowered birth weight who remain underweight up to 4 months of age but display delayed catch-up growth to reach normal weight by 30 weeks (Woodall et al., 1996), but display increased body fat percentage, hyperphagia and persistent hypertension already before catch-up growth takes place (Vickers et al., 2000). Offspring of dams exposed to global food restriction further display reduced leptin sensitivity, hyperinsulinemia, hypertriglyceridemia and an increased susceptibility to diet-induced obesity (Krechowec et al., 2006) as well as decreased locomotor activity (Vickers et al., 2003). Another popular model to induce IUGR is the maternal low protein diet, which produces similar adverse health outcomes in the offspring such as obesity, hyperglycemia, hyperinsulinemia, hypertension and exacerbated response to high-fat diets (Pinheiro et al., 2008; Bol et al., 2009; Sutton et al., 2010; Sarr et al., 2012). These animal models mirror the contrast between the human studies carried out in the Dutch and Stalingrad cohorts in that prolonged food restriction that prevents early catch-up growth seems to protect against many of the adverse long-term health effects of IUGR (Bieswal et al., 2006; Garg et al., 2012).

2.3.2 Postnatal undernutrition

In contrast to rodents who experienced undernutrition during prenatal development, rodents subjected to undernutrition during postnatal life have generally been observed to be protected from the development of obesity and metabolic disease. One model of postnatal undernutrition consists of raising rodent pups in large litters produced by grouping the pups of two litters born on the same day to one dam. Such so called “Large Litter” (LL) pups display reduced body weight gain and body fat percentage during the lactation period, and remain lighter and leaner into adulthood, with improved blood lipids and insulin sensitivity (López-Soldado et al., 2006).

Mice with polygenic sensitivity to diet-induced obesity have improved leptin sensitivity and reduced susceptibility to high-fat diet-induced obesity when raised in large litters (Patterson et al., 2010) , and large litter rearing protects from the adverse effects of maternal high-fat diet feeding during pregnancy and lactation (Sun et al., 2014). Another more common model of postnatal undernutrition is maternal restriction of calories or protein during lactation rather than gestation, similar to large litter rearing, offspring

from dams fed a restricted diet during lactation typically have lower weight and fat gain and enhanced insulin sensitivity (Palou et al., 2010; Garg et al., 2012). One possible explanation for the differences observed between these postnatally undernourished rodents and the animals that experience IUGR, may be the relative lack of rapid catch-up growth displayed by postnatally undernourished rodents (Velkoska et al., 2008a; Patterson et al., 2010). The notion that an accelerated early growth pattern, or a relative excess of nutrients during critical periods of development, might itself be a cause of later metabolic dysfunction will be explored next.

2.4 Perinatal overnutrition and adult disease

2.4.1 Epidemiology

While the vast majority of the early research understandably focused on the effects of low birth weight and undernutrition on later disease, it was recognized already in Barkers initial papers that there existed a U-shaped relationship between birth weight and disease, above average weight babies with a large abdominal circumference having an increased mortality from cardiovascular disease (Barker, 1995). Large for gestational age (LGA) babies, typically defined as having a birth weight > 4kg, have consistently been found to be at a higher risk for childhood and adult obesity (Yu et al., 2011), early development of MetS (Wang et al., 2007b) and type 2 diabetes (Harder et al., 2007).

LGA is strongly associated with gestational diabetes and maternal obesity, likely due to the fetal exposure to maternal hyperglycemia (Leipold et al., 2005; Aschwald et al., 2009) causing fetal hyperinsulinemia which may promote excessive growth. Maternal obesity is in of itself linked with various adverse metabolic outcomes in the offspring (O'Reilly and Reynolds, 2013) and it is likely that maternal obesity exposes the developing fetus to an excess of several circulating substrates aside from glucose such as triglycerides, free fatty acids and insulin.

The consistent association of abnormally high birth weights and maternal obesity and hyperglycemia with later onset of various metabolic diseases in the offspring suggested that perinatal overnutrition might also have some programming effect on the long-term metabolic state of the offspring. Although in the case of maternal obesity it is difficult to separate the effects of fetal overnutrition from genetic and socioeconomic factors, it was shown that obese women who underwent surgery to lose substantial amounts of weight prior to giving birth, had children who were significantly less likely to be born macrosomic or develop obesity later in life (Marceau et al., 2004; Barisione et al., 2012).

Besides high birth weight, rapid weight gain during early life may be another crucial factor in determining the susceptibility to adult metabolic disease. Eriksson and colleagues found that while being overweight as a child moderately increased the risk of cardiovascular disease, the risk was greatly exacerbated in overweight children who had

been smaller than average at birth (Eriksson et al., 1999). Rapid weight gain in infancy and early childhood has also consistently been found to increase the risk of later development of obesity, often independently of birth weight (Baird et al., 2005; Monteiro and Victora, 2005; Ong and Loos, 2006).

Since small birth weight often precipitates accelerated postnatal growth (ie "catch-up growth") it has been suggested that the adverse metabolic outcomes attributed to fetal malnutrition may actually be generalized to the adverse effects of rapid early growth (Lucas et al., 1999). Whether rapid gain during any particular postnatal period is especially predictive of adult disease remains unclear, since several studies find that abnormally accelerated growth during many different time spans up to several years after birth is associated with later obesity, but some have suggested that the first 6 months after birth is a period where children are particularly vulnerable to the effects of accelerated growth (Young et al., 2012). Although this period is coincidently when low birth weight infants are statistically most likely to experience catch-up growth, the particular importance of rapid growth in the first 6 months over later periods has been documented also in cohorts with normal birth weights (Ekelund et al., 2007).

2.4.2 Animal models of perinatal overnutrition

One popular model of perinatal overnutrition is that achieved by feeding female mice a high-fat diet for several months in order to render them obese and/or diabetic and subsequently breeding them to produce offspring. Similar to observations of maternal obesity in humans, diet-induced obesity by high-fat feeding in female rats has been shown to induce obesity and metabolic dysfunction in the offspring (Srinivasan et al., 2006; Samuelsson et al., 2008; Franco et al., 2012) an effect which is either due to the maternal obesity or the excess nutrition during gestation (Howie et al., 2013) rather than the high-fat diet itself (White et al., 2009).

Although most rodent studies apply the high-fat diet both during gestation and lactation, some studies have attempted to differentiate the effects on the fetus of maternal high-fat diet during only gestation or lactation by using cross-fostering techniques, with conflicting results. Masyama and Hiramatsu found that mouse offspring born to high-fat

fed dams but suckled by control dams were heavier and fatter with worse glucose tolerance than offspring born of control dams but suckled by high-fat fed dams, although both conditions worsened outcomes compared to offspring of dams fed a control diet (Masuyama and Hiramatsu, 2014).

In contrast, several groups have reported that only maternal high-fat feeding during lactation worsened metabolic outcomes for the offspring, while maternal high-fat feeding only during gestation did not increase adiposity and attenuated or abolished the adverse long-term metabolic effects compared to mice fed HFD during lactation (Sun et al., 2012a; Guberman et al., 2013; Vogt et al., 2014). It thus appears that rodents are particularly sensitive to the adverse effects of overnutrition during early postnatal life as compared to prenatal overnutrition.

As mentioned earlier, the differential effects of rapid growth early versus later in life has also been observed in rodent models, where feeding pregnant dams a low-protein diet only during gestation causes catch-up growth directly during the lactation period which leads to increased amounts of total and visceral fat mass in adulthood and a substantially shortened lifespan compared to offspring of mothers fed a control diet, whereas in contrast giving the mother the same low-protein diet also during lactation causes delayed catch-up growth where offspring only normalize their body weight by the 5th postnatal week. Such mice with delayed catch-up growth are not only leaner as adults (Coupé et al., 2012) but also have increased lifespan (Ozanne and Hales, 2004) compared to control mice, suggesting that excessive relative growth during early postnatal life is a crucial factor in the programming of metabolic disease.

We will now turn to what is arguably the most established rodent model for directly studying the effects of excessive nutrition and accelerated weight gain during early postnatal life - and also the subject of this thesis – the Small Litter model.

2.5 The Small Litter Model

2.5.1 Description

The Small Litter (SL) model is a variety on the litter size modification technique that has also been used to study undernutrition in the so-called "Large Litter" model described above. In short it consists of reducing the number of pups in a newborn litter to increase each remaining pups access to maternal milk. The typical procedure is to first normalize the number of pups in each litter to the average litter size for that species/strain (usually 7-10 pups) one or two days after birth, then on the third or fourth postnatal day sacrifice a number of pups to bring the litter size down (usually 3-4 pups depending on the protocol).

Since rodent pups do not self-regulate their milk intake, their consumption can be substantially altered depending primarily on the rate of milk production by the mother and the competition from the other pups in the litter. These are however interdependent variables; maternal milk production is dependent on the so called suckling stimulus - the product of the number of pups in the litter or total litter mass, and the duration of suckling (Russell, 1980), such that the mother will increase or decrease its food intake and subsequent milk production to suit the size of the litter. In the extreme this can lead to a severe downregulation of milk production if the litter is too small, leading to growth failure (Russell, 1980), which is why for the purposes of this model the minimum litter size is usually 3.

Never the less, in the more normal range of between 3 to 20 pups/litter, the total milk production varies less with litter size such that in mice who had their litter size manipulated at birth to create litters of between 3 to 18 pups, there was a nearly perfect inverse relationship between litter size and pup weight at weaning (Johnson et al., 2001), while in a rat model the milk available per pup nearly doubled when litter size was manipulated from 12 down to 2 (Kumaresan and Anderson, 1967). For a setup as in the present project for instance, with a normal mouse litter size set at 7 pups/litter and the reduced litter size at 3 pups/litter, the pups in the reduced litter might be expected to gain roughly 30-40% more weight than control pups between postnatal day 3 and

weaning at 22. The Small litter model thus constitutes a simple and reliable model for studying the effects of accelerated weight gain during the lactation period.

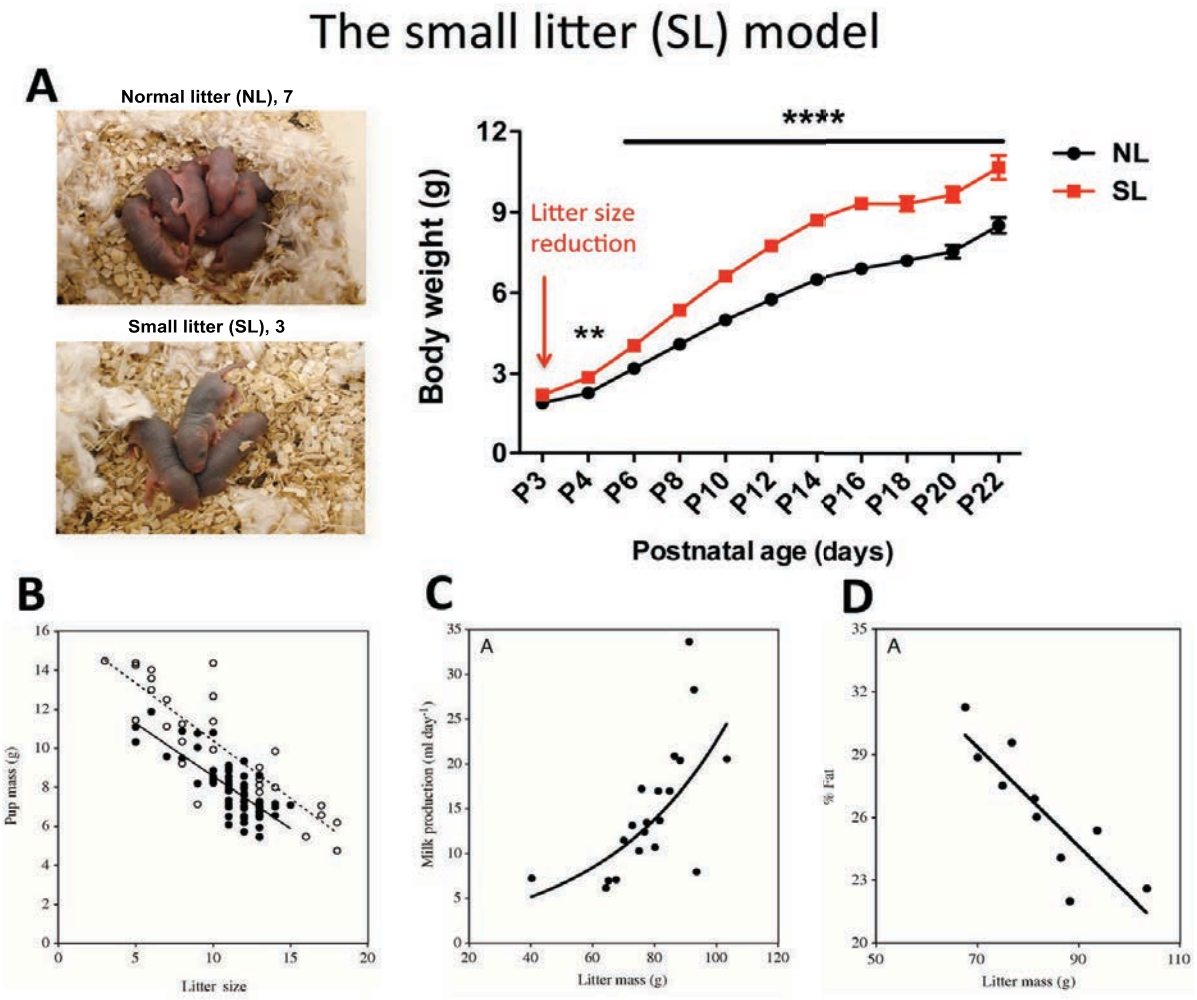

Figure 4: The small litter model and litter size effects on pup size, milk production and milk fat content. (A) Typical pre-weaning growth curve of C57BL6/J mice after litter size manipulation from 7 to 3 pups on postnatal day 3**,** from Colldén et al. 2014. (B) Effects of natural or manipulated litter size on individual pup weight at weaning, R^2 = 0.773 for manipulated and 0.596 for natural litters. (C, D) Effects of total litter mass on (C) maternal milk production, R^2 = 0.56 and (D) milk fat concentration, R^2 = 0.741. Adapted from Johnson et al. 2001.

2.5.2 History

Studies on the effects of litter size manipulation on somatic growth now dates back almost 60 years, when it was found that the milk intake of rat pups could be altered by manipulating litter size, and that an almost four-fold difference in pre-weaning growth could be obtained by decreasing or increasing the size of the litter from between 3 to 22 pups (Kennedy, 1957; Widdowson and McCance, 1960). This weight difference remained and even increased as the rats were weaned onto a regular chow diet. They further observed that this vast difference in growth rate seemed to alter the maturation of certain aspects of their physiology, sexual maturation was shown to depend mostly on the attainment of a certain size and was thus substantially advanced and retarded in rats raised in small and large litters, respectively, whereas biological events like functional vision and teeth eruption seemed to depend mainly on chronological age, while skeletal and muscular maturation was somewhat accelerated in the smallest litters, but still lagged behind the even faster increase in size.

Some years later the same model was used to demonstrate that raising rats in small litters not only affected their somatic growth but also disproportionately increased their fat mass. More importantly, the same study showed that the increased fat mass was partly due to an increase in adipocyte cell number (Knittle and Hirsch, 1968), thus showing that the number of fat cells could be altered by nutritional changes in early life. This was considered to have important implications for the understanding of obesity, since it was already known that there was an increase in adipocyte cell number in human obesity, and that attempts to lose weight primarily reduced the size of the adipocytes without affecting any change in cell number (Hirsch et al., 1966). This study thus provided an early clue as to how nutritional insults that had reversible effects in adulthood, could cause irreversible changes when occurring during early life.

By the early 1980s it had been well established that in addition to causing obesity with adipocyte hyperplasia (Cryer and Jones, 1979; Faust and Johnson, 1980; Bassett and Craig, 1988), small litter rearing was associated with hyperglycemia, hyperinsulinemia and increased hepatic lipogenesis with increased activity of hepatic enzymes involved in

lipid metabolism (Aubert et al., 1980; Duff and Snell, 1982). Studies in Germany from the lab of Günter Dörner later linked small litter rearing with more serious metabolic dysfunctions such as decreased glucose tolerance, hypertension and increased susceptibility to streptozotocin-induced diabetes (You et al., 1990; Plagemann et al., 1992). Dörners student Andras Plagemann was then among the first to study the metabolic alterations caused by small litter rearing in an integrated manner and relate them to the, at the time still relatively new concept of metabolic syndrome in humans (Plagemann A et al. 1999).

2.5.3 Pathophysiological and endocrine aspects

Aside from the by now well-established effects of small litter (SL) rearing on adiposity, blood pressure, glucose homeostasis and lipid metabolism (see Habbout et al. 2013 for review), it has been associated with numerous other pathological changes. SL mice display increased markers of myocardial oxidative stress (Habbout et al., 2012, 2013b), show signs of altered renal development and adult renal failure (Boubred et al., 2007, 2009; Yim et al., 2013), as well as early cardiac hypertrophy, late-onset cardiac fibrosis and microvascular dysfunction (Velkoska et al., 2008b; Moreira et al., 2009; Leite et al., 2012) and consequently are more susceptible to myocardial ischemia perfusion injury (Habbout et al., 2012). As adults they display several signs of alterations in regulation of inflammatory processes, such as increased airway hyper-responsiveness and lung inflammation (Ye et al., 2012) as well as an exacerbated febrile and inflammatory response to bacterial endotoxin (Clarke et al., 2012). They are more susceptible to non-alcoholic fatty liver disease (Ji et al., 2014) and high-fat diet-induced obesity (Glavas et al., 2010a).

Associated with these pathophysiological effects are numerous endocrine and neuroendocrine changes. Several studies have noted altered glucocorticoid signalling with increased corticosterone levels and increased adipose tissue glucocorticoid receptor expression in both developing and adult SL rats (Boullu-Ciocca et al., 2005; Hou et al., 2011).

Thyroid signalling has also been reported to be increased in developing SL rats, with higher circulating levels of TSH, T4 and the active T3 hormone (Rodrigues et al., 2009a), whereas adult 3-6 month old SL rats in contrast displayed lower levels of active T3, which was associated with a lower per mass energy expenditure (Aust et al., 1986; Rodrigues et al., 2009a). GH levels have also been reported to be elevated in both developing and adult SL mice, whereas IGF-1 is elevated only during postnatal development (Kappeler et al., 2009). Since thyroid, GH and IGF-1 are well-known to be involved in somatic growth and development, elevated postnatal signalling of these hormones likely explain why enhanced somatic growth with increased length and organ weights has frequently been observed in SL rodents (Chen et al., 2008).

Most studies have however focused on the effects on the neuroendocrine axis regulating energy balance. In a series of electrophysiological experiments on brain slices derived from adult rats raised in normal or small litters, Plagemann, Davidowa and Li examined the effects of small litter rearing on hypothalamic responses to a variety of neuropeptides involved in energy metabolism. Similar to most other models of overnutrition, small litter rearing has been shown to induce central leptin resistance (Davidowa and Plagemann, 2000a; Glavas et al., 2010a). Whereas leptin is known to stimulate satiety-related neurons in the VMH of normal rats, in SL rats the effect of leptin in the VMH was rather inhibitory (Davidowa and Plagemann, 2000b). Small litter rearing further induces central resistance to insulin, reducing the ability of insulin to inhibit ARH neurons and activate VMH neurons (Davidowa and Plagemann, 2001, 2007).

Small litter rearing also altered the central response to the CRF family-derived peptides CRF and SRP, causing a switch from predominantly stimulatory effects in the dorsomedial VMH and paraventricular nucleus of the hypothalamus to an inhibitory effect in SL rats (Davidowa and Plagemann, 2004). A similar switch in SL rats was seen in the effects of orexin on PVH neurons, from mainly stimulatory to inhibitory activity (Davidowa and Plagemann, 2005).The hypothalamic response to MCH was altered to produce a more consistently orexigenic stimulus in the SL rats compared to controls (Davidowa et al., 2002b), Small litter rearing blunted the inhibitory effect of amylin on orexigenic ARH neurons (Davidowa et al., 2004) and similarly altered the response in neurons of the PVH and DMH (Davidowa et al., 2006; Davidowa, 2007) compared to

controls. Similar alterations were observed in the signalling of the hypothalamic feeding-related neuropeptides AgRP, NPY, MCH, CART and aMSH in SL rats. Whereas CART, aMSH and NPY all stimulated neurons of the PVH in control rats, these peptides instead exerted inhibitory effects in the PVH of SL rats (Davidowa et al, 2003). In the VMH, the activation profile of AgRP was altered from a mix of stimulatory and inhibitory activity in control rats, to a predominantly inhibitory activity in SL rats (Li et al., 2002). Small litter rearing enhanced dopaminergic suppression of VMH neurons which was associated with increased VMH expression of D1 dopamine receptors (Davidowa et al., 2002a).

Adult SL rats also display lowered basal synthesis of pro-Thyroid-releasing-hormone (pro-TRH) in the anterior PVH, and are less responsive to fasting-induced changes in both pro-TRH synthesis and corticosterone secretion (Arechiga-Ceballos et al., 2014). To summarize these findings it seems that multiple components of the neuroendocrine system regulating feeding behaviour in SL rats are shifted towards a more orexigenic profile that could explain the hyperphagia commonly observed in SL rats.

As mentioned previously, a repeated finding in the early literature on small litter rearing was advanced sexual development. More recent studies have generally confirmed that SL rats show signs of advanced puberty, although the effect is sometimes sexually dimorphic (Castellano et al., 2011; Caron et al., 2012; Smith and Spencer, 2012; Sánchez-Garrido et al., 2013; Cui et al., 2014). There have also been inconsistent reports on the associated effects on the neuroendocrine components of reproductive function, some studies reporting that small litter rearing is associated with higher hypothalamic expression of GnRH (Cui et al., 2014) and Kisspeptin (Castellano et al., 2011), while others report reduced densities of hypothalamic GnRH and Kisspeptin fibers (Caron et al., 2012) and others still report no changes in hypothalamic Kisspeptin expression (Smith and Spencer, 2012). This may, in comparing Caron et al with the other studies, be due to species differences between mice and rats. We will next consider other possible species differences in the response to small litter rearing.

Adult physiological and endocrine effects of SL rearing

Physiological alteration	References
Obesity (+25-50% increased fat mass)	Aubert 1980, Rodrigues 2011, Glavas 2010, Li 2013, etc
Hyperglycemia/Hyperinsulinemia	Plagemann 1999, Cunha 2009, Habbout 2012
Hyperleptinemia	Habbout 2012, Cunha 2009, Hou 2011
Glucose intolerance	Plagemann 1999, Chenlin 2014, You 1990, Cunha 2009
Insulin resistance	Plagemann 1999, Glavas 2010, Cunha 2009, Ciocca 2007
Dyslipidemia	Kappeler 2008, Ciocca 2008, Plagemann 1999
Hypertension	Plagemann 1992, 1999, Kappeler 2008, Boubred 2007
Hyperphagia	Cunha 2009, Plagemann 1999, Ji 2014, Habbout 2012
Altered renal development/renal dysfunction	Boubred 2007, 2009, Yim 2013
Cardiac hypertrophy/fibrosis/vascular dysfunction, myocardial oxidative stress	Habbout 2012, 2013, Velkoska 2008, Leite 2012
Hyperreactive febrile/inflammatory response	Ye 2012, Clarke 2012, Liu 2013
Increased sensitivity to diet-induced obesity	Glavas 2010
Increased susceptibility to NAFLD	Ji 2014
Altered glucocorticoid signaling	Hou 2011, Ciocca 2008
Altered thyroid signaling	Aust 1986, Rodrigues 2009
Altered puberty/reproductive development	Castellano 2011, Caron 2012
Altered hypothalamic response to: Leptin	Glavas 2010, Davidowa 2000a, 2000b
- Insulin	Davidowa 2001, 2007
- CRF	Davidowa 2004
- Orexin	Davidowa 2005
- Amylin	Davidowa 2004, 2006, 2007
- AgRP/NPY/MCH/aMSH	Davidowa 2002, 2003, Li 2002

Table 1: Persistent pathophysiological and endocrine alterations in adult rodents reared in small litters. NAFLD = Non-alcoholic fatty liver disease.

2.5.4 Species, strain and sex considerations

Although some authors mention species or strain differences to account for some heterogeneous results observed in different studies of SL rodents (see for instance Habbout et al. 2013), no study has directly investigated differences between rats and mice or between different common strains of either species in the effects of small litter rearing. However, it has been noted that there are species and even strain differences in the maximum food intake of lactating dams and the consequent maximum litter size that they can support, from 26g of food / day and 16 pups / litter in MF-1 mice to 19g / day and 14 pups / litter in Swiss Webster mice, suggesting differences in milk production (Zhao et al, 2010). There have also been varying reports on the effects of litter size manipulation on growth rates in studies on different species and strains (compare for instance Zhao et al. 2013; Zhao et al, 2010; Johnson et al, 2001), although concurrent differences in other methodological variables like the extent and timing of litter size manipulation make such results difficult to interpret. Never the less, such data show that the gross effects of litter size reduction on accelerating growth rate during lactation are valid across a variety of rodent species and strains.

The vast majority of the research on SL rodents has been performed on rats, for instance, a PubMed search using 10 different common search terms for this model yielded 474 hits, adding the term “rats” yielded 291 hits, whereas adding the term “mice” yielded 100 hits, though many of the documented disturbances in SL rodents such as persistent overweight and obesity, glucose intolerance, hyperinsulinemia, leptin resistance and hypothalamic defects have been observed also in mice (Aubert et al., 1980; Glavas et al., 2010a; Ye et al., 2012; Habbout et al., 2013a). Concerning the effects of sex on small litter rearing, most publications have used only male pups for their studies, but a smaller number of papers have compared the response of male versus female rodents. Early alterations in renal development and worsening of inflammatory regulation appear to be similar in male and female SL rodents (Boubred et al., 2007; Clarke et al., 2012) however there is inconsistent data on whether female mice are less susceptible to obesity and insulin resistance following small litter rearing, some authors report an effect only in SL males but not females (Bassett and Craig, 1988), whereas

other report that female SL rodents are also susceptible to persistent overweight, increased adiposity, adipocyte hyperplasia, and hypothalamic alterations (Aubert et al, 1980; Li et al. 2013).

2.5.5 Methodological considerations

The primary methodological variations evident in the SL literature are the timing of the litter size reduction and the relative size of the small litter versus the control litter. In the early studies and also in quite a few more recent publications, the litter size was manipulated on the day of birth, but there are now more varied protocols where the litter size is merely standardized on postnatal day 0 (P0) to P1, and the litter subsequently reduced on P3 to P5. The significance of these methodological variations is not clear, since all have successfully been used to induce accelerated postnatal growth, but it is possible that delaying the litter size manipulation could reduce the risk of substantially reducing maternal milk production in some situations, since there were early reports using this protocol with immediate litter size reduction that failed to induce accelerated weight gain (Wurtman and Miller, 1976). The difference in size between the litters defined as control and SL is also an important factor since the number of pups has a strong linear relationship with postnatal growth rate, and so the SL phenotype may be augmented or muted depending on the relative size compared to the control litter.

A third variable that may factor in the success of generating a strong phenotype is the number of past litters the dam has nursed, since milk production may not be optimal either in virgin females or females who have already nursed 3 or more litters (Zhao, 2011).

2.5.6 Mechanisms of programming by SL rearing

We will first consider the mechanisms whereby rearing rodents in small litters induce accelerated postnatal weight gain. As described in section 2.5.1, reducing litter size has been found to increase the milk available to each pup in a nearly linear fashion (Wainwright et al., 1989; Johnson et al., 2001), and postnatal weight gain in SL rodents

compared to controls has been found to be highly correlated with milk energy intake (Fiorotto et al., 1991), but other factors may contribute to the accelerated growth. For instance, there is evidence that rearing a reduced number of pups increases the amount of maternal care and bodily contact with the pups (Grota and Ader, 1969; Priestnall, 1972; Guerra and Nunes, 2001; McGuire and Bemis, 2007). Neonatal rodents are highly dependent on maternal contact to preserve their body heat, and excessive heat loss may negatively impact their growth. Pups raised at cold temperatures (5-10C) have been found to be significantly lighter at weaning than pups raised at 23C, irrespective of maternal heat loss (Johnson and Speakman, 2001; Paul et al., 2010). On the other hand, moderate litter size reduction probably does not affect heat loss to such an extreme extent, and some authors have failed to find an effect of degree of maternal care on postnatal growth (Champagne et al., 2003).

A third factor that may contribute to the effects of small litter rearing is a change in the milk composition induced by reduced maternal demands. Fat is the dominant energy source in rodent maternal milk, with a fat concentration at least 3-4 times higher than cows milk (Meier et al., 1965). The fat content of maternal milk is inversely related to the total litter mass (Johnson et al., 2001) and is thus typically observed to be increased in dams raising small litters (Šefčíková et al., 2011; Liu et al., 2013) and this may be related to the greater preservation of maternal fat stores with decreasing litter size (Zhao et al., 2013). Since milk is a source of various hormones such as leptin (Smith-Kirwin et al., 1998), ghrelin (Kierson et al., 2006) and insulin-like growth factors (Prosser, 1996), which may be particularly concentrated in milk fat (Savino et al., 2011), and dietary fat content is known to affect a variety of metabolic parameters and physiological functions, the potential significance of the higher milk fat content observed in SL rodents cannot be dismissed.

The short- and long-term metabolic effects of small litter rearing in rodents may thus plausibly be attributed to at least three different factors

- Increased global intake of calories and macronutrients
- Increased dietary fat content
- Increased exposure to milk-derived hormones

Proceeding now to some possible physiological alterations that may be involved in the long-term metabolic alterations seen in SL rodents. As previously described, small litter rearing has consistently been shown to induce adipocyte hyperplasia, or increase in fat cell number. Adipocyte hyperplasia is currently believed to be one of the key factors that determine the level of body fat an individual is programmed to carry and may explain why it is so difficult for obese individuals to achieve long-term fat loss (Spalding et al., 2008), and it is plausible that this could at least in part contribute to the persistently elevated body fat levels that SL rodents exhibit in adulthood. However, other metabolic derangements such as insulin resistance are thought to be more related to the size of individual adipocytes (Salans et al., 1968; Roberts et al., 2009; Meissburger et al., 2011), while increased adipocyte number may actually be protective against the development of insulin resistance in obesity (Gustafson et al., 2013). Since SL rodents display not only adipocyte hyperplasia but also hypertrophy (increased cell size) there are likely other developmental changes that are driving the appearance of this and related metabolic dysfunctions in SL rodents.

The gut microbiota has recently emerged as a new factor in the development of obesity (DiBaise et al., 2012), following observations that mice lacking intestinal bacteria are leaner than conventional mice and can be induced to gain weight by restoring their gut flora (Bäckhed et al., 2004). It was later discovered that there are differences in the relative populations of various bacterial species in obese and lean animals (Ley et al., 2005) and humans (Ley et al., 2006), more specifically a reduction in *Bacteroidetes* and increase in *Firmicutes*. It was further shown that transferring the microbiota of an obese animal to a germ-free animal would make them gain more weight than if they instead received the biota of a lean animal (Turnbaugh et al., 2006).

This has also been suggested to be a factor in the early development of obesity since differences in gut flora populations exist between infants who become overweight at 7 years vs those who remain at a normal weight (Kalliomäki et al., 2008). Interestingly, a high-fat diet was shown to be able to rapidly induce the same type of changes in the gut microbiome that had been observed in obesity, independently of obesity itself (Hildebrandt et al., 2009; Murphy et al., 2010). In this context, given both their increased energy intake and increased dietary fat content, it is perhaps not surprising that small litter rearing has been found to cause alterations in the gut microbiota similar to those seen in other models of obesity (Šefčíková et al., 2011), with an increase in *Lactobacillus* and reduction in *Bacteroidete* species. These differences further not only persist but actually become greater after the mice have been weaned onto a standard chow diet for several weeks. The mechanisms whereby diet-induced alterations in gut microflora have been proposed to contribute to the development of metabolic disease involve increases in intestinal energy absorption, but possibly more important, increases in intestinal endotoxin production, which could contribute to systemic inflammation (Erridge et al., 2007; Siebler et al., 2008). Perhaps because of this link, intestinal endotoxemia has been implicated at the core of the development of MetS and related pathologies like heart disease and diabetes (Cani et al., 2007, 2008; McIntyre et al., 2011), and even adipocyte hyperplasia (Luche et al., 2013).

Although the gut microbiota thus seems like a clear suspect in the development of obesity, there is still question about the relationship of relative changes in bacterial species to obesity, since more recent studies have failed to verify the same differences observed in earlier studies between lean and obese humans (Duncan et al., 2008; Schwiertz et al., 2010).

In addition to systemic inflammation, hypothalamic inflammation has recently gained much attention as another contributing factor to the extensive metabolic derangements associated with obesity (Purkayastha and Cai, 2013; Thaler et al., 2013). This hypothesis emerged after observing that feeding mice a high-fat diet provoked an inflammatory response in the hypothalamus that was associated with hypothalamic insulin resistance (De Souza et al., 2005). It was later demonstrated that hypothalamic inflammation is rapidly induced by a high-fat diet and in fact precedes any significant weight gain

(Thaler et al., 2012a), suggesting that hypothalamic inflammation could be a contributor rather than consequence of metabolic dysfunction. The same study also observed increased levels of microglial cells, a marker of neuronal injury that is commonly observed subsequent to inflammation, in the hypothalami of obese human subjects. Some evidence suggests this effect of high-fat diets is mediated primarily by saturated fatty acids (Milanski et al., 2009). Activating inflammatory signalling pathways in the hypothalamus has been shown to induce hypertension in the absence of obesity (Purkayastha et al., 2011) and blocking similar inflammatory signalling pathways in the hypothalamus has been shown to ameliorate hepatic insulin resistance and hepatic fat accumulation in high-fat fed rodents (Milanski et al., 2012), which demonstrates that hypothalamic inflammation may be an independent contributor to systemic metabolic derangements.

This inflammatory process is followed by proliferation of microglial cells and other signs of neuronal injury within a week of high-fat diet initiation, and is associated with a long-term disappearance of anorexigenic POMC-containing neurons and mitochondrial abnormalities (Thaler et al., 2012a), suggesting that initiation of hypothalamic inflammation may lead to long-term structural changes that could plausibly contribute to persistent metabolic dysfunction. Elevated levels of the pro-inflammatory cytokine IL-6 as well as increased densities of activated microglia has also been observed in the hypothalami of adult rodents exposed to overnutrition by small litter rearing (Tapia-González et al., 2011), which suggests that early induction of hypothalamic inflammation may be one mechanism by which SL rearing causes long-term metabolic dysfunction. This would be consistent with previous data because not only are SL rodents exposed to a relatively higher-fat diet enriched particularly in saturated fatty acids during lactation as described before, but they also display a several fold elevation of circulating leptin during lactation (Schmidt et al., 2001) which is itself a cytokine with potent pro-inflammatory effects (Lord, 2006) and has been shown to directly activate microglial cells and modulate their morphology (Pinteaux et al., 2007; Tang et al., 2007; Lafrance et al., 2010).

Aside from modulating the activity of microglial cells, hormones such as leptin are known to have direct effects on the developing hypothalamus. As described previously,

SL rodents display multiple abnormalities in their hypothalamic responses to various local and circulating hormones that is believed to underlie their phenotype at least in part. To briefly recapitulate, they demonstrate a switch from a stimulatory to inhibitory response of VMH neurons to leptin (Davidowa and Plagemann, 2000b), a switch from predominantly stimulatory effects of CRF in the dorsomedial VMH and paraventricular nucleus of the hypothalamus to an inhibitory effect in SL rats (Davidowa and Plagemann, 2004), a similar switch in the effects of orexin on PVH neurons, from mainly stimulatory to inhibitory activity (Davidowa and Plagemann, 2005), and finally whereas CART, aMSH and NPY all stimulated neurons of the PVH in control rats, these peptides instead exerted inhibitory effects in the PVH of SL rats (Davidowa et al, 2003). These altered responses was in addition observed also in dissociated hypothalamic preparations, suggesting that small litter rearing causes structural changes in the hypothalamic neuronal circuits that control energy balance. Small litter rearing is further known to cause major alterations in the circulating levels of several hormones, such as leptin and insulin (Schmidt et al., 2001), that act in the hypothalamus and are known to be involved in the postnatal development of the hypothalamus (see section 4). Temporary alterations in the levels of circulating hormones induced by postnatal overnutrition may thus be a contributing factor to the altered hypothalamic development, and consequently involved in the long-term metabolic perturbations caused by SL rearing.

Before exploring this hypothesis in more detail, we will now turn to the hypothalamus, describing its structure and central role in the control of energy balance, and then proceed to its perinatal development and how this development may be altered by nutritional and hormonal factors during postnatal life.

3. Hypothalamic regulation of energy balance

3.1 The Hypothalamus

3.1.1 History

The hypothalamus is an ancient brain structure present in all vertebrate species. Centered on the third ventricle, it is comprised of a number of anatomically distinct neuronal nuclei that all contain different neuronal populations and control different metabolic processes such as the regulation of temperature, blood pressure, energy metabolism, reproduction, and feeding behaviour. It is the principal secretory neuroendocrine organ and works in concert with the pituitary gland to link the central nervous system to the endocrine system, receiving inputs from many other parts of the CNS as well as parts of the peripheral nervous system such as those innervating the heart and intestines. Neurons of the hypothalamus primarily control metabolic processes by secreting different releasing hormones into the median eminence that are then transported via the hypophyseal portal system to the anterior pituitary to either stimulate or inhibit the secretion of pituitary hormones into the blood stream, but also by their numerous projections to autonomic neurons in the brain stem and spinal cord, in addition to numerous central projections to other parts of the brain.

Recognition of the essential role of the brain in regulating various bodily processes goes back to Aristotle, and the third ventricle and its surrounding tissues was identified as a key region in the control and integration of bodily functions as early as the 14th century by medieval anatomist Mondino de Liuzzi (Toni, 2000). The term “hypothalamus” itself was coined in 1893 by Wilhelm His and by then many of its subanatomical divisions such as the lateral hypothalamus, the supraoptic nucleus, and the preoptic region had already been defined (Swaab and Schade, 2011). The invention of stereotactic devices in the early 20th century allowed for further functional elucidation of individual hypothalamic regions as it was found that lesions in distinct hypothalamic structures could induce diabetes. Around the same time many researchers began to use electrical stimulation and dye tracing studies to further define both the functions of the hypothalamic subregions, and explore how the hypothalamus was able to communicate with the pituitary.

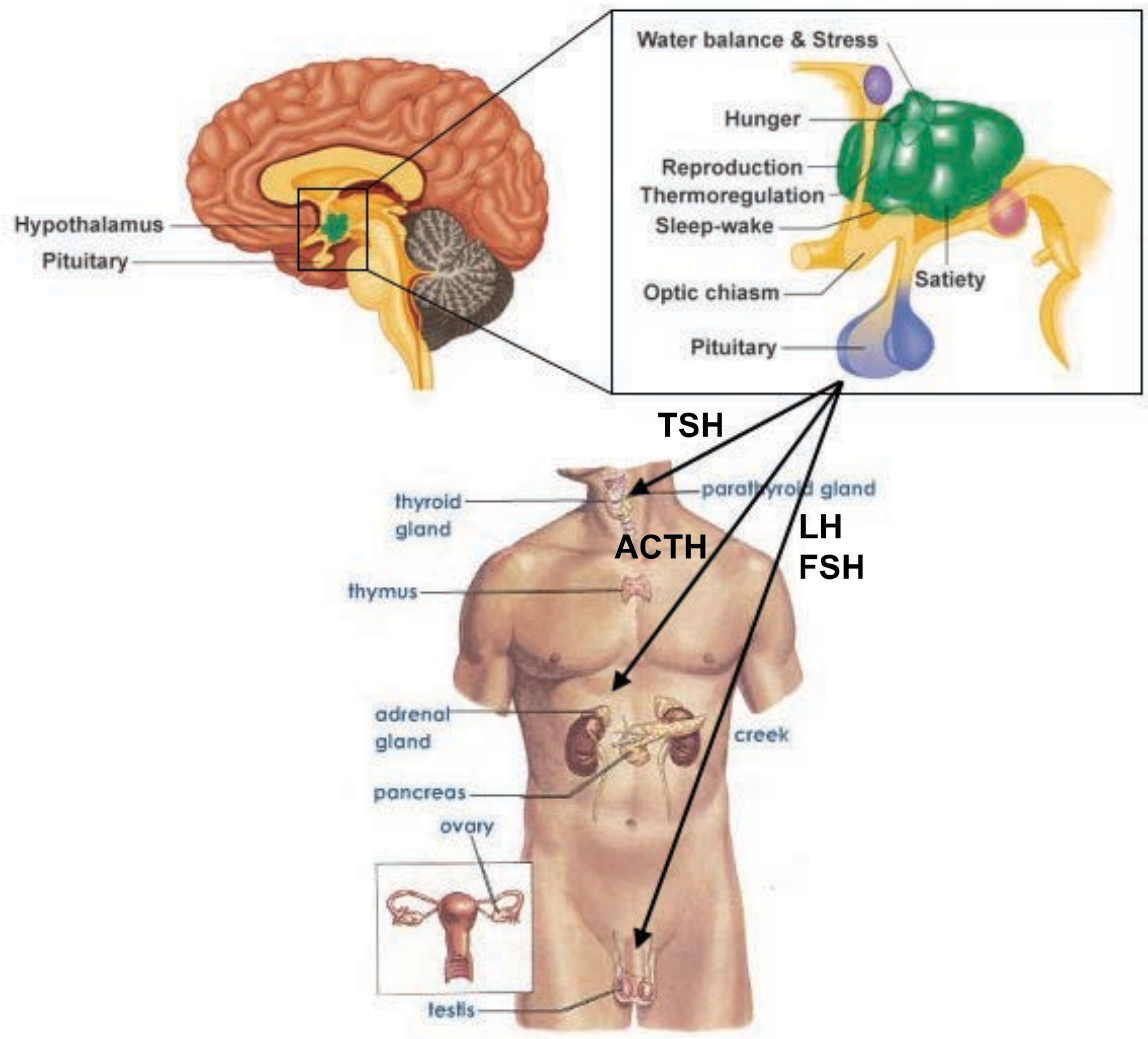

Figure 5: The hypothalamus controls various physiological functions such as feeding, energy metabolism, reproduction and sleep/wake-cycles. One important role of the hypothalamus is secretion of releasing hormones that act to stimulate production of pituitary hormones that in turn act on the peripheral glands to control metabolic functions.

The essential role of the hypothalamus in the regulation of energy metabolism was first established in 1940 by a series of stereotactic lesion experiments by Hetherington and Ranson. They found that these lesions, which did not disturb the pituitary, but caused extensive bilateral damage to the dorsomedial, ventromedial and arcuate nuclei as well as the lateral hypothalamic area, would quickly result in as much as a doubling of body weight and tremendous increase in body fat stores (Hetherington and Ranson, 1940). Anand and Brobeck later noted that lesions specifically to the lateral hypothalamic area could severely inhibit feeding even to the point of death by starvation (Anand and Brobeck, 1951), thus emerged the “dual center” hypothesis of brain control of feeding,

with the lateral hypothalamic area serving to stimulate feeding and the ventromedial nucleus serving to inhibit feeding. Gold and colleagues later challenged these hypotheses, as they had demonstrated that smaller lesions targeting specifically the VMH were not sufficient to produce hyperphagia. Although Golds conclusions about the VMH have themselves later been questioned (King, 2006), he did in the same paper demonstrate that lesions targeting the connections to the PVH were also effective in producing hyperphagia (Gold, 1973).

In the 1960s, Debons and colleagues demonstrated that systemic injection of the toxic glucose analog gold thioglucose caused localized damage to the VMH and ARH that led to hyperphagia and obesity (Debons et al., 1962). Olney showed that systemic high-dose injections of monosodium-glutamate caused similar hypothalamic damage that however seemed to be more restricted to the arcuate and preoptic nuclei. He reported that these mice were paradoxically hypophagic yet obese, in addition to a variety of other abnormalities like stunted growth, lethargy, female sterility, elevated corticosterone levels, and hepatic fat accumulation (Olney, 1969) suggesting that the hypothalamus was a crucial regulator not only of feeding behaviour, but also growth and energy homeostasis and reproduction. We will in the remainder of this chapter discuss the different nuclei of the hypothalamus, with a special focus on the network of nuclei that control feeding and energy metabolism.

3.1.2 The Arcuate Nucleus (ARH)

The Arcuate nucleus is a small triangular nucleus located right at the base of the third ventricle. It is situated adjacent to the median eminence (ME) – a circumventricular organ that contains a dense network of fenestrated blood vessels (see section 3.2.1 for more details). The ARH is thus strategically located to be able to respond to circulating metabolic signals that have a facilitated access to the ARH through the ME. Consistent with a key function as integrator of peripheral signals of energy balance, the ARH has a uniquely high concentration of receptors for multiple circulating hormones derived from peripheral organs such as leptin (Huang et al., 1996), ghrelin (Zigman et al., 2006) and insulin (Marks et al., 1990). The ARH contains many distinct populations of neurons. There are for instance neuroendocrine cells that secrete either dopamine or growth-

hormone-releasing hormone (GHRH) into the hypophyseal portal system to regulate prolactin (Blum et al., 1987) and growth hormone signalling (Bluet-Pajot et al., 1998) via the pituitary. The other two major neuronal populations in the ARH reciprocally regulate energy metabolism and feeding behaviour in opposing directions. Food intake-stimulating neurons produce the orexigenic peptides Neuropeptide Y (NPY) and Agouti-related protein (AgRP), whereas neurons that inhibit feeding when stimulated produce the anorexigenic peptides alpha-melanocyte stimulating hormone (aMSH) and cocaine-and-amphetamine-related transcript (CART).

NPY is a 36-amino acid peptide that is widely expressed in the brain, but in the hypothalamus cell bodies producing NPY are most concentrated in the ARH (Chronwall et al., 1985). It is often considered to be the most potent known endogenous orexigenic signal. Its production is dynamically regulated by feeding state to be increased by fasting and inhibited by feeding (Kalra et al., 1991; Mizuno et al., 1999). NPY-producing neurons of the ARH project primarily to other hypothalamic regions including the DMH, VMH, PVH and LHA where it acts to directly stimulate food intake (Clark et al., 1985; Beck, 2006). Animals overexpressing NPY in the hypothalamus develop hyperphagia and obesity (Tiesjema et al., 2009; Ruohonen et al., 2012) while conversely animals that lack NPY display hypophagia and delayed feeding (Sindelar et al., 2005), and ablating NPY/AgRP neurons in adult mice can cause a complete arrest of feeding leading to rapid starvation (Luquet et al., 2005). NPY is also upregulated by glucocorticoids (Shimizu et al., 2008, 2010) and stress (Kuo et al., 2007) and appears to have a role in stress-induced obesity. NPY acts by binding to a family of G-protein-coupled receptors of which 4 subtypes have been identified, Y1, 2, 4 and 5. Of these, Y1 (Lopez-Valpuesta et al., 1996) and Y5 (Schaffhauser et al., 1997) are the primary subtypes implicated in the orexigenic effects of NPY, being highly expressed in hypothalamic regions targeted by neural projections from ARH NPY neurons (Undén et al., 1984; Fetissov et al., 2004).

AgRP is 132-amino acid peptide that is only expressed by neurons in the ventromedial ARH, where it is more than 95% co-localized with NPY-expressing neurons (Broberger et al., 1998b). It is similarly to NPY considered to have potent orexigenic actions, mice overexpressing AgRP develop obesity (Graham et al., 1997) while acute and chronic ICV AgRP injections have been shown to both induce hyperphagia and reduce energy

expenditure (Rossi et al., 1998; Goto et al., 2003; Small et al., 2003). Some studies suggest that AgRP may have a stronger influence on energy expenditure than feeding behaviour, since inhibiting hypothalamic AgRP expression by RNA interference increases energy expenditure and induces weight loss without affecting food intake (Makimura et al., 2002). AgRP is considered to act both as an antagonist and an inverse agonist of the melanocortin receptors MC3R and MC4R (Fong et al., 1997; Haskell-Luevano and Monck, 2001), by blocking the effects of their endogenous agonist aMSH, and suppressing their constitutive activity (Adan and Kas, 2003), since AgRP has been found to alter long-term energy balance also in neuron-specific POMC KO mice (Tolle and Low, 2007). Although there is some evidence that the hyperphagic effect of AgRP involves other mechanisms (Hagan et al., 2000; Kim et al., 2002). Similarily to NPY, AgRP is increased by fasting (Palou et al., 2009) and suppressed by feeding, both peptides are also inhibited by leptin and stimulated by ghrelin (Korner et al., 2001; Chen et al., 2004a; van den Top et al., 2004).

NPY/AgRP-expressing cell bodies are predominantly found in the ventromedial ARH, although AgRP-containing nerve terminals are found in numerous extrahypothalamic regions, most AgRP-positive fibers are found in hypothalamic sites. Aside from the ARH itself, the PVH has the largest density of NPY/AgRP-positive fibers, where AgRP appears to act to suppress the anorexigenic action of PVH neurons via the inhibitory neurotransmitter GABA (Atasoy et al., 2012). AgRP neurons also project to the LHA where they innervate both MCH and orexin-containing neurons (Elias et al., 1998), as well as the DMH, whereas in the VMH, AgRP-IR fibers have been reported to be conspicuously absent (Broberger et al., 1998b), although AgRP-positive neurons may make contact on neurons located in the outer shell of the VMH (Fu and van den Pol, 2008).

Alpha-melanocyte-stimulating hormone (aMSH) is a 13-amino acid peptide belonging to the melanocortin peptide family. It is derived from the pro-opio-melanocortin (POMC) precursor polypeptide, which in the hypothalamus is expressed primarily in neurons of the ventrolateral ARH. aMSH acts as the main endogenous agonist of the melanocortin receptors MC3R and MC4R to inhibit feeding. MC3R and 4 receptors are proposed to exert a tonic inhibitory effect on feeding (Fan et al., 1997). Mice lacking the MC3R (Chen

et al., 2000a) or MC4R receptor (Huszar et al., 1997) or the POMC gene (Challis et al., 2004) develop obesity , which in the latter case can be reversed by administration of aMSH (Yaswen et al., 1999).

POMC neurons project widely in the brain, including most hypothalamic nuclei, but with particularly dense projections to the PVH, where they innervate TRH-positive neurons (Fekete et al., 2000a) and regulate the expression of CRH (Fekete et al., 2000b). MC4R receptors are densely expressed in the PVH (Mountjoy et al., 1994; Kishi et al., 2003), whereas MC3R receptors are predominantly present in the VMH (Chen et al., 2000a; Begriche et al., 2011). However, some authors have suggested that the role of MC3R in mediating the effects of melanocortin agonists is limited (Marsh et al., 1999; Chen et al., 2000b), and that POMC-neurons exert their effects on feeding behaviour and metabolism predominantly through their projections to cells in the PVH (Cowley et al., 1999).

aMSH-containing neurons in ARH also co-express the peptide cocaine-and-amphetamine-regulated transcript (CART) (Vrang et al., 1999). Although CART is expressed in several areas of the brain, it is concentrated in the hypothalamus, where it is predominantly located in the ARH and PVH with lesser levels in the VMH, LH and DMH (Murphy, 2005; Keller et al., 2006). Similar to aMSH, ICV injections of CART potently reduces food intake (Keller et al., 2006). However, its effects on body weight regulation seem to be more complex than a simple feeding inhibitor, it has also been found to sometimes stimulate food intake (Abbott et al., 2001) and also stimulate expression of mitochondrial uncoupling proteins in muscle and adipose tissue, (Wang et al., 2000), presumably causing an increase in energy expenditure. It was further found that injections of CART into the ARH stimulated both food intake, UCP-1 expression and thermogenic response to adrenergic stimulation, whereas mice overexpressing CART in the ARH displayed hyperphagia but also reduced weight gain, suggesting that CARTs effects on energy expenditure may overcompensate for its stimulatory effect on feeding (Kong et al., 2003). Consistent with a role for hypothalamic CART in regulating energy expenditure, CART-positive neurons innervate TRH-expressing neurons in the PVH and regulates TRH response to fasting (Fekete et al., 2000c).

Several other neurotransmitters are expressed by ARH neurons, such as Kisspeptin, Dynorphin and Neurokinin B which are believed to interact to regulate reproductive function (Wakabayashi et al., 2010; Goodman et al., 2013) as well as Galanin, known to be involved in nociceptive sensing (Gundlach et al., 2001; Sun et al., 2003) and Neurotensin, which may regulate fluid homeostasis (Rosas-Arellano et al., 1996). In addition to these functions, these neurotransmitters may also influence feeding behavior, with Galanin and Dynorphin having stimulatory (Walker et al., 1980; Schick et al., 1993) and Kisspeptin and Neurotensin having inhibitory (Hawkins et al., 1986; Stengel et al., 2011) effects on feeding. Nonetheless, the two distinct NPY/AgRP and POMC/CART-expressing populations, with their extensive projections to other hypothalamic nuclei with important functions in the regulation of feeding and metabolism, are considered to be the neurons that predominantly underlie the role of the ARH in the control of energy homeostasis.

3.1.3 The Paraventricular Nucleus (PVH)

Because a large portion of the NPY/AgRP and POMC/CART cell bodies in the ARH project to the PVH, the communication between the ARH and PVH is believed to be an important part of the hypothalamic system that regulates energy homeostasis. The PVH is located rostral to the ARH at either side of the top of the third ventricle. It is divided into two anatomical subregions defined by their neuronal morphology. The magnocellular part contains large neuroendocrine neurons that express Vasopressin and Oxytocin and project to the posterior pituitary, whereas the parvocellular part contains small neuroendocrine cells that project to the median eminence where they secrete TRH and CRH into the hypophyseal portal to act on the anterior pituitary. The PVH is thus the stage for the first part of the HPT and HPA-axes, which lie at the heart of the central control of metabolism.

TRH, or Thyrotropin-releasing hormone is a tripeptidal hormone that stimulates the secretion of Thyroid-stimulating hormone (TSH) and prolactin from the anterior pituitary. TSH in turn stimulates the production of the primary thyroid hormones thyroxine (T4) and triiodothyronine (T3) from the thyroid gland. These thyroid hormones, T3 in particular, have a fundamental influence on energy homeostasis, since

they control the intensity of the metabolic rate and substrate turnover in almost every tissue in the body and powerfully regulate functions such as heart rate, body temperature, growth and development (Fisher et al., 1982; Hulbert, 2000; Morreale de Escobar et al., 2004), and tissue regeneration and differentiation (Pascual and Aranda, 2013) . This signal network that translates neuronal activity in the hypothalamus to changes in the circulating levels of thyroid hormones is known as the Hypothalamic-Pituitary-Thyroid (HPT) axis. This system is normally regulated by a negative feedback loop to keep circulating levels stable, wherein TRH secretion in the PVH is suppressed by elevated levels of T3 and T4 in the CSF binding to the Thyroid hormone receptor beta-2 present on PVH TRH neurons (Abel et al., 2001 p.2). However, this system is additionally regulated by input from ARH NPY/AgRP and POMC/CART neurons that innervate TRH neurons, which can thus suppress TRH secretion during fasting/starvation (Mihály et al., 2000; Lechan and Fekete, 2004). In addition to regulating thyroid hormone secretion, TRH and TSH have been shown to themselves act to suppress feeding when injected peripherally or centrally (Vijayan and McCann, 1977; Lin et al., 1983; Choi et al., 2002).

The other major function of parvocellular PVH neurons is the secretion of Corticotropin-releasing hormone (CRH) and Vasopressin. CRH is a 41-amino acid peptide that acts together with Vasopressin to stimulate secretion of adrenocorticotropic hormone (ACTH) in corticotropes of the anterior pituitary in response to stress. ACTH in turn acts to stimulate secretion of corticosteroids, primarily cortisol, from the adrenal glands. This endocrine signal cascade between the PVH of the hypothalamus to the secretion of adrenal steroid hormones is known as the Hypothalamic-pituitary-adrenal (HPA) axis, and is a major component in the central response to stress. Like the HPT axis, the HPA axis is regulated by a negative feedback since cortisol can directly inhibit CRH and Vasopressin secretion by acting on glucocorticoid receptors present on paraventricular CRH cells (Uht et al., 1988). Since one of the primary functions of cortisol is to liberate energy stores in response to stress, increased energy demands or hypoglycaemia, CRH secretion is also regulated by glucosensing neurons in the hindbrain via adrenergic/noradrenergic inputs (Ritter et al., 2003). CRH neurons also receive direct input from NPY-containing afferents from the ARH (Li et al., 2000) which acts to stimulate CRH secretion (Dimitrov et al., 2007). CRH in turn can directly suppress

feeding when administered centrally (Drescher et al., 1994), possibly by regulating the activity of ARH NPY neurons since these neurons express the CRH receptor (Campbell et al., 2003).

In addition to the neuronal populations expressing CRH or TRH, the PVH also contains both magnocellular and parvocellular neurons that secrete Oxytocin. While oxytocin is most widely known for its diverse roles in sexual and mating behaviour, including lactation, sexual arousal, orgasm, pair-bonding, maternal bonding and pro-social behaviour (Macdonald and Macdonald, 2010) there is mounting evidence that it also influences feeding behaviour and energy metabolism. Both peripheral and ICV injections of oxytocin inhibit food intake (Arletti et al., 1990; Olson et al., 1991), and oxytocin receptor-deficient mice develop obesity (Takayanagi et al., 2008), while a relative deficiency of hypothalamic oxytocin neurons seems to be a consistent characteristic in genetically obese humans suffering from the Prader-Willi syndrome (PWS) (Swaab et al., 1995) as well as in mouse models of PWS (Dombret et al., 2012). Consistent with its putative role as a satiety hormone, the VMH is among the regions found to have a strong binding affinity for oxytocin (Tribollet et al., 1988).

To summarize, the PVH is a crucial site for the central control of energy homeostasis owing largely due to its regulation of the HPA and HPT axes via CRH and TRH-secreting neurons. The extensive neural connections between the ARH and the PVH may thus hold a special importance for the integration of peripheral signals in the regulation of metabolism.

3.1.4 The Ventromedial Nucleus (VMH)

As mentioned previously, the VMH was early on identified as the “satiety center” of the hypothalamus. Although there was early controversy about the role of the VMH in feeding behaviour, the research predominantly point to the VMH having a key function in the suppression of feeding and regulation of body weight (King, 2006). Despite being the first region to gain attention for its role in central control of feeding, the mechanisms underlying this are still today less clearly defined than for other hypothalamic nuclei. Located just dorsolaterally of the ARH, the VMH contains a large number of glucosensing

neurons, and it is perhaps most well known for its role in the central response to hypoglycaemia (Routh, 2010). In addition it receives input from glucosensing neurons in other parts of the brain, and so thus likely serves as an integrative center of signals on glucose levels and orchestrates the counter-regulatory response that aims to keep glucose levels stable in situations of increased energy demands (Routh, 2003). A key role for the VMH in glucose homeostasis is suggested by the acute hyperinsulinemia that can result only minutes after a lesion (Berthoud and Jeanrenaud, 1979). More general metabolic defects have also been observed following VMH lesions, which are independent of food intake (Han, 1968).

The VMH contains a high density of both MC3R and MC4R melanocortin receptors and is a target of both AgRP and POMC-positive fibers (Fu and van den Pol, 2008), which both seem to excite their respective target neurons. In obese MC3R KO mice, restoring MC3R expression specifically in the VMH has only a modest effect on obesity but a greater impact on normalizing metabolic homeostasis (Begriche et al., 2011). The VMH also has a high content of brain-derived neurotrophic factor (BDNF), which has been postulated to be the downstreams effector of the effects of melanocortin signalling in the VMH (Xu et al., 2003), with MC4R signalling stimulating BDNF production which in turn acts to lower food intake and increase energy expenditure (Wang et al., 2007a, 2010).

Aside from BDNF, the VMH also contains receptors for histamine, dopamine and noradrenaline, all of which appear to be involved in the regulation of feeding by VMH (Shimazu et al., 1986; Fetissov et al., 2002; Magrani et al., 2004).

In summary, there is an extensive literature that demonstrates the diverse roles of the VMH in the regulation of feeding and metabolic regulation, particularly of glucose homeostasis, although the underlying neuronal circuits and mechanisms remain to be clearly elucidated.

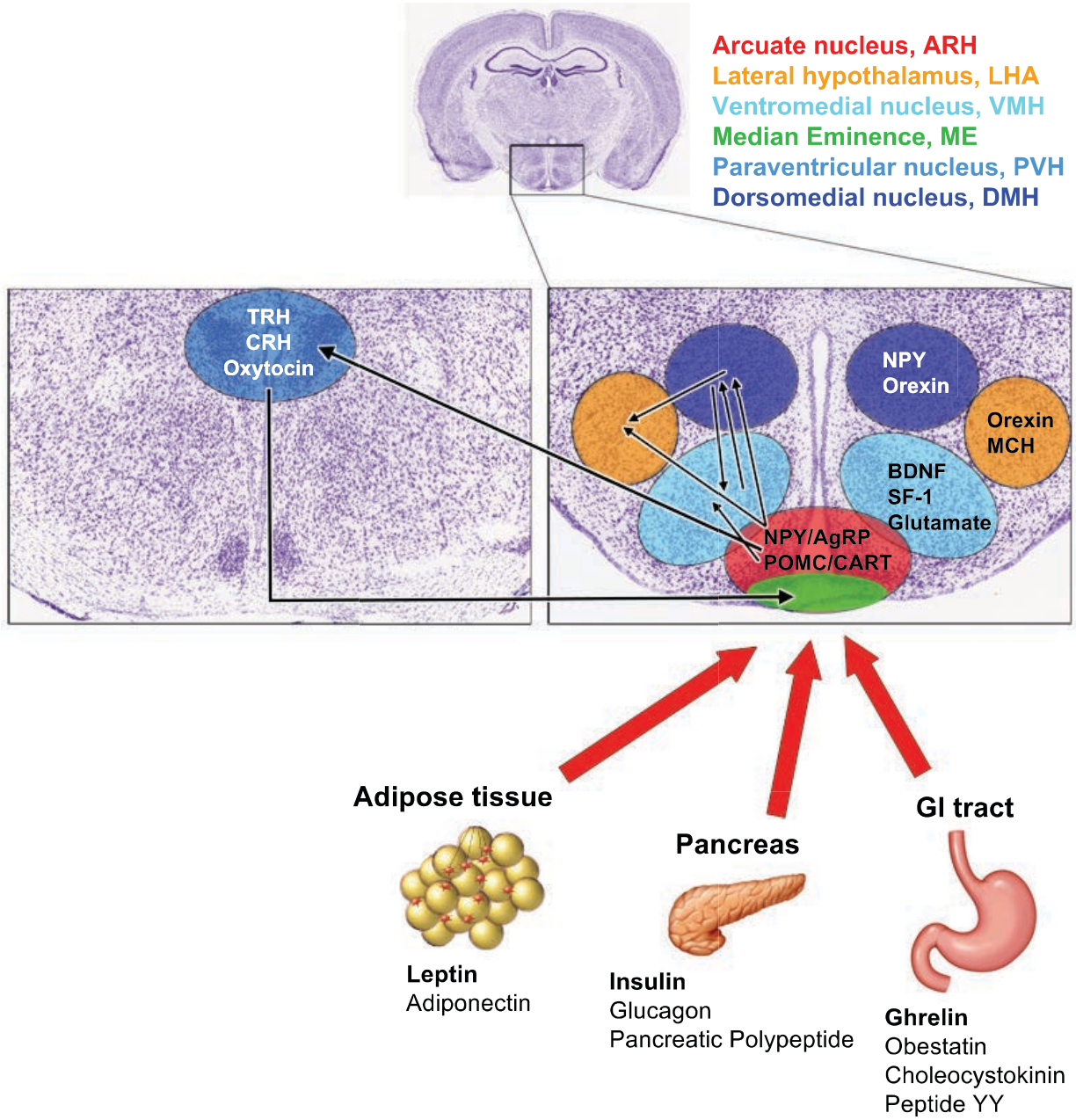

Figure 6: Hypothalamic control of feeding and energy metabolism. Peripheral signals secreted from various organs, particularly leptin, insulin and ghrelin, act on the hypothalamic arcuate nucleus to control the electrical activity of NPY/AgRP and POMC/CART neurons, these neurons in turn project to the nearby ventromedial, dorsomedial, lateral and paraventricular nucleus which are involved in the downstreams regulation of metabolism. Arcuate projections to the paraventricular nucleus may be particularly important in the control of energy metabolism by regulating activity of the HPA and HPT axes via TRH and CRH neurons.

3.1.5 The Dorsomedial Nucleus (DMH)

The DMH, located at the top of the third ventricle at the level of the ARH, was recognized early on to influence both feeding behaviour and metabolism. Electrical stimulation of the DMH was initially shown in sheep to cause hyperphagia (Forssberg, 1954) while chemical disinhibition of DMH neurons was later found to provoke non-shivering thermogenesis and raise body temperature (Dimicco and Zaretsky, 2007). DMH lesions in contrast induce hypophagia and reduced linear growth with reduced fat mass (Bernardis, 1970). Consistent with a role in stimulating feeding, the DMH is one of few hypothalamic nuclei to express the orexigenic peptide Orexin (Nambu et al., 1999), and further contains an important population of NPY-producing neurons. Knockdown of NPY expression specifically in the DMH was found to inhibit fat gain in response to high-fat diet feeding and enhance thermogenic capacity (Chao et al., 2011), while DMH-specific overexpression of NPY was found to cause hyperphagia and obesity (Yang et al., 2009). The DMH is abundantly innervated by AgRP and POMC fibers that are found in close apposition to NPY-positive cell bodies (Chen et al., 2004b). Although these DMH NPY cells do not themselves express the melanocortin receptor MC4R, it has been found that MC4R KO mice have elevated expression of NPY in the DMH (Kesterson et al., 1997), while injections of MC4R agonists reduce food intake and NPY expression in DMH and stimulate mitochondrial uncoupling protein expression in brown adipose tissue, consistent with increased thermogenic capacity (Chen et al., 2004b). Collectively these data suggest the DMH has an important role in both feeding and energy homeostasis and in particular regulation of thermogenesis, which may partly be mediated by inputs from the ARH via NPY.

3.1.6 The Lateral Hypothalamus (LHA)

As previously mentioned, the LHA was among the first hypothalamic regions studied in relation to body weight regulation, Anand and Brobeck reported in 1951 that lesions of the LHA could cause severe hypophagia to the point of death by starvation (Anand and Brobeck, 1951), and later research has consistently confirmed the role of the LHA in the stimulation of feeding. Indeed, the LHA is the region found to most potently respond to nucleus-specific injections of NPY (Stanley et al., 1993). The LHA contains two separate populations of neurons that express the orexigenic peptides melanin-concentrating

hormone (MCH) and Orexin (Broberger et al., 1998a). Nerve terminals containing NPY/AgRP are abundantly present in close proximity to MCH and Orexin-containing cell bodies, and they are further innervated by aMSH-positive fibers (Elias et al., 1998), suggesting that information from the ARH may be communicated to the LHA to modulate activity of MCH/Orexin neurons. Both MCH (Verret et al., 2003) and Orexin (Kantor et al., 2009) may be involved in the sleep-wake cycle and circadian regulation of metabolism. Orexin neurons are themselves regulated by light and darkness stimuli (Marston et al., 2008), and they have been found to be important for the induction of locomotor activity induced by food anticipation (Akiyama et al., 2004; Mieda et al., 2004).

3.2 Peripheral inputs onto hypothalamic feeding circuits

3.2.1 Access of peripheral signals to the hypothalamus

To efficiently fine-tune the balance of energy expenditure and energy intake according to the constantly changing metabolic state of the organism, the hypothalamic regions regulating these functions have evolved to receive continuous feedback from many different circulating factors that originate from peripheral organs. However, the brain is separated from the general circulation by the blood-brain-barrier (BBB). The BBB is composed of a layer of endothelial cells lining the blood vessels of the brain that form tight junction complexes, making the brain virtually impermeable to circulating molecules with the exception of water, some gases and small hydrophilic substances (Mitchell and Hatch, 2011). The brain takes up the circulating energy substrates it needs through various transport systems that are specific for glucose, amino acids, pyruvate, etc (Pardridge and Oldendorf, 1975; Cremer et al., 1979; Pardridge et al., 1990; Boado et al., 1999), and certain hormones such as insulin and leptin may also pass the BBB through similar saturable transport mechanisms (Banks et al, 1997; Hileman et al. 2000; Hileman et al. 2002).

However, such saturable transport systems may not be appropriate for regions of the brain that depend on rapid and sensitive feedback on the concentrations of circulating factors, and indeed it had long been suspected that there be a privileged pathway bypassing the BBB whereby certain hormones can more rapidly access the

hypothalamus. There are several regions in the brain known as circumventricular organs that are densely populated by fenestrated blood vessels, and as such are considered to lack an intact BBB. In the hypothalamus, the ARH is located adjacent to the circumventricular organ known as the median eminence (ME). The ME is known to be a central component of the hypophyseal portal system which connects the hypothalamus with the pituitary, as many hypothalamic neuroendocrine cells project to the ME to secrete hormone releasing factors such as TRH, CRH, GHRH and GnRH which then enter the portal system to act on the anterior pituitary.

More recently it was demonstrated that the ME represents a plausible candidate as a rapid-access pathway for metabolic hormones to the ARH, since it was shown to contain fenestrated capillaries that extend into the ventromedial ARH (Ciofi et al., 2009; Schaeffer et al., 2013), which incidentally contains the most dense concentration of cells expressing receptors for leptin, insulin, ghrelin. Schaeffer and colleagues further demonstrated in mice that small molecules < 20 kDa can passively diffuse into the ventromedial ARH through the fenestrated capillaires of the ME, including hormones that target the ARH such as ghrelin. Interestingly, the ability of peripherally administered ghrelin to diffuse into the ARH via this pathway was shown to depend on feeding state, as diffusion was substantially enhanced by fasting the mice for 24 hours, and then reduced to baseline levels by refeeding the mice (Schaeffer et al., 2013). Langlet and colleagues further showed that 24 hour fasting is associated with structural re-organization of the ME capillary network, causing increased fenestrations of the ME vessels extending into the ARH. This structural plasticity appeared to be mediated by a reduction in blood glucose, as the ME contains specialized endothelial cells called tanycytes that may act as glucosensors and locally secrete the vascular endothelial growth factor (VEGF) in response to hypoglycemia, VEGF in turn driving the vascular re-organization (Langlet et al., 2013a).

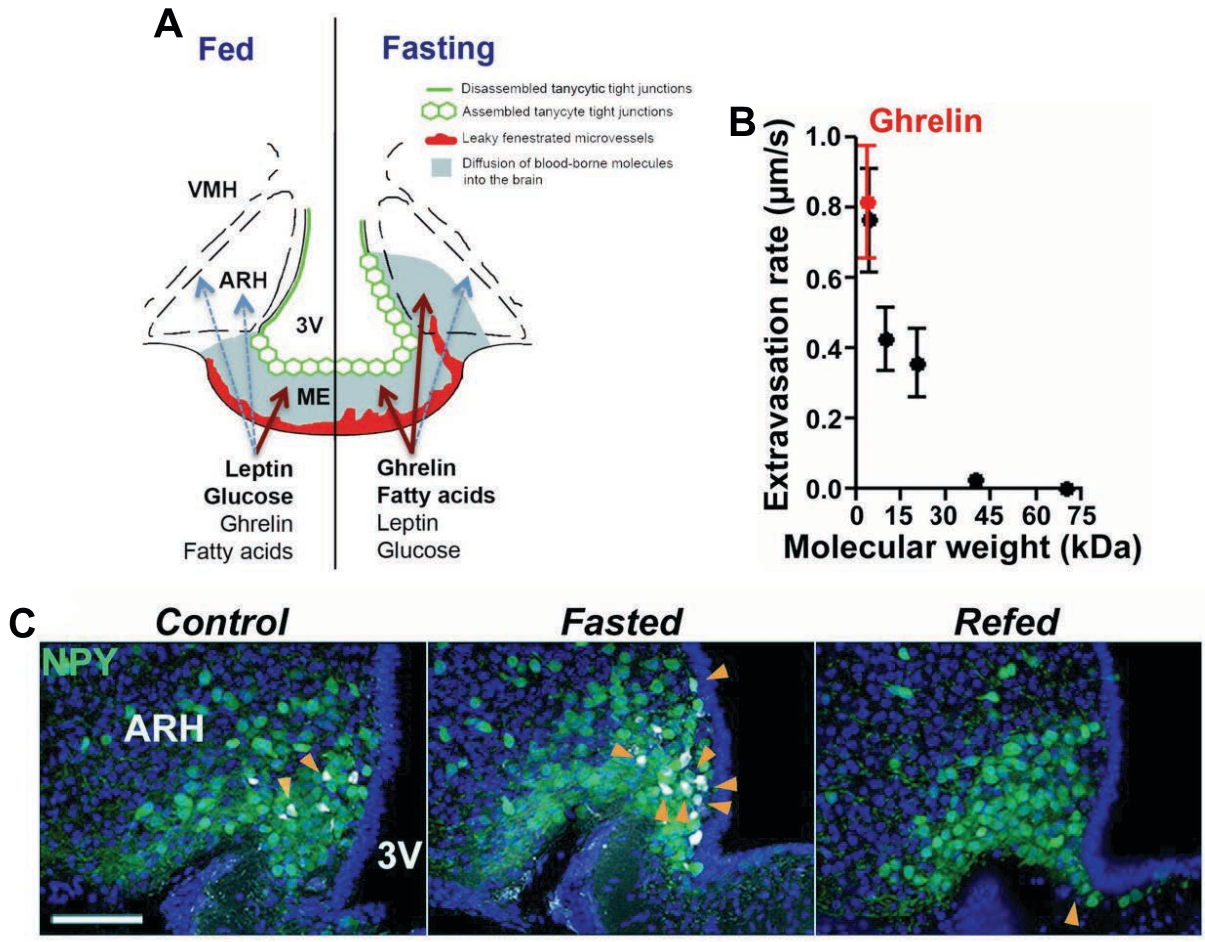

Figure 7: Diffusion of metabolic substrates into the ARH through the median eminence. (A) Model of feeding-dependent diffusion through the ME. Substrates such as leptin, ghrelin and glucose may freely diffuse across the fenestrated microvessels of the ME, these are re-organized during 24-hour fasting to permit easier access into the ARH. (B) Size-dependent diffusion of subtrates, molecules larger than 20kDa cannot diffuse through this pathway. (C) Diffusion of peripherally injected fluorescent ghrelin (white) into the ARH is dependent on feeding, ie enhanced by fasting and reduced by re-feeding. From Prevot et al 2013 and Schaeffer et al. 2013.

The ME thus represents a likely pathway for the rapid access of peripheral hormones to the ARH, and consistent with the observation of hypothalamic resistance to the action of metabolic hormones such as ghrelin in obesity (Briggs and Enriori, 2010), the efficiency of this pathway was shown to be modulated by feeding state. The concept of central hormone resistance will be revisited in section 3.2.4. We will now turn to a more detailed description of some of the various circulating factors that provide direct input to the hypothalamic feeding circuits.

3.2.2 Glucose

Maintaining circulating glucose levels at a steady level to ensure a stable energy supply throughout the tissues of the body is one of the most critical aspects of metabolic homeostasis, it is thus logical that the hypothalamic system that regulate energy metabolism would be exquisitely sensitive to fluctuations in glucose levels to be able to initiate proper counter-regulatory responses. It was first shown by Oomura and Anand (Anand et al., 1964; Oomura et al., 1964) that the VMH and LHA contained neurons capable of responding to changes in glucose levels. Oomura described two kinds of glucose-sensing neurons; those that were excited and those that were inhibited by rising glucose levels, and these types of cells are now known to be expressed in several hypothalamic nuclei. Aside from the VMH and LHA, glucose sensing neurons are found in the ARH (Muroya et al., 1999), in parvocellular neurons of the PVH (Melnick et al., 2011; Chaleek et al., 2012), and the suprachiasmatic nucleus (Hall et al., 1997).

Extracellular brain glucose levels range from around 10-30% of the concentration in the blood circulation (Vries et al., 2003), and closely mirror fluctuations in blood glucose levels (Silver and Erecinska, 1994). Although this includes the hypothalamus, which may normally experience a range of 0.7 – 1.4 mmol, the ARH may be exposed to levels much closer to the blood concentration, owing to its close proximity to the ME. While many hypothalamic glucose responsive neurons are relatively insensitive to fluctuations in glucose far above the normal range in the brain (Burdakov et al., 2005; Song and Routh, 2005), neurons in the ARH can respond to much higher fluctuations between 5 – 20 mmol (Fioramonti et al., 2004). In the LHA, rising glucose levels appear to inhibit Orexin neurons but excite MCH neurons (Burdakov et al., 2005). In the ARH, a large fraction of the NPY-positive neurons are excited by a fall in glucose (Muroya et al., 1999; Fioramonti et al., 2007), whereas data on the response of POMC neurons are inconclusive, some showing no response at all (Wang et al., 2004; Fioramonti et al., 2007), while others show the majority are excited by rising glucose (Ibrahim et al., 2003).

In the PVH, about equal numbers of glucose sensitive neurons are either hyperpolarized or depolarized by a fall in glucose (Melnick et al., 2011). Although the VMH is the region

most associated with glucose sensing, the nature of these neurons are still poorly characterized. However the VMH seems to have an important role in the counter-regulatory response to general hypoglycaemia, since hypoglycaemia localized to the VMH triggers a rapid increase in circulating glucose associated with a massive increase in counter-regulatory hormones such as glucagon and epinephrine (Borg et al., 1995), while glucose infusion into the VMH can block the counter-regulatory response to systemic hypoglycaemia (Borg et al., 1997). Glucose infusion into the VMH stimulates sympathetic activity to brown adipose tissue, suggesting the VMH is also involved in the thermogenic response to hyperglycemia (Sakaguchi and Bray, 1987). In conclusion, many components of the hypothalamic feeding circuits respond to fluctuations in glucose levels to induce either feeding or increase energy expenditure, and neuronal glucosensing is by now considered to be an important factor in the central control of metabolism (B. E. Levin, 2006).

3.2.3 Lipids

Another circulating energy substrate that can provide important information about the metabolic state are fatty acids. Postprandially, the major circulating source of lipids are fatty acids derived from the liver, which are bound to lipoproteins such as LDL or VLDL, whereas during fasting, the circulating levels of free fatty acids (FFA) released from adipose tissue increases. The activity of hypothalamic neurons is known to respond to FFAs. ICV infusion of oleic acid reduces the expression of NPY and AgRP proteins in the ARH with consequent reduction in food intake and glucose production (Obici et al. 2002; Morgan et al, 2004). Inducing a cellular accumulation of FFAs by ICV injection of CPT1 which inhibits the transport of FFAs from the cytosol to mitochondria, causes a similar reduction in food intake, suggesting that it may be the intracellular concentrations of FFAs that may act as a satiety signal (Obici et al., 2003).

3.2.4 Ghrelin

Structure and synthesis

Ghrelin was identified in 1999 by Kojima and colleagues as an endogenous ligand of the Growth-hormone-secretagogue receptor (GHSR) (Kojima et al., 1999). The name derives from *ghre* meaning "to grow" since ghrelin was at first believed to act primarily to stimulate secretion of growth hormone (GH), since the GHSR is densely expressed in the pituitary but also found on GHRH-positive neurons in the ARH (Tannenbaum et al., 1998)

The *ghrelin* gene gives rise to the 117-amino acid precursor peptide pre-proghrelin, which is cleaved to proghrelin, which is in turn cleaved to produce the mature 28-amino acid ghrelin peptide. Ghrelin is further post-translationally modified by the attachment of an acyl group to its third serine residue, this modification of "des-acyl ghrelin" is necessary for it to be able to bind to the GHSR, and "acyl ghrelin" was thus for long considered to be the active form of the hormone, however it has become increasingly recognized that des-acyl ghrelin is a hormone in its own right, and may even be able to activate the GHSR, albeit substantially less potently than acyl ghrelin (Heppner et al., 2014). The acylation of des-acyl ghrelin is catalysed by the enzyme ghrelin-O-acyltransferase (GOAT) prior to secretion (Yang et al., 2008).

Ghrelin was first isolated from the stomach and is the tissue where it is predominantly located in adult animals, being synthesized by X/A-like cells of the oxyntic mucosa in the fundus of the stomach (Date et al., 2000), although it is also produced in lesser quantities in the duodenum, and to an even smaller extent in the jejunum, ileum and colon. During development, the lungs and pancreas also produce ghrelin. In fetal life ghrelin production is even substantially greater in the pancreas compared to the stomach (Chanoine and Wong, 2004), but their concentration of ghrelin gradually diminishes throughout embryonic life to become extinguished by adulthood (Volante et al., 2002; Santos et al., 2006; Arnes et al., 2012), although some have found that ghrelin expression in the pancreas persists in adulthood (Date et al., 2002). Some authors have also reported widespread distribution of ghrelin mRNA in human peripheral tissues

(Gnanapavan et al., 2002). Kojima and colleagues initially reported that ghrelin was also produced in small quantities in the hypothalamus, however the presence of ghrelin in the CNS has since been a controversial issue and most recent data point to that the mature ghrelin peptide is probably not present in significant quantities in the brain (Furness et al., 2011) .

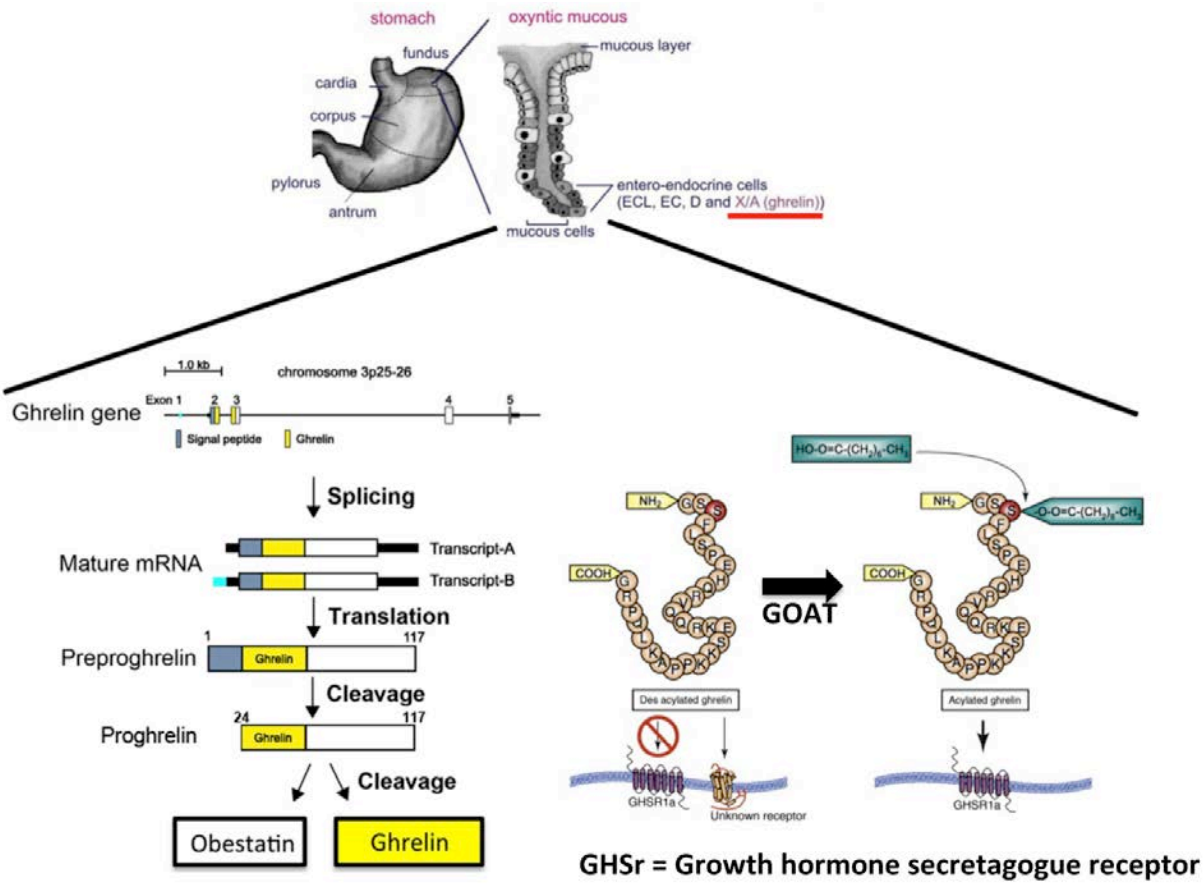

Figure 8: Ghrelin synthesis and processing. Ghrelin is secreted primarily by X/A-like cells of the oxyntic mucosa in the stomach. The mature ghrelin gene mRNA gives rise to the preproghrelin precursor which goes through several cleaving steps to eventually produce either ghrelin or obestatin. The mature ghrelin peptide is subsequently acylated by GOAT prior to secretion, permitting its binding to the GHSR. Adapted from Authesserre et al. 2009, Kojima and Kangawa 2005, and Andrews 2010.

Physiological roles of ghrelin

In addition to its potent stimulation of pituitary GH release (Kojima et al., 1999; Takaya et al., 2000), it was soon discovered that ghrelin was involved in the hypothalamic control of feeding. Both ICV (Nakazato et al., 2001) and peripheral (Wren et al., 2001a, 2001b) injections of ghrelin potently stimulates food intake and body weight gain in rats and humans in a dose-dependent manner, and independently of GH signalling (Tschöp et al., 2000). Consistent with the high density of GHSR receptors in the ARH, intranuclear ghrelin injection has the strongest effect in the ARH. Both ICV and IP ghrelin induces ARH cFos expression (Nakazato et al., 2001; Wang et al., 2002) and upregulates NPY and AgRP mRNA expression without affecting POMC expression (Kamegai et al., 2001), although electrophysiological experiments indicate that in addition to stimulating NPY neurons, ghrelin can indirectly inhibit the activity of POMC neurons by affecting GABA release onto these neurons (Cowley et al., 2003) The effect of ghrelin on feeding is abolished in mice KO for NPY and AgRP or mice treated with NPY/AgRP antagonists, demonstrating that ghrelin acts to stimulate feeding primarily via the activation of the orexigenic ARH neurons (Nakazato et al., 2001; Chen et al., 2004a). Peripheral ghrelin also stimulates Fos expression in the PVH (Rüter et al., 2003). Consistent with a role for ghrelin in regulating normal feeding behaviour, circulating ghrelin levels are increased by fasting, rise shortly prior to anticipated meal time, and are suppressed post-prandially (Cummings et al., 2001; Toshinai et al., 2001a; Tschöp et al., 2001a; Natalucci et al., 2005).

Beyond stimulating GH release and food intake, ghrelin has been found to influence numerous other physiological processes. It stimulates gastric acid secretion and motility (Masuda et al., 2000) and has protective cardiovascular effects (Nagaya and Kangawa, 2003). Ghrelin also has potent anti-inflammatory actions, it inhibits endotoxin-induced release of IL-6, IL-beta and TNF-alpha (Waseem et al., 2008; Beynon et al., 2013) while enhancing release of the anti-inflammatory cytokine IL-10 (Baatar et al., 2010) and attenuates the development of pancreatitis and oxidative brain damage (Dembinski et al., 2003; Erşahin et al., 2010). Interestingly, ghrelin has also been shown to be able to counteract many of the adverse metabolic effects induced by high-fat diet feeding, presumably due to its anti-inflammatory effects (Barazzoni et al., 2011, 2014), and was

further shown to directly inhibit the pro-inflammatory effects of leptin (Dixit et al., 2004).

Some evidence suggests that not only acyl ghrelin but also des-acyl ghrelin can induce these anti-inflammatory effects (Delhanty et al., 2013). Des-acyl ghrelin was initially thought to be biologically inactive owing to its inability to bind to the GHSR, but has been shown to have some hormonal effects of its own. Although there is conflicting data (Neary et al., 2006; Toshinai et al., 2006) several studies report an anorexigenic effect of des-acyl ghrelin (Asakawa et al., 2005; Chen et al., 2005), and it seems that des-acyl ghrelin can antagonize some of the effects of acyl ghrelin (Delhanty et al., 2012). Thus in interpreting the effects of ghrelin it may be important to consider not only circulating acyl ghrelin levels but also the acyl:des-acyl ghrelin (A/DAG) ratio. Studies have consistently shown obese subjects to have lower total ghrelin levels (Tschöp et al., 2001b), but they may in contrast have an increased A/DAG ratio. Whereas total ghrelin levels are usually inversely associated with obesity, insulin resistance and metabolic syndrome, A/DAG is in contrast positively associated with these metabolic derangements (Barazzoni et al., 2007; St-Pierre et al., 2007; Pacifico et al., 2009; Rodríguez et al., 2009), and des-acyl ghrelin may improve glycemic control in obese diabetics by suppressing the action of acyl ghrelin (Özcan et al., 2014).

One important role of ghrelin may be in glucose regulation, since ghrelin is one of the most potent endogenous stimulators of GH secretion, which in turn functions to liberate fatty acids during fasting to prevent hypoglycaemia and muscle catabolism (Møller and Jørgensen, 2009) and in high doses may even induce glyconeogenesis (Ghanaat and Tayek, 2005). Indeed, growing mice who lack the GOAT enzyme necessary for acylation of ghrelin display a profound inability to maintain glycemia during severe calorie restriction (Zhao et al., 2010a). This effect may depend on the age of the mice since the same severe results were not observed in studies using more mature mice (Gahete et al., 2012; Yi et al., 2012), although adult ghrelin and GHSR KO mice still maintain lower glucose levels during calorie restriction (Sun et al., 2008a).

The multiple interactions of ghrelin with both the hypothalamus, the GH-axis and the pancreas may explain the disparate results that have been obtained on the effects of

ghrelin on glycemia and insulinemia. Administration of ghrelin has been shown to inhibit pancreatic insulin secretion (Tong et al., 2010; Peng et al., 2012b) and induce hyperglycemia, (Broglio et al., 2001) and peripheral insulin resistance independently of GH secretion (Dezaki et al., 2004; Vestergaard et al., 2008). Since ghrelin also stimulates the release of the counter-regulatory hormones cortisol, epinephrine and glucagon in addition to GH, its actions may be interpreted as a coordinated response to maintain glycemia during energy deficits, which may explain why it is commonly observed to induce adverse metabolic effects such as hyperglycemia and insulin resistance when administered under normally fed conditions, whereas in contrast antagonizing or knocking out ghrelin or GHSR improves glucose tolerance (Esler et al., 2007) and insulin sensitivity (Chacko et al., 2012). It was further shown that the ratio of ghrelin to somatostatin, which is high during fasting but lower in fed conditions, influences whether ghrelin will have an inhibitory or stimulatory action on insulin secretion (Park et al., 2012b).

Regulation of ghrelin secretion

It is not clear exactly how ghrelin secretion is regulated. As mentioned, circulating levels are increased in fasting, suppressed by feeding and are inversely correlated with glycemia, insulin resistance and fat mass. Ghrelin is normally secreted in a ultradian pattern with peaks prior to meal times and troughs postprandially, however this pattern is lost in diet-induced obesity which also induces chronically elevated levels of insulin, lipids and glucose (Perreault et al., 2004). Glucose has mostly been shown to suppress ghrelin (McCowen et al., 2002; Shiiya et al., 2002; Baldelli et al., 2006). Insulin may be the more important factor since it also suppresses ghrelin (Flanagan et al., 2003; Murdolo et al., 2003) even in the face of insulin-induced hypoglycaemia (Saad et al., 2002; Kim et al., 2007), although some studies do report that insulin has no effect or even a stimulatory effect on ghrelin (Toshinai et al., 2001b; Caixás et al., 2002). The discrepancy may be due to dose of insulin used since severe hypoglycaemia would be expected to increase ghrelin. Somatostatin may also directly suppress ghrelin secretion (Shimada et al., 2003).

Leptin, whose signalling and secretion patterns are typically the mirror opposite of ghrelin, was found to directly inhibit ghrelin secretion from isolated rat stomach

(Kamegai et al., 2004). Ghrelin was in turn shown to antagonize leptin signalling in nodose ganglionic neurons by increasing SOCS-3 expression (Heldsinger et al., 2014). Similar to ghrelin, GHSR expression is increased by fasting and suppressed to normal levels by re-feeding, is stimulated by ghrelin and suppressed by leptin (Kim et al., 2003; Nogueiras et al., 2004; Kurose et al., 2005).

The half-life of circulating acyl ghrelin is very short, on the order of 8-10 minutes, primarily due to enzymatic hydrolysis of the unstable acyl group to form des-acyl ghrelin (Hosoda and Kangawa, 2012), and also due to rapid binding to ghrelin targets throughout the body, acyl ghrelin thus makes up only about 5-15% of the total amount of circulating ghrelin.

The ghrelin receptor

The primary target of acyl ghrelin is the growth hormone secretagogue receptor (GHSR) 1a. The GHSR is a typical 7-transmembrane G-protein-coupled receptor widely present in the brain, being most densely expressed in the pituitary, followed by the hypothalamus, with lower levels in the hippocampus, pons, midbrain and medulla oblongata (Katayama et al., 2000). Inside the hypothalamus, the highest concentrations are found in the ARH, where it is highly co-localized to NPY/AgRP-expressing cells (Willesen et al., 1999) although certain species variations exist (Zigman et al., 2006). Both in the hypothalamus and pituitary, the GHSR is expressed throughout embryonic and postnatal development at levels comparable to adulthood, although increasing slightly with age (Katayama et al., 2000). GHSR seems to also be present in numerous peripheral tissues, including the pancreas (Guan et al., 1997; Kageyama et al., 2005), adrenal cortex (Andreis et al., 2003; Carraro et al., 2004), testis (Gaytan et al., 2004) ovaries (Rak et al., 2009) and the heart (Beiras-Fernandez et al., 2010).

Upon being activated by a ligand, the GHSR induces an increase in intracellular calcium levels by the activation of G-protein subtype Gaq/11 which stimulates the production of inositol triphosphate (IP3), activation of Protein Kinase C (PKC) and Phospholipase C (PLC) with a concomitant release of calcium (Chan et al., 2004). In addition, GHSR activation also induces the phosphorylation of ERK1/2 as well as induction of the PI-3

kinase (PI3K) and phosphorylation of Akt (Grey and Chang, 2009; Rak-Mardyla and Gregoraszczuk, 2010; Chen et al., 2013).

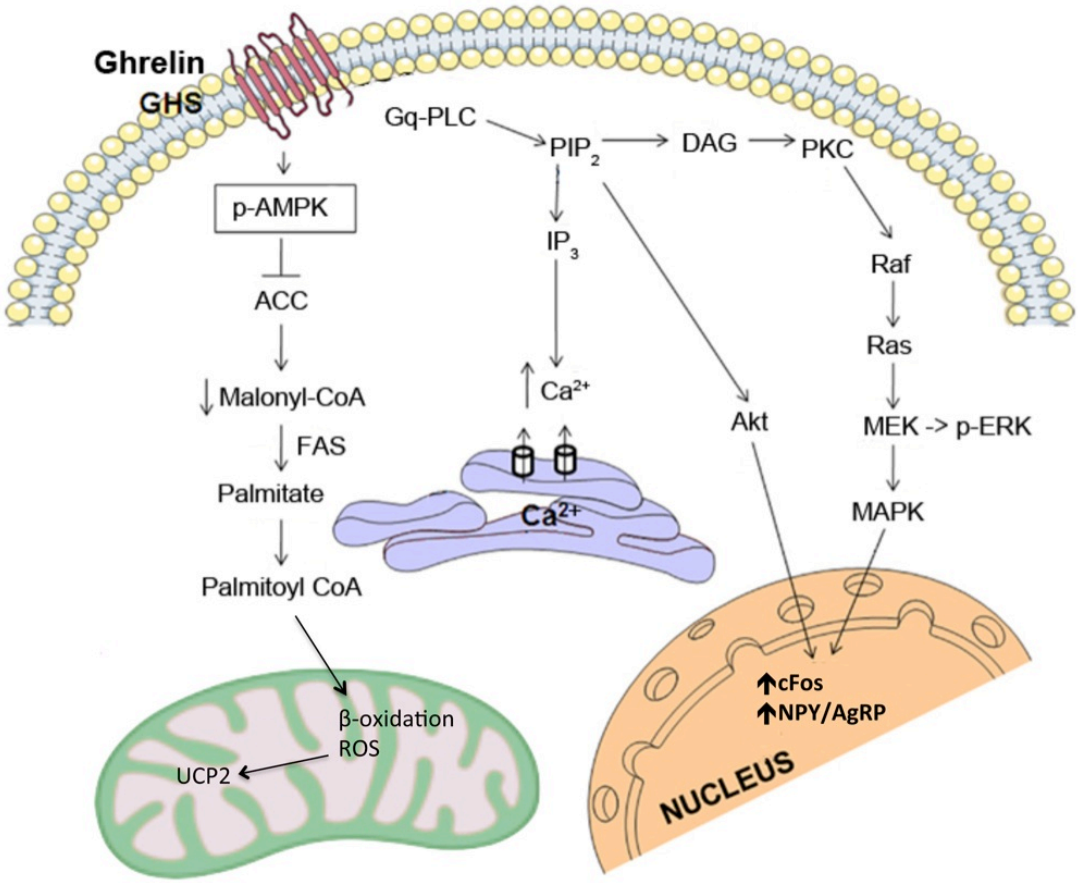

Figure 9: Intracellular signaling cascades activated by ghrelin binding to the Growth-hormone secretagogue receptor (GHSR). Upon stimulation by ghrelin, several pathways are activated: 1) AMPK phosphorylation which leads to enhanced mitochondrial fatty acid oxidation, 2) PLC-mediated phosphorylation of PIP2 which leads to release of intracellular calcium stores via IP3, 3) phosphorylation of Akt, and 4) phosphorylation of ERK via the MAPK/ERK pathway. Transcription of cFos and NPY/AgRP are upregulated following GHSR binding, likely by different mechanisms. Adapted from Frago et al. 2011.

ARH NPY neurons are activated via AMPK-mediated calcium release (Kohno et al., 2011) and ghrelin seems to activate this pathway (Kohno et al., 2008). This transduction mechanism may further involve changes in mitochondrial respiration mediated by UCP2 (Andrews et al., 2008). AMPK is an intracellular energy sensor that when stimulated

switches off ATP-consuming pathways and turns on ATP-generating pathways such as glucose uptake and fatty acid oxidation. Downstreams actions of AMPK include the disinhibition of CPT1, which transports fatty acids into mitochondria for oxidation. Ghrelin causes an increase in hypothalamic fatty acid concentration as a substrate for mitochondrial fatty acid oxidation and concurrently increases expression of CPT1 to facilitate fatty acid oxidation (López et al., 2008). Activation of UCP2 may thus buffer the ghrelin-activated cells against excess ROS production by the fatty acid oxidation, providing a mechanism whereby NPY/AgRP neurons can maintain a high ghrelin-induced firing rate during low energy states, whereas POMC neurons with their lack of GHSR receptor-induced UCP2 activation cannot buffer against ROS as well and thus cannot maintain neuronal firing during starvation (Briggs and Andrews, 2011). Both insulin and leptin may inhibit ghrelin-induced calcium release in NPY neurons, for leptin this involves PI3K and phosphodiesterase 3 signaling, but not STAT3 signaling (Kohno et al., 2007; Maejima et al., 2011). The GHSR has also been found to have a high level of constitutive activity (Holst et al., 2003) and can ligand-independently increase Ca2+ influx when transfected into cells.

Regulation of central ghrelin sensitivity

Although it has been shown that diet-induced obesity impairs the ability of ghrelin to activate hypothalamic neurons and stimulate food intake (Perreault et al., 2004; Briggs and Enriori, 2010) , and also that there is an inverse relationship between body weight and the extent of transport of ghrelin across the BBB (Banks et al., 2008), relatively little is known about the molecular mechanisms of ghrelin resistance. Diet-induced obese mice display reduced response not only to peripheral but also ICV ghrelin, probably due at least in part to reduced ghrelin-induced stimulation of NPY/AgRP neurons, suggesting that central ghrelin resistance is not only mediated by impaired transport from periphery to brain but also involves intracellular signalling defects (Briggs and Enriori, 2010). Briggs and colleagues proposed that hyperleptinemia was the critical factor inducing ghrelin resistance in this model since leptin-deficient ob/ob mice fed a HFD remain ghrelin sensitive in spite of severe obesity and glucose intolerance, while mice on HFD diet pair-fed to chow-fed controls also remain ghrelin sensitive, showing that neither obesity, HFD or glucose intolerance per se affects ghrelin sensitivity (Briggs et al., 2014). It was shown that ghrelin binding induces rapid desensitization of the GHSR

by endocytosis and intracellular recycling of the ghrelin/GHSR complex wherein it may take up to 6 hours for baseline surface ligand binding capacity to recover (Camiña and Carreira, 2004). However the relevance of this to physiological ghrelin resistance is unclear since in obesity circulating ghrelin levels are not elevated but depressed, ghrelin also stimulates the expression of GHSR, which may compensate for ghrelin-induced GHSR recycling. The ability of IP ghrelin to stimulate ARH neurons is enhanced by fasting (Hewson and Dickson, 2000)

3.2.5 Leptin

Leptin is a 146-amino acid hormone that is synthesized predominantly by adipose tissue. It was discovered by Jeffrey Friedman in 1994 as a result of identifying the mutated gene in the genetically obese ob/ob mouse, a mutant strain that is normal weight at birth but develops hyperphagia, hyperglycemia, hyperinsulinemia and severe obesity by adulthood, often attaining weights three times as high as normal mice. Treating ob/ob mice with leptin normalizes their food intake, body weight, fat mass and glycemic control, in addition to increasing spontaneous activity levels, body temperature and basal metabolic rate (Pelleymounter et al., 1995). Results such as these have clearly established leptin as one of the most important hormonal regulators of energy homeostasis. Leptin acts on the leptin receptor (OB-R) of which several splice forms exist. The long isoform OB-Rb is primarily expressed in the hypothalamus and is considered the primary leptin receptor, since db/db mice who lack only the long form leptin receptor display virtually the same phenotype as ob/ob mice, restoring OB-Rb expression in the hypothalamus of db/db mice is sufficient to mediate the effects of leptin on feeding, energy homeostasis and reproduction (Kowalski et al., 2001; de Luca et al., 2005). The functions of the remaining shorter isoforms are less clear, the OB-Ra isoform is abundantly expressed in the BBB and data showing it facilitates transcellular transport of leptin suggest it may act to transport leptin into brain targets (Hileman et al., 2000). Nonetheless, though Ob-Ra KO mice show a slight reduction in CSF/plasma ratio of leptin, the overall metabolic effects of OB-Ra deficiency are modest compared to OB-Rb deficiency (Li et al., 2013d), as are the effects of complete lack of OB-R isoforms compared to the lack of only OB-Rb (Osborn et al., 2010). OB-Rb forms a functional dimer receptor complex which upon binding leptin, induces intracellular signalling

primarily via the Janus Kinase/signal transducer and activator of transcription (JAK/STAT) pathway, leading to phosphorylation of STAT3, but also phosphorylation of several other proteins such as ERK, erbB2 and IRS1 leading also to induction of PI3K -> Akt signaling (Bjørbaek et al., 1997; Eisenberg et al., 2004; Myers, 2004).

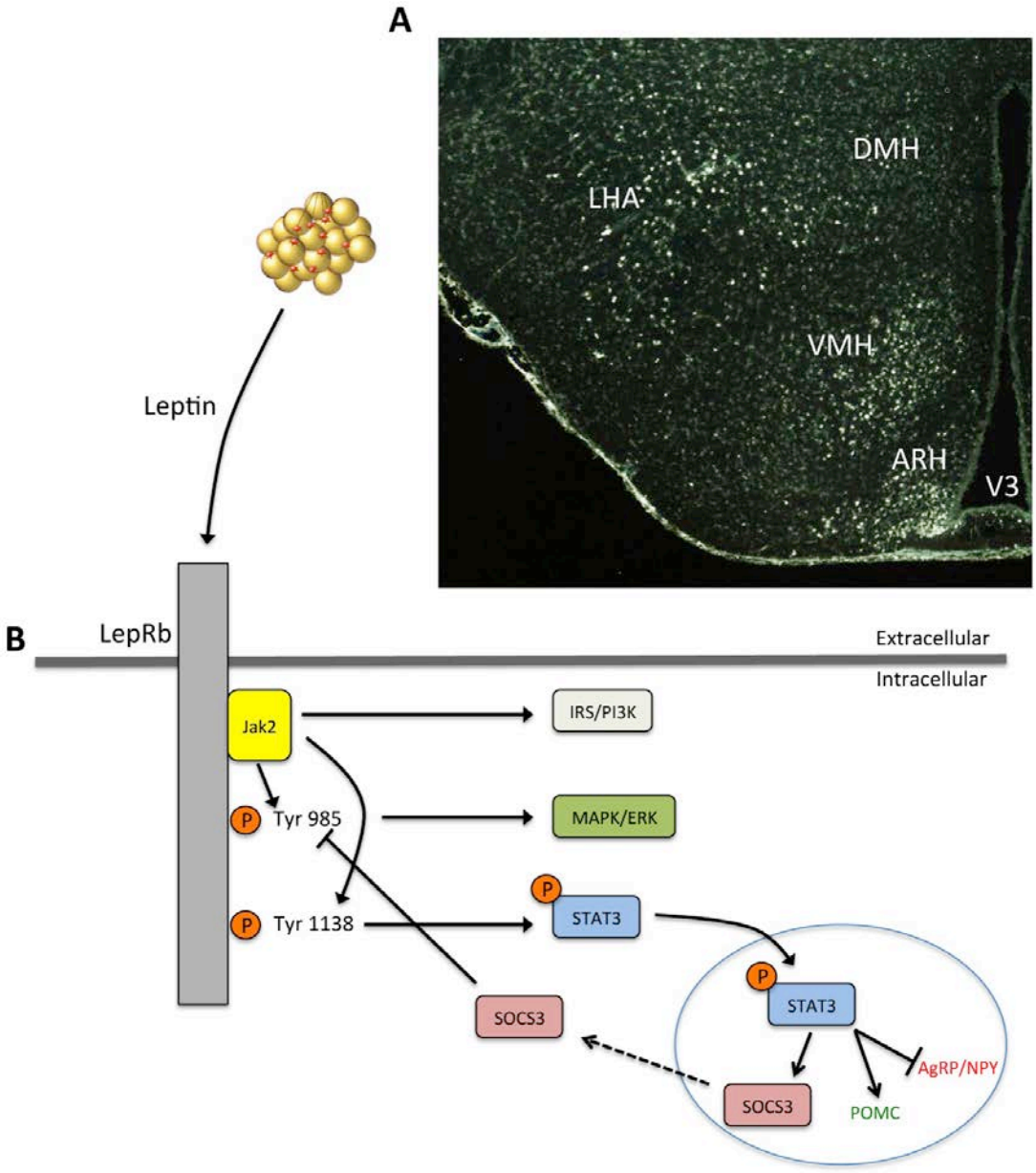

Figure 10: Leptin is secreted from adipocytes to act primarily on POMC and NPY/AgRP neurons of the arcuate nucleus. (A) Hypothalamic distribution of long form leptin receptor (OB-Rb) mRNA at the level of the ARH visualized with FISH (source Allen Brain). (B) Upon binding to the OB-Rb, leptin activates the Jak2 kinase which phosphorylates tyrosine residues 985 and 1138, in addition to activating the IR/PI3K pathway directly. Tyr 985 activates the MAP/ERK pathway, while Tyr 1138 goes on to phosphorylate STAT3. STAT3 subsequently enters the nucleus and upregulates the transcription of POMC and SOCS3, and downregulates transcription of NPY/AgRP.

In the hypothalamus, OB-Rb is most concentrated in the ARH and VMH, with lesser expression in the DMH and LHA (Baskin et al., 1999). In the ARH, they are expressed on both NPY/AgRP and POMC neurons (Cowley et al., 2001), and leptin regulates both of these populations in opposite directions (Morrison et al., 2005; Hill et al., 2008) via a mechanism that involves PI3K signalling.

Circulating leptin levels primarily correlates with adiposity and is increased or reduced by weight gain or loss, respectively, while fasting or overfeeding of carbohydrates in particular can respectively cause an acute decrease or increase in leptin levels (Ahrén et al., 1997; Dirlewanger et al., 2000). In humans, plasma leptin follows a circadian fluctuation, usually starting to rise at 11pm to reach a peak by 2am (Langendonk et al., 1998).

The observation that leptin levels are typically greatly increased in obese subjects, whereas the strong anorectic effects of leptin suggests this should drive them to lose weight, led to the idea that persistent obesity is caused by leptin resistance. The concept of central leptin resistance has since been extensively studied and the physiological mechanisms have been more clearly defined than for ghrelin.

Mechanisms of central leptin resistance may be roughly divided into two categories, diminished transport of leptin into the hypothalamus, and impaired intracellular leptin signalling. Leptin resistance caused by diet-induced obesity seems to involve both transport defects and impaired intracellular signalling (El-Haschimi et al., 2000), although the extent to which transport defects and intracellular signalling defects contributes to leptin resistance seems to vary, in some models of obesity animals retain sensitivity to centrally administered leptin (Halaas et al., 1997; Van Heek et al., 1997). In the former category, it has been observed that the ratio of leptin present in the CSF vs blood is greatly diminished in obesity, whereas obese humans have a more than three-fold elevation in circulating leptin levels, their CSF leptin levels were increased by only 30% (Caro et al., 1996).

There are several possible pathways for peripheral leptin to get inside the brain. For one, leptin is transported across the BBB by a saturable transport system (Banks et al.,

1996), and obesity is associated with reduced BBB leptin transport (Banks et al., 1999). One mechanism for this may be elevated circulating triglycerides, which are elevated in obesity and have been shown to inhibit BBB leptin transport (Banks et al., 2004), furthermore mice who lack the capacity to synthesize triglycerides have increased leptin sensitivity (Chen et al., 2002).

As mentioned previously, short isoforms of the leptin receptor are expressed in the BBB and have been implicated in BBB leptin transport (Kastin et al., 1999), however, circulating factors such as triglycerides or leptin seem to be able to inhibit BBB leptin transport without altering BBB OB-Ra expression (Nonaka et al., 2004), and alterations in leptin transport is not correlated with changes in BBB OB-R expression (Hileman et al., 2002). Leptin may also be able to access hypothalamic targets such as the ARH and VMH via the ME. Tanycytes of the ME express several OB-R isoforms, and Balland and colleagues described a pathway whereby plasma leptin can be captured and internalized by ME tanycytes to be released into the CSF. This transport mechanism was found to require functional leptin receptor signalling and tanycytic phosphorylation of ERK (Balland et al., 2014), and was impaired in diet-induced obese mice and db/db mice.

Numerous intracellular signalling defects that may contribute to leptin resistance have also been described. The intracellular signalling cascade following Ob-R activation is subject to negative feedback regulation by several proteins such as SOCS3, PTP1B and TCPTP, which all act in different ways to inhibit activity of the kinase Jak2 (Zhou and Rui, 2013). Elevated hypothalamic levels of TCPTP and SOCS-3 are observed in obesity with leptin resistance (Bjørbaek et al., 1998 p.-3; Loh et al., 2011) and genetic or pharmacological blockade of these proteins enhance leptin sensitivity and its associated metabolic effects (Cheng et al., 2002; Zabolotny et al., 2002; Howard et al., 2004; Mori et al., 2004; Kievit et al., 2006; Loh et al., 2011; Tsou et al., 2012). However some results suggest also the PI3K -> Akt pathway may be affected in the development of leptin resistance (Metlakunta et al., 2008, 2011).

SOCS-3 expression is directly regulated by STAT3 activation in a negative feedback loop (Ernst et al., 2009), and some studies suggest that chronically elevated leptin signalling at the Ob-R is one cause of leptin resistance by activation of this feedback loop (Ernst et

al., 2009; Knobelspies et al., 2010; Gamber et al., 2012). One further possible mechanism of leptin resistance is alteration in the receptor expression density, however, hypothalamic leptin receptor expression seems to be mostly, but not always (Liu et al., 2007) unaffected by diet-induced obesity (Madiehe et al., 2000; Sahu et al., 2002; Enriori et al., 2007). It has however been found to be downregulated by chronic leptin treatment (Proulx et al., 2002).

3.2.6 Insulin

Insulin is a 51-amino acid hormone that plays a central role in glucose regulation, by storing circulating glucose in muscle and fat tissue and causing a shift from fat oxidation to fat storage and thereby removing excess glucose from circulation to prevent hyperglycemia. Insulin is primarily produced by pancreatic beta cells and its secretion is stimulated by the ingestion of protein or glucose. Insulin has however also been demonstrated to exert some effects on the hypothalamic feeding center where it seems to have anorexigenic actions. ICV or intranuclear VMH insulin infusions reduce food intake and body weight (Woods et al., 1979; McGowan et al., 1990; Air et al., 2002), whereas reducing hypothalamic insulin receptor content or infusing insulin antibodies causes hyperphagia and fat gain (Strubbe and Mein, 1977; Obici et al., 2002a).

Consistent with these effects, insulin was shown to electrically excite POMC neurons (Qiu et al., 2014) and inhibit NPY expression in the ARH (Schwartz et al., 1992; Sato et al., 2005). However, insulin appears to mediate at least some of its hypothalamic actions via insulin receptors in the VMH as well (Klöckener et al., 2011). Insulin activates the IRS1-> PI3K pathway which appears to be crucial to for its actions both in ARH and VMH neurons (Niswender et al., 2003; Klöckener et al., 2011), and further acts with ERK signalling in ARH neurons (Streiff et al., 2014). Insulin, leptin and ghrelin thus all act on both NPY/AgRP and POMC neurons of the ARH and appear to share several common signalling pathways in these neurons.

3.2.7 Other hormones

Choleocystokinin: or CCK is a peptide hormone secreted by the small intestine. Its secretion is stimulated primarily by ingestion of fats and its primary purpose is to stimulate the secretion of digestive enzymes and bile acids from the pancreas and gallbladder. CCK also has a hunger-suppressive effect when injected ICV or IP (Krinsky et al., 1979; Schick et al., 1986; Hirosue et al., 1993). It is known to slow the firing rate of a subset of ARH neurons (Burdakov and Ashcroft, 2002) and induce phosphorylation of STAT3 in the ARH and cFos in ARH and PVH, as well as synergistically increase leptin-induced pSTAT3 (Merino et al., 2008).

Peptide YY: or PYY is a 36-amino acid hormone secreted by the colon and ileum in response to feeding, and is conversely decreased by fasting. PYY has a close structural homology to NPY and binds to the Y2 receptor in the Y receptor family. Y2 receptor expressing cells are present in the ARH, stimulation of these receptors has an anorexigenic effect, and intranucleuar injection of PYY into the ARH inhibits the increase in hoarding and feeding normally stimulated by food deprivation (Teubner and Bartness, 2013). Peripheral injection of PYY likewise inhibits food intake and weight gain (Batterham et al., 2002; Adams et al., 2006) and inhibits fasting-induced activation of ARH neurons (Riediger et al., 2004).

Pancreatic polypeptide: Or PP is a 36-amino acid hormone secreted from the pancreas. It is a close analog of PYY but acts on the Y4 receptor instead. Like PYY it has central appetite-suppressing effects by acting on hypothalamic neurons, mostly in the ARH, but appears to act by different pathways (Hankir et al., 2011), with PYY activating primarily the dorsal ARH, the DMH and PVH, whereas PP activates the lateral ARH, DMH, VMH and LHA (Shi et al., 2013), inhibiting orexin in the LHA and upregulating BDNF in the VMH (Sainsbury et al., 2010).

Glucagon: Glucagon is a 29-peptide hormone secreted by the pancreas that is primarily involved in glucose regulation and is in many ways the mirror opposite of insulin. It is secreted in response to fasting or hypoglycaemia to raise glucose levels by causing the

liver to convert stored glycogen into glucose. A moderate amount of glucagon binding capacity is evident in the hypothalamus (Hoosein and Gurd, 1984) and hypothalamic glucagon signalling may be involved in central control of glucose homeostasis by regulating hepatic glucose production (Mighiu et al., 2013), although it was also paradoxically reported to act in the PVH to increase thermogenesis and glucose utilization (Atrens and Menéndez, 1993).

Glucagon-like peptide 1: or GLP-1 is derived from the same precursor as glucagon but is unlike glucagon primarily secreted from the intestines. It is stimulated by various nutrients like protein, fat and carbohydrates and acts to enhance glucose-induced insulin secretion while inhibiting the actions of glucagon. Like several other hormones secreted in response to food intake, it has been found to function as a satiety signal in the hypothalamus, and it has recently received much attention for its potential anti-diabetic and anti-obesity effects (Shirazi et al., 2013). GLP-1 receptors are densely expressed in the ARH, where GLP-1 acts to stimulate POMC neurons (Ma et al., 2007) and also prevents the fasting-induced decrease in ARH POMC and CART mRNA expression (Seo et al., 2008). GLP-1 also stimulates the majority of PVH CRH-positive neurons and a smaller portion of oxytocinergic PVH neurons (Larsen et al., 1997).

Obestatin: Obestatin is a 23-amino acid hormone derived from alternative cleavage of the proghrelin precursor that also produces ghrelin. While early studies showed the hormone to have an anorexigenic effect, most recent studies have failed to repeat these findings and the physiological role of obestatin is currently unclear (Hassouna et al., 2010). Some authors reported that obestatin can antagonize ghrelin-induced GH secretion and feeding (Zizzari et al., 2007) although others have failed to demonstrate any role for obestatin in energy homeostasis (Nogueiras et al., 2007) while other authors suggested a role for obestatin in regulating thirst and fluid balance (Samson et al., 2007, 2008). Obestatin may have an important role in glucose and lipid metabolism (Gargantini et al., 2013) and other functions in the regulation of cell proliferation and apoptosis have been suggested, but more research is needed to clarify its actions and to identify its receptor (Trovato et al., 2014).

4. Environmental control of developmental programming

4.1 Perinatal development of the hypothalamus

The perinatal development of the hypothalamic feeding circuits can be roughly divided into three stages. The embryonic origin of the hypothalamus is the region of the neural tube called the diencephalon; the first stage thus comprises the birth of the hypothalamic neurons and their migration from the diencephalic neuroepithelium into their respective hypothalamic zones. The second phase comprises the final differentiation of the cells and the growth of their axonal fibers, and the third and final phase consists of axon growth termination and synaptogenesis to form the fully functional neuronal networks.

4.1.1 Embryonic development

In both humans and rats, the first phase comprising the migration of neurons and formation of the differentiated hypothalamic regions has been described to occur in three distinct migratory waves between embryonic day 12 (E12) and 19 in rodents, and approximately during the first half of gestation in humans. Neurons that populate the lateral hypothalamus migrate first, followed by the development of the paraventricular, dorsomedial and ventromedial nuclei, followed by the arcuate nucleus which forms quite late, and the development of the tanycytic epithelium comprising the ME which forms even later and only matures completely after birth (Altman and Bayer, 1978a, 1978b, 1978c). Although later studies tracking the terminal differentiation of neurons using BrdU concluded that the vast majority of the neurons in the ARH, VMH, DMH, PVH and LHA are born at the same time around E12 (Ishii and Bouret, 2012). A peak in the birth number of hypothalamic neuroendocrine cells that secrete TRH, CRH, GHRH, somatostatin and dopamine is observed around E12-13, whereas cells immunoreactive for TH or GHRH in the presumptive ARH are observed first at E15 (Markakis, 2002). Fibers immunoreactive for CRH and GHRH projecting to the ME can be observed already by E16-18 (Daikoku et al., 1984; Markakis, 2002).

Thus, in the rodent brain, certain parts of the hypothalamic neuroendocrine network are already relatively mature prior to birth. In the mouse ARH, immunoreactivity for POMC is evident in neurons of the ARH as early as E10,5 whereas NPY expression is only observed at E13,5. Curiously, BrdU studies show that both populations of neurons are born at the same time, and that actually the NPY/AgRP neurons originate from a subset of POMC-expressing neurons whose expression of POMC is extinguished between E14-18 and instead begin expressing NPY/AgRP (Padilla et al., 2010).

4.1.2 Postnatal development

In contrast to the projections from neuroendocrine cells of the PVH and to a lesser extent the ARH that extend to the ME, the axonal fibers that project from the ARH to the adjacent hypothalamic nuclei the PVH, DMH and LHA are virtually undeveloped at birth. Bouret and colleagues tracked the postnatal development of these fibers with ARH injections of the anterograde tracer DiI, and observed that the ARH projections to the DMH, PVH and LHA only start to develop by the end of the first postnatal week and only attain a projection pattern resembling that of adults by the end of the third postnatal week (Bouret et al, 2004). The DMH is the first nucleus to become innervated by ARH fibers starting around P6, with the fiber density increasing by about six-fold by P12. The PVH is next, with significant amount of fibers only appearing by P10 and attaining a mature pattern by P16, whereas the LHA only begins to be innervated by P12 and attains a mature pattern by P18. Consistent with these findings, peripheral leptin administration can substantially induce cFos in the ARH already at P6, whereas leptin fails to induce cFos in the PVH until P10 and in the LHA significant amounts of leptin-induced cFos is not observed until P16 (Bouret et al., 2004a).

Collectively these data imply that the hypothalamic feeding circuits that serve to integrate peripheral signals of energy balance via the ARH are not mature at birth in mice, and only develop during the early postnatal period, to mature approximately around the time the pups are mature enough to be weaned from milk-only feeding. The final phase comprising termination of axon growth and synaptogenesis is poorly described, but appears to progress quite slowly, with the number of synapses in the ARH at P20 only approaching half of the quantity present at P45 when synaptic connections

resemble those seen in adults (Matsumoto and Arai, 1976). There is some evidence that the polygenic susceptibility to develop obesity is associated with differences in the structure of hypothalamic feeding circuits, since mice bred to be resistant to diet induced obesity display greater densities of POMC and AgRP IR fibers in the PVH than mice who are more susceptible to obesity, both during development and as adults (Bouret et al., 2008), and furthermore display differences in synaptic reorganization of POMC axon terminals in response to a high-fat diet (Horvath et al., 2010).

It should be noted that owing to the significantly longer gestation period compared to rodents, in primates the first phase of neurogenesis, migration and formation of the hypothalamic nuclei takes place far earlier during embryonic development compared to rodents, with the peak of hypothalamic neuron births occurring in the first quarter of the gestational period in macaques, which in rodents only occur after midgestation (Markakis, 2002). Similarly, the developmental events taking place in the postnatal period in rodents such as the formation of ARH-PVH AgRP fibers are already well in progress by the late second trimester in primates (Grayson et al., 2006), and are considered to occur in the last trimester and be completed before birth in humans (Koutcherov et al., 2003).

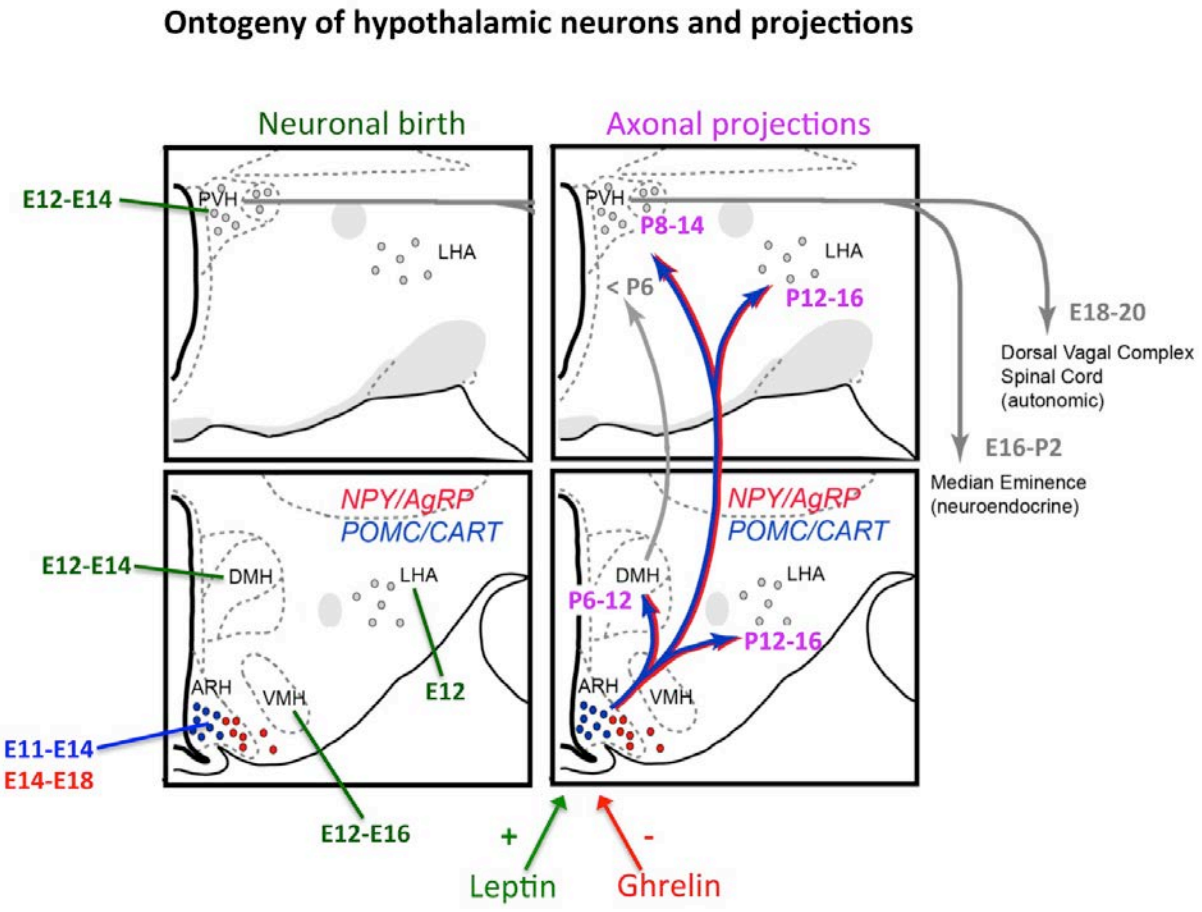

Figure 11: Birth and axonal development of the hypothalamic feeding circuits in rodents. Hypothalamic neurons populating the ARH, DMH, VMH, LHA and PVH undergo final differentiation and migrate from the neuroepithelium to reach their destination around embryonic day 11 to 19, although migration is largely complete already by E12. Projections from the PVH to the median eminence are mostly complete before birth, whereas the NPY/AgRP and POMC projections extending from the ARH reach their hypothalamic targets in the DMH, LHA and PVH around P6 to P16. Leptin and ghrelin have been found to have a stimulatory and inhibitory influence, respectively, on the growth of ARH axonal projections. Adapted from Ishii and Bouret 2012, Bouret et al 2004a, b, Steculorum et al. 2014, and Padilla et al 2010.

4.2 Endocrine regulation of hypothalamic development

4.2.1 Leptin

The first clues that leptin was involved in brain development came more than a decade before it was even discovered. Several groups had reported that leptin-deficient ob/ob rodents displayed various defects in brain development, including reduced total brain weight and volume as well as reduced neuronal soma size, altered dendritic organization of VMH and LHA neurons, significantly decreased brain myelination and accelerated neuronal degeneration in the ARH and VMH (Bereiter and Jeanrenaud, 1979, 1980; Garris, 1989). It was later found that administration of leptin to ob/ob mice could induce brain growth apparently by increasing the number of brain cells (Steppan and Swick, 1999). Although in adult mice leptin has a crucial role in maintaining thyroid signalling, which in turn has a fundamental effect on brain development and maintenance (Sher et al., 1998), so it was not clear whether the brain defects in ob/ob mice were directly due to leptin or due to the congenital hypothyroidism they suffer (van der Kroon et al., 1982), or indeed due to indirect affects on any of the other myriad endocrine systems regulated by leptin. Later studies have provided more direct evidence that leptin is involved in embryonic brain development. OB-Rb is widely expressed in the embryonic mouse brain; central leptin injections stimulate the proliferation of neuroepithelial and cortical plate cells in vivo and leptin treatment maintains neural progenitor cells in vitro (Udagawa et al., 2000, 2006a, 2006b). Leptin also induces cell proliferation and differentiation in fetal hypothalamic neurospheres (Desai et al., 2011), indicating that leptin may influence hypothalamic development during embryogenesis.

Considering the immaturity of the hypothalamic neuronal circuits on which leptin primarily acts to regulate energy homeostasis, it is not surprising that leptin has little effect on metabolic regulation during the early postnatal period. Not only does neither peripheral nor central leptin administration alter food intake, oxygen consumption, body weight, or fat mass (Mistry et al., 1999; Proulx et al., 2002) until at least P17, but leptin-deficient ob/ob mice, who become massively obese as adults, display essentially normal growth rates up until the third postnatal week (Bouret and Simerly, 2007).

However, leptin does modulate the activity of ARH neurons during this period as evidenced by altered expression of NPY and POMC (Proulx et al., 2002) as well as cFos induction by leptin (Bouret et al, 2004). Furthermore, several groups independently observed a brief surge in the circulating levels of leptin between the first and second postnatal week, temporally coinciding with the postnatal growth spurt of ARH axonal projections (Devaskar et al., 1997; Rayner et al., 1997; Ahima et al., 1998). The leptin surge is accompanied by a surge in the expression of OB-Rb in the ARH, which is expressed at low levels between P2-P6 to then undergo a more than 4-fold increase by P10 (Bouret et al., 2012).

As suggested by Ahima and colleagues, it was indeed found by Bouret and colleagues that leptin acts as a signal for the postnatal maturation of the endocrine axis. They showed that leptin deficient ob/ob mice have severely impaired growth of ARH fibers innervating the PVH, LHA and DMH that persists into adulthood, and that these impairments can be largely reversed by leptin injections during early postnatal life, but not in adulthood (Bouret et al., 2004b). Leptin appeared to directly stimulate the growth of axonal fibers since the same results were obtained by administering leptin to ARH explants in vitro.

Several groups have shown that temporally restricted leptin treatment during postnatal life can have persistent metabolic effects, either ameliorating the adverse effects of fetal undernutrition (Vickers et al., 2005a; Attig et al., 2008a, 2013a) which is known to cause hypoleptinemia with a premature surge in leptin related to catch-up growth. Or, leptin treatment can itself induce adverse effects such as obesity, hyperphagia and leptin resistance if used to simulate a premature surge (de Oliveira Cravo et al., 2002; Yura et al., 2005, 2008; Passos et al., 2009a; Marques et al., 2010; Samuelsson et al., 2013). Attig and colleagues showed that leptin may in addition to its effects on the hypothalamus also directly promote normal growth of several peripheral organs such as pancreas, kidneys, spleen and thymus in undernourished fetuses (Attig et al., 2013a)

Further evidence of leptin's involvement in the postnatal programming of metabolism comes from studies using newly developed leptin antagonists. Attig and colleagues showed that leptin antagonism from P2-13 had no effect on growth trajectories on a

normal chow diet, but impaired leptin sensitivity and exacerbated weight gain on a HFD diet (Attig et al., 2008b). In contrast, Beltrand and colleagues showed that leptin antagonism between P3-P7 tended to decrease weight and fat gain on standard chow diet and significantly reduced weight gain on a HFD diet (Beltrand et al., 2012). The disparate results may be due to differences in dosing, or it may more probably be due to sex differences since Attig used females and Beltrand males, and the effect of neonatal leptin antagonism on hypothalamic neurotrophic factors and neuropeptide levels was shown to differ markedly between males and females (Mela et al., 2012).

The intracellular mechanisms implicated in leptin's stimulation on postnatal hypothalamic development remain largely unexplored. Several of the intracellular signalling cascades activated by leptin such as JAK/STAT3, MAPK -> ERK and PI3K -> Akt are known to be involved in the process of axonal growth in several types of neurons (Doherty et al., 2000; Conway, 2006; Zhou and Snider, 2006). The growth-stimulatory effect of leptin in the postnatal hypothalamus was shown to depend on STAT3 and ERK signaling (Bouret et al., 2012) but leptin may also stimulate axonal growth in other types of neurons through PI3K and PKC signalling (Valerio et al., 2006). Bone-morphogenetic proteins (BMP) are a family of growth factors that have recently been implicated in the development of neuronal axons via their activation of the Smad 1/5/8 effectors which act as transcriptional factors to promote neurite extension (Hegarty et al., 2013, 2014; Kelly et al., 2013), and signalling through BMP receptors was recently shown play a role in the postnatal development of ARH axonal fibers and to be essential for leptin-stimulated neurite growth of ARH neurons (Peng et al., 2012a).

4.2.2 Ghrelin

As mentioned, the pancreas and lungs are major secretors of ghrelin during fetal life, and GHSR mRNA is widely expressed by fetal tissues (see Steculorum and Bouret 2011 for review), suggesting that ghrelin may be involved in embryonic development. Maternal administration of ghrelin during pregnancy results in higher birth weights, while immunization against ghrelin during pregnancy results in lower birth weights (Nakahara et al., 2006). While mice lacking either the GHSR or ghrelin display virtually normal growth and development, they have been shown to resist the development of

diet-induced obesity when fed a HFD during adolescence (Zigman et al., 2005), but not adulthood (Sun et al., 2008b). These results suggest that ghrelin is involved in the programming of metabolic regulation, but that compensatory mechanisms exist that allow normal development of the system regulating energy homeostasis in the long-term. Ghrelin may additionally have an age-dependent effect on the development of peripheral organs such as the pancreas and gastrointestinal system, since ghrelin administration to newborn rats causes reduced pancreatic and gastric growth with reductions in DNA synthesis and enzyme contents, whereas ghrelin treatment in older rats caused increased growth of the pancreas and the gastric system with associated increases in DNA synthesis (Dembiński et al., 2005; Warzecha et al., 2006). This may be linked to ghrelin's ability to potently stimulate GH secretion already from postnatal day 10 and onwards (Pinilla et al., 2003).

Ghrelin can stimulate neurogenesis in neurons of the dorsal vagal complex and nucleus of the solitary tract in adult rats (Zhang et al., 2004, 2005) and can also promote neurogenesis during fetal development in the spinal cord (Sato et al., 2006) as well as the hypothalamus both during late embryonic development and shortly after birth (Inoue et al., 2010). Interestingly there appeared to be age-related changes in the ability of both des-acyl and acyl ghrelin to promote neurogenesis, as des-acyl ghrelin had the strongest promotional effect during embryogenesis, whereas only acyl ghrelin was capable of stimulating neurogenesis in the postnatal hypothalamus and spinal cord (Inoue et al., 2010).

Although ghrelin potently modulates feeding and neuronal activity in hypothalamic feeding centers in adulthood, it has similarly to leptin been found to not have any effect on feeding during early postnatal life (Piao et al., 2008; Steculorum and Bouret, 2011a). However, the GHSR is expressed in the ARH at high levels from early postnatal life, and ghrelin can modulate the activity of ARH neurons in neonatal rats as early as P7, as evidenced by cFos induction and ghrelin-induced changes in NPY and POMC mRNA levels both in vivo and in vitro (Goto et al., 2006; Steculorum and Bouret, 2011a).

Steculorum and colleagues have extensively studied the role of ghrelin in the postnatal development of the hypothalamus and metabolic programming (Steculorum et al.,

Under revision) and their major findings will be summarized in the following paragraphs. Similar to leptin, circulating ghrelin levels in mice display a distinct secretion pattern during neonatal life, staying at low levels during the first two postnatal weeks to thereafter gradually increase and reach adult levels by the end of the third postnatal week. This pattern appears to be developmentally regulated and not a result of changes in feeding since serum ghrelin levels are not correlated with stomach milk content either at P6 or P14 and at P14 is not altered by short-term fasting. Peripheral ghrelin is capable of inducing both cFos and ERK phosphorylation in the ARH as early as P6, suggesting it could act as a signal for postnatal hypothalamic development. Indeed, they showed that antagonizing the signalling of ghrelin from P4 to P22 enhanced the growth of ARH axonal projections into the PVH, DMH and LHA, causing persistent elevations in the densities of AgRP and aMSH IR fibers in adulthood, which was associated with accelerated postnatal weight gain and persistently increased body weight, fat mass, food intake and fasting glycemia as well as leptin resistance in adulthood.

Conversely, treating neonates with ghrelin between P4 and P22 caused persistent reductions in the densities of ARH axonal projections in the PVH and LHA, which was also associated with accelerated post-treatment weight gain and persistent elevations in fasting glycemia, insulin and leptin. They confirmed the inhibitory effect of ghrelin on ARH axonal growth in vitro by observing that ARH explants treated with ghrelin displayed significantly reduced neurite outgrowths. Although they further observed that ghrelin was able to inhibit the leptin-induced phosphorylation of STAT3 in vivo and block the neurite-growth promoting effects of leptin in vitro, suggesting ghrelin may act to inhibit axonal growth both directly and indirectly by blocking the signalling of leptin. Finally, they observed that ghrelin-KO mice initially displayed the same increased fiber density as mice subjected to neonatal ghrelin antagonism, but that their fiber densities had declined to normal levels by P35, lending support to the idea that compensatory signalling pathways are activated in ghrelin-KO mice that can eventually restore normal development.

4.2.3 Insulin

Considering the central role of insulin in energy homeostasis and the fact that disturbances in insulin signalling are among the most prominent and consistently found abnormalities in most models of perinatal programming such as maternal diabetes, and perinatal under- or overnutrition, insulin has long been recognized as one the main hormonal mediators of perinatal metabolic programming. Insulin seems to play an important, yet not essential role in embryonic development since insulin-deficient pups are smaller and lighter at birth yet otherwise morphologically normal, with the exception of some pancreatic abnormalities (Duvillié et al., 1997; Terauchi et al., 2000),. Pancreatic insulin secretion in response to glucose is already highly developed during late fetal and early neonatal life (Mendonça et al., 1998). Maternal insulin cannot pass the placental barrier whereas maternal glucose readily passes into the fetus to stimulate insulin secretion (Desoye et al., 2011). This suggested that the cause of the metabolic abnormalities in foetuses of diabetic women, such as macrosomia and pancreatic abnormalities might be due to maternal hyperglycemia causing fetal hyperinsulinemia (Farquhar, 1962).

Plagemann and colleagues later found that fetal hyperinsulinemia caused by gestational diabetes or fetal insulin administration lead to numerous persistent alterations in the hypothalamus of the offspring, such as increased number of orexigenic NPY and Galanin-positive neurons in the ARH and a reduction in the mean nucleus size, cell density, and nucleus:cytoplasm ratio of the ARH, VMH and PVH (Plagemann et al. 1998; Plagemann et al. 1999; Plagemann et al. 1999; Plagemann et al. 1999), and that this pharmacologically induced hypothalamic hyperinsulinemia was associated with increased body weight, glucose intolerance, hyperinsulinemia and hypertension in the adult rats (Harder et al., 1998). They further found that insulin treatment during early postnatal life also had long-lasting metabolic effects like obesity, glucose intolerance, hyperinsulinemia and increased susceptibility to STZ-induced diabetes (Plagemann et al. 1991; T. Harder et al. 1999). Like leptin, insulin can stimulate cell differentiation in brain explants and hypothalamic neurospheres, although to a lesser extent than leptin, and also primarily stimulates astrocyte differentiation in contrast to leptin (Schechter and Abboud, 2001;

Desai et al., 2011). Similar to leptin, insulin was also found to stimulate neurite growth in vitro (Schechter et al., 1999) and insulin was found to synergistically enhance the neurite-promoting effects of estrogen (Toran-Allerand et al., 1988).

Steculorum and colleagues investigated the effects of fetal hyperinsulinemia induced by STZ-induced maternal diabetes on the development of hypothalamic feeding circuits (Steculorum and Bouret, 2011b). In addition to persistent obesity, hyperphagia, hyperglycemia, hyperinsulinemia and leptin resistance, they reported that the densities of AgRP and aMSH IR fibers in the PVH were reduced by more than half in the adult offspring of diabetic dams, suggesting that like ghrelin and leptin, insulin has an important influence on the perinatal maturation of the hypothalamic feeding circuits and metabolic programming. Vogt and colleagues further reported that hypothalamic insulin signaling may play a role in the programming of glucose regulation in offspring of mice fed a HFD during lactation, since the found that knocking out the insulin receptor specifically in POMC neurons enhanced glucose tolerance and glucose-stimulated insulin secretion, probably by altering the development of POMC projections to the pre-autonomic part of the PVH (Vogt et al., 2014), which are implicated in the autonomic control of the pancreas (Marino et al., 2011).

4.3 Role of perinatal nutrition in hypothalamic development

4.3.1 Pre- and postnatal undernutrition

The effects of IUGR induced by maternal restriction of calories or protein on hypothalamic development are relatively well understood, although there is significant heterogeneity in the results, probably due to the myriad different protocols used to induce IUGR. As mentioned previously IUGR results in a premature leptin surge associated with catch-up growth that is presumed to be causally involved in its effects on metabolic programming (Yura et al., 2005), these authors found that both IUGR induced by maternal calorie restriction and artificial premature leptin surge were associated with increased densities of NPY and CART-positive nerve terminals in the PVH.

Coupé and colleagues found that whereas maternal protein restriction exclusively during gestation with subsequent rapid postnatal catch-up growth was associated with early leptin resistance, it did not have deleterious effects on the growth of AgRP and aMSH IR fibers in the PVH compared to control rats. In contrast, pups subjected to maternal protein restriction during both gestation and lactation displayed impaired development of AgRP and especially aMSH IR fibers in the PVH that was associated with neonatal hypoleptinemia (Coupé et al., 2010). Paradoxically, the former group experiencing rapid catch-up growth with normalized hypothalamic development was shown to have substantially elevated visceral fat levels in adulthood whereas the latter group displaying defects in hypothalamic maturation were protected from obesity (Coupe et al., 2012), although heightened sensitivity to leptin and melanocortin agonists and reduced sensitivity to NPY in IUGR rodents may help explain these findings (Stocker et al., 2012).

Delahaye and colleagues found that 50% maternal calorie restriction in the last week of gestation throughout lactation caused severely reduced leptin levels in the offspring during lactation which was associated with a reduction in PVH POMC fibers at P21, but no difference in AgRP fibers (Delahaye et al., 2008). IUGR in primates has been found to upregulate CRH expression and downregulate leptin receptor expression in the fetal hypothalamus (Li et al., 2013b), as well as cause alterations in fetal ARH NPY and POMC expression consistent with a priming for increased appetitive drive after birth (Li et al., 2013a). Fukami and colleagues found that maternal restriction only during late gestation caused a persistent alteration in the hypothalamic balance of orexigenic and anorexigenic mediators such as AMPK, pAkt, NPY and POMC mirroring the state observed during fasting in normal mice, to favour persistently elevated appetitive drive in adult IUGR mice (Fukami et al., 2012).

Finally, unpublished data from Bouret and colleagues demonstrate that postnatally undernourished mice raised in large litters display impaired growth of ARH axonal projections into the PVH during postnatal development that persists into adult life (Bouret et al, unpublished data). In summary these data are consistent with the idea that perinatal undernutrition, particularly if it proceeds into postnatal life, causes neonatal hypoleptinemia which impairs development of hypothalamic feeding circuits. Prenatal

undernutrition coupled with rapid postnatal catch-up growth may in contrast cause an exaggerated and premature elevation of leptin that is nonetheless associated with normalization of hypothalamic fiber development compared to pups that do not experience catch-up growth. However, the observation that rapid catch-up growth causes adult-onset obesity and leptin resistance whereas growth restriction without catch-up growth is protective from obesity, makes the connection between hypothalamic circuit development and later metabolic outcomes in perinatally undernourished pups difficult to interpret.

4.3.2 Pre and postnatal overnutrition

In both rodents and primates, offspring of mothers fed a high-fat diet during pregnancy show alterations in hypothalamic development. Chang and colleagues reported that maternal HFD stimulated the proliferation of neural precursor cells in the embryonic neuroepithelium which resulted in increased number of cells expressing the orexigenic peptides galanin, dynorphin and enkephalin in the PVH and orexin and MCH in the LHA (Chang et al., 2008). Kirk and colleagues reported that maternal HFD is also associated with an exaggerated neonatal leptin surge and later central leptin resistance in offspring, as well as decreased density of PVH AgRP fibers (Kirk et al., 2009). Hypothalamic SOCS3 and STAT3 levels are increased by maternal HFD, suggesting that perinatal overnutrition causes hyperleptinemia with resulting exaggerated hypothalamic leptin signalling and central leptin resistance due to activation of negative feedback signals (Rajia et al., 2010). Maternal HFD is also associated with increased hypothalamic expression of the "fat mass and obesity associated" (FTO) gene (Caruso et al., 2011). Grayson and colleagues studied the effects of maternal HFD in non-human primates and found a marked reduction in AgRP-positive fibers in the PVH of the HFD primate foetuses during the third trimester, as well as a modest increase in POMC mRNA expression, and strong upregulation in markers of hypothalamic inflammation (Grayson et al., 2010).

As described in section 2.5.3, postnatal overnutrition by small litter rearing is associated with numerous alterations in the responses of hypothalamic neurons in several different nuclei to various neuroendocrine signals, suggesting widespread changes in the neural

connectivity of the hypothalamus in postnatally overnourished mice (Davidowa et al., 2003b). Small litter rodents further display changes in distinct hypothalamic neuronal populations such as increased number of galanin-positive neurons and decreased number of CCK-positive neurons in the PVH (Plagemann et al., 1998b, 1999d). Unpublished data from Bouret and colleagues (Bouret et al., 2007) show that mice reared in small litters also have permanently reduced densities of AgRP and aMSH positive fibers in the PVH.

Similar to mice born to obese dams, pups reared in small litters display numerous endocrine alterations during postnatal life that may be contributing to their abnormal hypothalamic development. Rodents raised in small litters have repeatedly been shown to be hyperinsulinemic and hyperleptinemic from an early age even in the absence of hyperglycemia (Plagemann et al., 1999e) and at P7 can already display as much as a 4-fold increase in insulin levels and a 7-fold increase in leptin (Schmidt et al., 2001). Data from our lab suggest that the observed increase in leptin in SL pups does not reflect a global increase in leptin throughout the lactation period but rather a greatly exaggerated leptin surge that reaches a peak around the same date as it does in normal pups at about 7-fold higher concentration, then gradually declines to settle at about a 2-fold elevation in plasma leptin around P22. In light of the previously described effects of the hormones leptin and ghrelin on the postnatal maturation of the hypothalamus, there is surprisingly little data on the possible relation of such endocrine abnormalities to the development of the hypothalamus and the postnatal programming of metabolism in postnatally overnourished mice.

The subject of the present thesis was thus two-fold: the first was to make a detailed characterization of the effects of postnatal overnutrition on the ghrelin system during postnatal development, and to investigate whether changes in postnatal ghrelin signalling makes a causal contribution to the adult phenotype of SL mice. The second was to investigate the contribution of neonatal hyperleptinemia to the long-term metabolic alterations caused by postnatal overnutrition.

Aim of the study

Aim of the study

It is by now clearly established that overnutrition during critical periods of brain development can have lasting consequences on the metabolic programming of an organism, influencing its susceptibility to develop obesity or metabolic diseases such as diabetes and heart disease in adulthood. The hypothalamus has a critical role in controlling the metabolic processes which, if dysregulated, can lead to metabolic disease, and disruptions in hypothalamic development is known to cause persistent adverse metabolic effects.

Following the discoveries that disturbances in the signalling of the nutritionally regulated hormones ghrelin and leptin can influence the postnatal development of the hypothalamus and metabolic programming in rodents, the aim of this thesis was to investigate the effects of postnatal overnutrition on the signalling of ghrelin and leptin during postnatal development on both the peripheral and central levels. Both leptin and ghrelin display distinct patterns of secretion during development that are believed to be significant for their developmental role. Thus, we further aimed to assess how disturbances in the postnatal signalling of ghrelin and leptin are causally related to the postnatal programming of the metabolic phenotype observed in postnatally overnourished rodents.

The first part of my thesis was thus dedicated to studying how the ghrelin system is affected by postnatal overnutrition and particularly how its postnatal secretion pattern might be altered, but also to how postnatal overnutrition might affect its ability to act as a signal in the hypothalamus. We further aimed to investigate if the metabolic phenotype displayed by overfed mice might be altered by pharmacological intervention to normalize any observed defect in ghrelin signalling.

Postnatally overfed rodents are known to exhibit hyperleptinemia during postnatal development, and excessive leptin signaling has been suggested to be implicated in the early development of leptin resistance. The second part of my thesis was thus dedicated to investigating the contribution of postnatal hyperleptinemia to the adverse metabolic

programming observed in postnatally overfed mice, by assessing the long-term metabolic effects of partial neonatal leptin antagonism, as well as the acute effect of leptin antagonism on central leptin signalling during postnatal development in postnatally overfed mice.

Results: Article 1

Article 1: Neonatal overnutrition causes early alterations in the central response to peripheral ghrelin

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Postnatal overnutrition in rodents is known to cause persistent alterations in the adult metabolic phenotype, such as increased susceptibility to obesity, insulin resistance and hypertension (Plagemann et al., 1999e). This may be related to abnormal development of the hypothalamic neuronal circuits that regulate energy metabolism and autonomic functions, since numerous studies suggest aberrant hypothalamic function in postnatally overnourished rodents (Davidowa et al., 2003b).

Recent findings from our lab have demonstrated that the stomach-derived hormone ghrelin, which acts to regulate feeding in the adult hypothalamus, is involved in the postnatal development of the hypothalamus and programming of metabolic function (Steculorum et al., Under revision). Since ghrelin signalling is nutritionally regulated in adult animals both at the peripheral and central level, the purpose of the present study was to study how ghrelin secretion and the central response to ghrelin was affected by overnutrition during early postnatal development and assess the contribution of abnormal ghrelin signalling to the adverse metabolic programming observed in postnatally overnourished rodents.

Neonatal Overnutrition Causes Early Alterations in the Central Response to Peripheral Ghrelin

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Abbreviations : AgRP, agouti-related peptide; ARH, arcuate nucleus ; DMH, dorsomedial nucleus ; GOAT, ghrelin O-acyltransferase; GHSR, growth hormone secretagogue receptor; HFHS, high-fat/high-sucrose diet ; LHA, lateral hypothalamic area ; MBH, mediobasal hypothalamus; ME, median eminence; NL, normal litters ; NPY, neuropeptide Y ; P, postnatal day ; POMC, pro-opiomelanocortin ; PVH, paraventricular nucleus ; SL, small litter

Abstract

Objective: Excess nutrient supply and rapid weight gain during early life are risk factors for the development of obesity during adulthood. This metabolic malprogramming may be mediated by endocrine disturbances during critical periods of development. Ghrelin is a metabolic hormone secreted from the stomach that acts centrally to promote feeding behavior by binding to growth hormone secretagogue receptors in the arcuate nucleus of the hypothalamus. Here, we examined whether neonatal overnutrition causes changes in the ghrelin system.

Methods: We used a well-described mouse model of divergent litter sizes to study the effects of postnatal overfeeding on the central and peripheral ghrelin systems during postnatal development.

Results: Mice raised in small litters became overweight during lactation and remained overweight with increased adiposity as adults. Neonatally overnourished mice showed attenuated levels of total and acyl ghrelin in serum and decreased levels of *Ghrelin* mRNA expression in the stomach during the third week of postnatal life. Normalization of hypoghrelinemia in overnourished pups was relatively ineffective at ameliorating metabolic outcomes, suggesting that small litter pups may present ghrelin resistance. Consistent with this idea, neonatally overnourished pups displayed an impaired central response to peripheral ghrelin. The mechanisms underlying this ghrelin resistance appear to include diminished ghrelin transport into the hypothalamus.

Conclusions: Early postnatal overnutrition results in central resistance to peripheral ghrelin during important periods of hypothalamic development. Because ghrelin signaling has recently been implicated in the neonatal programming of metabolism, these alterations in the ghrelin system may contribute to the metabolic defects observed in postnatally overnourished mice.

Keywords

Ghrelin, hypothalamus, nutrition, programming, hormone, tanycytes

1. Introduction

Over the past three decades, the prevalence of obesity and type II diabetes has increased at an alarming rate, including among children and adolescents [1, 2]. Epidemiological data have suggested that excess nutrition and growth during pre- and/or post-natal life may contribute to the etiology of obesity and related diseases in later life, particularly in an environment with a wide availability of calorie-dense foods [3, 4]. The results of experiments in a variety of animal models also support a link between the perinatal nutritional environment and the programming of energy balance “set points” [5-8]. Because of the importance of postnatal organ development, including that of the brain, animal models of postnatal metabolic programming have been developed to specifically target this developmental period. An animal model that has proven very useful for the study of postnatal overfeeding is a reduction of litter size. Pups raised in small litters (SL) display accelerated growth during the pre-weaning period, and these animals remain overweight throughout life [9-12]. In addition, postnatally overfed animals show accelerated and exacerbated weight gain and altered glucose tolerance when fed an obesogenic diet [10, 12].

The precise biological substrates that mediate the effects of early postnatal overfeeding on later metabolic health are not fully understood. However, there is a growing appreciation that the developmental programming of hypothalamic systems involved in energy balance regulation by the perinatal environment represents a possible cause of obesity and related diseases. The rodent hypothalamus develops primarily during the postnatal period under the influence of intrinsic and environmental cues (see [13] for a review). Perturbations in the development of projections from the arcuate nucleus of the hypothalamus (ARH) are a common feature of animals subjected to nutritional insults during perinatal life, including in postnatally overfed mice [14-19]. Changes in the circulating levels of metabolic hormones, such as the adipocyte hormone leptin, in response to nutritional challenges that occur during early life represent a likely cause for the nutrition-induced

alterations in hypothalamic development. For example, maternal obesity and postnatal overnutrition increase leptin levels throughout postnatal life and cause central leptin resistance during critical periods of hypothalamic development [10, 16, 20]. In contrast, maternal malnutrition during pregnancy and/or lactation blunts the naturally occurring postnatal leptin surge [14, 15, 21]. Remarkably, daily leptin treatment during early postnatal life in pups born to malnourished dams normalizes their metabolic abnormalities [22], indicating that the developmental actions of leptin contribute to the adult metabolic phenotype.

The gut-derived hormone ghrelin is also particularly well suited to transmit signals to the developing brain in response to alterations in the nutritional environment. In adults, circulating ghrelin levels are influenced by nutritional status [23-26], and neonatal ghrelin plays an important role in hypothalamic development and lifelong metabolic regulation [27]. However, whether ghrelin levels are regulated in response to nutritional challenges during early life and whether neonatal nutrition influences the hypothalamic reponse to ghrelin remain unknown. In the present study, we used the small litter model to determine whether overnutrition during early postnatal life influences the development of the ghrelin system and the sensitivity of hypothalamic neurons to ghrelin.

2. Material and Methods

2.1. Animals

Offspring of C57BL6 mice (Charles River Laboratory) produced in our mouse colony were used in these studies. Litters were normalized to 7 pups per litter on postnatal day 1 (P1), with 4 male and 3 female pups per litter. On P3, some litters were culled to 3 pups per litter (2 male + 1 female pups, SL = small litter) to induce postnatal overnutrition, whereas the control litters were maintained with 7 pups/litter (NL = normal litter). Animals were fed standard chow following weaning unless otherwise specified. Only male pups were used for the studies. Each experimental group in all experiments consisted of offspring from at least 3 litters. The animal usage was in compliance with and approved by the Institutional Animal Care and Use Committee of the University of Lille and the Saban Research Institute of the Children's Hospital of Los Angeles.

2.2. Physiological measurements

Pups were weighed once every two days from P4 to P22 and once weekly after weaning using an analytical balance. Body composition analysis (fat/lean mass) was performed at P120 using a LaTheta 100 X-ray Computed Tomography scanner. Food intake and respiratory exchange ratio (RER) were monitored at P90 using a combined indirect calorimetry system (TSE systems). Briefly, after adaptation for 3 days, O_2 and CO_2 production were measured for 3 days to determine the respiratory exchange ratio. In addition, food intake was determined continuously by the integration of weighing sensors fixed at the top of the cage from which the food containers were suspended into the sealed cage environment. Blood glucose levels were measured on P180 after overnight fasting using a glucometer (OneTouch Ultra, Johnson & Johnson). For the high-fat/high-sucrose (HFHS) challenge, mice were divided into two groups on P120: a first group was fed a standard chow diet for 8 weeks (also referred as "control" mice). A second group was fed for 8 weeks with a high-fat diet (60% fat wt/wt) plus sucrose in the drinking water (20% wt/vol)

(also referred as “HFHS” mice).

2.3. Ghrelin assays

Pups were decapitated on P8, P12, P14, P16, P22 and P60 and trunk blood was collected in a chilled tube containing Pefabloc (AEBSF, Roche Diagnostics). The collected serum was also acidified with 1 N HCl to achieve a final concentration of 0.05 N and then stored at -20 C until use. Total and acyl ghrelin levels in the serum were assayed using ELISA kits (Millipore). Acyl ghrelin levels were also characterized in mouse neonates injected with ghrelin. For this, P16 mice were injected with various doses of ghrelin (5, 10, and 50 ug/kg) or saline, and 15 minutes after injection the trunk blood was collected as described above.

2.4. Real-time qPCR analysis

The ARH (including the median eminence), DMH, and stomach of P14, P16, P22, and P60 mice (n = 4-6 per group) were dissected. For the ghrelin-induced changes in neuropeptide expression, P14 mice were given an intraperitoneal injection of ghrelin (Phoenix Pharmaceuticals, 2 mg/kg) or vehicle alone (0.9% NaCl) (n = 3-5) and were sacrificed 2 hours later. Total RNA from the ARH and DMH was isolated using the Arcturus PicoPure RNA Isolation Kit (Life Technologies). Total RNA from the stomach was isolated using the RNeasy Lipid Tissue kit (Qiagen). cDNA was generated using the high-capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative real-time PCR analysis was performed using TaqMan Fast Universal PCR MasterMix. The mRNA expression was calculated using the 2^{-ddCt} method after normalization to *gapdh* (Mm99999915_g1) as a housekeeping gene. The inventoried TaqMan Gene expression assays for *GHSR* (Mm00616415_m1), *Ghrelin* (Mm00445450_m1), *Goat* (Mm01200389_m1), *Pomc* (Mm00435874_m1), *Agrp* (Mm00475829_g1), *Npy* (Mm03048253_m1) were used. All assays were performed using an Applied Biosystems StepOnePlus real-time PCR system.

2.5. Chronic neonatal injection of ghrelin

Starting at P12, pups were treated twice daily with intraperitoneal injections of ghrelin (Phoenix Pharmaceuticals, 10 ug/kg) for a total of 10 days. Controls received equivolume injections of vehicle (0.9% NaCl).

2.6. cFos analysis

On P14, P16, P22, and P60 mice were given an intraperitoneal injection of ghrelin (Phoenix Pharmaceuticals, 2 mg/kg) (n = 4-7) or vehicle alone (0.9% NaCl) (n = 3-9) and were then perfused 2 hours later with a solution of 4% paraformaldehyde. A separate cohort of mice received an intracerebroventricular injection of ghrelin at P14. For this purpose, 1 ul of ghrelin (240 ug/ml) or vehicle (0.9% NaCl) was stereotactically infused over 3 minutes into the lateral ventricle (1 mm lateral to Bregma 0, depth of 3 mm) under isoflurane anesthesia. Pups were perfused 90 minutes later with a solution of 4% paraformaldehyde.

The brains were then frozen, cut into 30-um thick sections, and processed for cFos immunostaining using standard procedures [28]. Briefly, after pretreatment overnight in a mixture of 0.3% Triton X-100 and 2% normal goat serum, sections were incubated for 48 hours at 4 C in a rabbit primary antiserum directed against the N-terminal domain of Fos (Ab-5, Oncogene; 1:2,000). The primary antibody was localized with Alexa Fluor 488 Goat anti-Rabbit IgG (Invitrogen; 1:200). The sections were counterstained using bis-benzamide (Invitrogen; 1:3,000) to visualize the cell nuclei and coverslipped with buffered glycerol (pH 8.5).

Two sections through the ARH and DMH from animals of each experimental group (n = 4-7 animals per group) were imaged using a Zeiss AxioImager Z1 Microscope equipped with a 20X objective. Slides were numerically coded to obscure the treatment group. The number of cFos-immunopositive cells in the ARH and DMH were manually counted using the point tool of the ImageJ analysis software (NIH). The average number of cells counted in two sections from each mouse was used for statistical comparisons.

2.7. Assessment of ghrelin uptake

Fluorescent bioactive ghrelin (Cisbio Bioassays, 25 nmol/mouse) [29] was injected intravenously, and mice were sacrificed 5 minutes later to the assess tanycytic ghrelin uptake by fluorescence microscopy as previously described [30]. The primary antibodies used for these studies were a rat anti-PECAM (1:200, generous gift from Dr. Britta Engelhardt, Theodor Kocher Institut, Bern, Switzerland) and a rabit anti-GFAP (Dako Cytomation; 1:2,000).

For the *in vitro* analysis of the ghrelin transport, tanycytes were isolated from the median eminence of the hypothalamus of P10 rats as described previously [29, 30]. The internalization of ghrelin was assayed using clathrin immunoprecipitation in tanycytes after 15 minutes of ghrelin treatment and using clathrin immunolabeling of tanycytes cultured on coverslips and treated for 15 minutes with fluorescent ghrelin (Cisbio Bioassays, 50 nM) [29, 30].

To determine the ability of ghrelin to be transported to the MBH, mice were intraperitoneally injected with ghrelin (Phoenix Pharmaceuticals, 2 mg/kg) and sacrificed 45 minutes later. The MBH was then rapidly microdissected and subjected to western blotting as previously described [30]. The primary antibodies used for western blotting were anti-ghrelin (AbCam, 1:500) and anti-ERK (Cell Signaling, 1:1,000).

2.8. Statistical analyses

All values are expressed as the means ± SEM. Statistical analyses were conducted using GraphPad PRISM (version 5.0d). Statistical significance was determined using unpaired two-tailed Student's t-tests and a two-way ANOVA followed by the Bonferroni multiple comparisons post hoc test. A one-way ANOVA, followed by the Tukeys multiple comparisons test was used for the western blot analysis. *P*-values less than 0.05 were considered to be statistically significant. Experimental units used for statistical comparisons correspond to the number of litters.

3. Results

3.1. Small litter rearing causes metabolic disturbances

To manipulate nutrient intake specifically during postnatal (pre-weaning) life, we used a well-described mouse model of divergent litter size. To this aim, litter size was manipulated beginning on P3 such that small litters (overfed) had 3 pups and normal litters (normally fed) had 7 pups. Small litter rearing was associated with changes in growth rates as revealed by a significant increase in pre-weaning body weight in overfed animals compared with normally fed mice (Figure 1A). As early as P4, the pups raised in small litters displayed heavier body weights than did the control animals (Figure 1A). Neonatally overfed animals remained heavier after weaning, and this elevated body weight persisted into adulthood (Figure 1B). The cumulative food intake was significantly higher in adult SL mice compared with control mice (Figure 1C). We also evaluated adiposity and found that SL mice displayed an elevated fat mass compared with NL mice (Figure 1E). In addition, the fasting glucose levels were significantly elevated in the adult SL mice fed a chow diet (Figure 1H). However, SL mice were comparable to NL mice with regard to respiratory quotient (Figure 1D), locomotor activity, oxygen consumption (VO2), and carbon dioxide production (VCO2) (data not shown). To determine whether neonatal overnutrition programs diet-induced obesity, we exposed SL and NL mice to a high-fat/high-sucrose (HFHS) regimen starting at 17 weeks of age. SL mice exposed to an HFHS regimen displayed a greater increase in body weight compared with NL mice (Figure 1F). Differences in body weight were detected as early as 1 week after HFHS feeding began and persisted throughout the HFHS exposure (Figure 1F). This elevated body weight was accompanied by greater adiposity (Figure 1G). However, SL mice exposed to an HFHS diet had a similar increase in fasting glucose levels compared with SL mice fed a chow diet (Figure 1H).

3.2. Neonatal overnutrition influences the pattern of postnatal ghrelin secretion

Given the importance of feeding status on ghrelin secretion in adults [23-26], we next investigated circulating total and acyl ghrelin levels in neonatally overfed mice. Serum ghrelin levels increased gradually after birth to reach adult-like levels by 3 weeks of life in normally fed mice (Figures 2A and 2B) [27, 31, 32]. However, mice raised in SLs displayed attenuated total ghrelin levels at P12 and P16 (Figure 2A) and attenuated acyl ghrelin levels at P16 and P22 (Figure 2B). In addition the ratio of acyl to total ghrelin was diminished in SL pups at P16 compared with NL mice (Supplementary Figure 1). Stomach levels of *Ghrelin* and *Goat* (the ghrelin activating enzyme) mRNA were increased in control pups between P14 and P22; however, levels remained unchanged in overfed pups (Figures 2C and 2D). Notably, these changes in circulating ghrelin and *Ghrelin* mRNA expression appear restricted to early postnatal life because in adult mice no difference in circulating ghrelin levels or stomach expression patterns was evident between control and neonatally overfed mice.

3.3. Neonatal ghrelin injections have moderate effects on the metabolic phenotype of SL mice

Neonatally overnourished pups displayed a reduction in circulating ghrelin levels during early postnatal life. Based on these observations, we next investigated whether neonatal ghrelin injections in SL mice rescue the metabolic phenotype of these mice. Ghrelin was administered twice daily in pups from P12 to P22, *i.e.*, when endogenous ghrelin levels are low in SL mice. We determined through pilot studies that a dose of 10 ug/kg normalizes the ghrelin levels in SL mice (Figure 3A). Neonatal administration of ghrelin to SL mice did not result in changes in body weight or body composition (Figure 3B-E). Similarly, SL mice treated neonatally with ghrelin displayed similar weight and fat gain when challenged for 8 weeks with an obesogenic (HFHS) diet (data not shown). However, the fasting glycemia of SL mice neonatally injected with ghrelin was significantly reduced compared with saline-treated pups (Figure 3F).

3.4. Postnatal overnutrition alters the ability of peripheral ghrelin to modulate ARH neurons

Our findings indicate that neonatal ghrelin treatment is relatively ineffective at influencing metabolic outcomes in SL mice. A possible explanation for this lack of response is that brain regions that normally convey the metabolic effects of ghrelin are insensitive to ghrelin during neonatal life and that SL mice may present hormonal resistance. To test this hypothesis, we first examined the mRNA expression of the ghrelin receptor *GHSR* in the arcuate nucleus of the hypothalamus (ARH)/median eminence (ME) of SL and NL mice. The rationale for focusing on this region, specifically, is because the ARH is a key site for mediating the effects of ghrelin on feeding [33-35], and under normal conditions neonatal ghrelin acts on the ARH to influence its development [27]. The ARH/ME of P14 and P60 SLs mice contained levels of *GHSR* mRNA that were similar to those found in NL mice (Figure 4A). However, neonatal overnutrition caused a reduction in *GHSR* mRNA levels at P16 and P22 (Figure 4A). We next studied the ability of peripheral ghrelin to activate ARH neurons. We used cFos expression as a surrogate marker of neuronal activation [36]. Peripheral ghrelin injection caused a marked increase in the number of cFos-immunoreactive cells in the ARH of control (NL) mice at P14, P16, and P22 (Figure 4B). In NL pups, cFos induction was found in 35% and 25% of NPY and POMC neurons, respectively, (Supplementary Figure 2B-C) and only a few number of cFos-immunoreactive cells was found in the ME (Supplementary Figure 2A). The induction of cFos immunoreactivity following peripheral ghrelin administration was significantly attenuated in the ARH of SL pups (Figure 4B). Nevertheless, neonatal ghrelin administration caused a significant increase in *GHSR* mRNA levels in the ARH/ME of both NL and SL mice (Figure 4C). In addition, there were no differences in ghrelin-induced cFos expression between SL and NL mice at P60 (Figure 4B), indicating that the altered ghrelin response observed in SL mice is restricted to early postnatal life.

Within the mature ARH, ghrelin acts primarily on AgRP/NPY neurons to stimulate food intake [34, 35]. Previous studies indicated that in normally fed animals, ghrelin

administration stimulates *Agrp* and *Npy* mRNA expression [26, 37, 38]. Consistent with these data, we found that peripheral administration of ghrelin to NL mice at P14 increased the expression of *Agrp* and *Npy* mRNA (Figures 4D and 4E). However, the same ghrelin treatment did not result in changes in *Agrp* and *Npy* mRNA levels in the ARH of P14 SL mice (Figures 4D and 4E). Additionally, ghrelin injection at P14 did not change *Pomc* mRNA expression in either NL or SL mice (Figure 4F).

To determine whether neonatal overfeeding also affected the ability of peripheral ghrelin to modulate other non-ARH neurons, we examined ghrelin-induced cFos immunoreactivity in the DMH, which is another hypothalamic nucleus known to contain high levels of ghrelin receptors [27, 39, 40]. In contrast to the reduction in *GHSR* mRNA expression observed in the ARH of SL pups, *GHSR* mRNA levels appeared to be unaltered in the DMH of SL neonates (Figure 5A). Additionally, peripheral ghrelin administration did not result in cFos induction in the DMH of either NL or SL neonates (Figure 5B). Furthermore, consistent with previous report showing that *GHSR* expression is low in non-ARH nuclei during postnatal development [27], ghrelin administration did not result in a significant induction of cFos expression in the VMH, LHA, and PVH of SL and NL pups at P14 and P16 (data not shown).

3.5. Postnatal overnutrition alters ghrelin access to the brain

The mechanisms underlying hormonal resistance remain elusive but likely include defective transport of hormones across the blood-brain barrier to the cerebrospinal fluid [41, 42] or to their sites of action within the brain [43]. To test the hypothesis that ARH neurons of SL mice can respond to ghrelin, we evaluated the ability of central ghrelin injection to activate cFos immunoreactivity in the ARH. In sharp contrast to the peripheral administration of ghrelin, intracerebroventricular injection of ghrelin induced similar cFos immunoreactivity in the ARH of both NL and SL pups at P14 (Figure 6). These observations indicate that the ARH neurons of SL mice retain the ability to respond to ghrelin and suggest that ghrelin transport from the periphery to the brain may be defective in neonatally overnourished mice.

It was recently shown that tanycytes, which are specialized hypothalamic glial cells located in the median eminence (ME), play a critical role in transporting peripheral hormones, such as leptin, from the periphery to the ARH [30]. Notably, by P10 tanycytes appear to be fully developped and display an adult-like morphology [44]. To determine whether tanycytes are also important for ghrelin uptake, we intravenously administered fluorescently labeled bioactive ghrelin [29] to wild-type mice. We observed that fluorescent ghrelin was exclusively present in ME tanycytes 5 minutes after injection (Figure 7A). We further explored the role of tanycytes in transporting ghrelin *in vitro* using primary cultures of tanycytes from neonatal rats. The results indicate that after 15 minutes, cultured tanycytes internalized fluorescent ghrelin through clathrin-coated vesicles (Figures 7B and 7C). These *in vivo* and *in vitro* observations support a role for ME tanycytes in transporting ghrelin from the periphery to the brain in the same manner as other hormones such as leptin [30]. To examine whether neonatal overnutrition alters the ability of ghrelin to be transported to the ARH, we performed western blots of the MBH (containing the ARH) from P14 SL and NL mice 45 minutes after an intraperitoneal ghrelin injection. Significant amounts of exogenous ghrelin were detected in the MBH of NL neonates (Figure 7D). However, the same ghrelin treatment did not result in the presence of ghrelin in the MBH of SL pups (Figure 7D), suggesting that neonatal overfeeding alters ghrelin access to the MBH. To investigate if the defective ghrelin transport observed in SL mice can be attributed to structural abnormalities in tanycytes, we compared the overall organization of tanycytes in the ARH/ME of NL and SL mice at P14. The gross morphology and the number of tanycytes appeared to be similar between NL and SL pups (Supplementary Figure 3), suggesting that the alteration in ghrelin transport in overnourished pups likely involves non-structural tanycytic perturbations such as intracellular defects.

4. Discussion

A variety of peripheral signals contribute to the regulation of food intake and energy homeostasis in adult life and it has been suggested that metabolic hormones are capable of transmitting signals to the developing organism in response to alterations in the nutritional environment and may underlie potential maladaptive responses to early metabolic perturbations. In the present study, we show that postnatal overnutrition alters the ability of the gut-derived hormone ghrelin to act on the hypothalamus during an important period of growth and development. Neonatal overfeeding silences the ghrelin system by reducing circulating ghrelin levels, attenuating ghrelin uptake from the periphery to the brain, diminishing ghrelin receptor expression and decreasing the ability of peripheral ghrelin to activate arcuate neurons.

Our findings are generally consistent with data suggesting that small litter rearing causes accelerated postnatal weight gain that leads to persistent increased weight and obesity, especially when animals are exposed to an obesogenic diet [10, 12]. Our data indicate that neonatal overnutrition has marked effects on circulating ghrelin levels during important periods of growth and development. A similar reduction in ghrelin levels has previously been reported in adult obese individuals [25, 26]. One exception to the negative correlation between body weight and circulating ghrelin level occurs in Prader-Willi Syndrome. This disorder, which is primarily characterized by severe hyperphagia and morbid obesity, is associated with markedly elevated ghrelin levels, including during early life [45, 46]. During postnatal development, ghrelin is expressed in a variety of tissues, including the stomach and pancreas [47]. The present study suggests that the reduction in ghrelin levels observed in SL mice is, at least in part, due to altered production of ghrelin by the stomach. The observation that the acyl:total ghrelin ratio and *Ghrelin* mRNA levels are lower in SL mice despite higher levels of *Goat* mRNA is intriguing because it suggests that substrate

availability may be a more important factor in production of acyl ghrelin in SL mice or that GOAT activity may be markedly reduced in SL mice.

Despite being hypoghrelinemic, SL mice display an attenuated central response to peripheral ghrelin. Exogenous ghrelin administration in SL pups results in an impaired induction of cFos immunoreactivity and does not increase *Npy* mRNA levels, in contrast to the observations in NL mice. However, direct injection of ghrelin into the brain results in similar cFos activation in both SL and NL. Similarly, Davidowa and colleagues reported that direct exposure of ARH neurons to ghrelin results in a similar electrical response in both overnourished and control pups [48]. These data suggest that the ARH neurons of SL animals can respond to ghrelin but that neonatal overnutrition alters the ability of peripheral ghrelin to reach these neurons. Supporting this hypothesis, our western blot analysis shows that peripheral ghrelin injection results in an increased ghrelin content in the ARH of NL mice; SL mice do not exhibit this effect. The cellular mechanisms involved in the attenuated ghrelin transport likely involve alteration in ghrelin uptake by tanycytes. These ependymoglial cells have recently emerged as critical regulators of hormone transport into the brain, including into the ARH [30, 49]. In addition, diet-induced obesity in adults alters the ability of these cells to transport hormones, such as leptin, into the brain [30].

The relative contribution of these early alterations in the ghrelin system to the ultimate phenotype of SL pups remains to be investigated. However, based on recent reports indicating that neonatal ghrelin plays a lifelong role in energy balance regulation, it is likely that these perturbations in ghrelin secretion and action may contribute to the metabolic defects observed in postnatally overnourished mice. Recent data indicate that in addition to its regulatory role in mature animals, ghrelin acts during early postnatal life as a signal that can influence hypothalamic development. Neonatal ghrelin blockade results in enhanced densities of ARH projections. In contrast, abnormally elevated levels of ghrelin during postnatal life attenuate the normal development of ARH projections [27]. Remarkably, both

neonatal ghrelin blockade and neonatal hyperghrelinemia are associated with the same metabolic phenotype, *i.e.*, elevated body weight and hyperglycemia [27]. In our studies, normalizing the ghrelin levels in SL neonates did not result in marked changes in metabolic outcomes, which is consistent with the idea that the brain of neonatally overfed mice is relatively insensitive to ghrelin. Nevertheless, neonatal ghrelin injections improve glucose levels in SL mice. Because SL mice display central resistance to peripheral ghrelin, the effects of neonatal ghrelin on glucose levels likely involves a peripheral action, such as a direct action in the pancreas. Dembinski and colleagues previously reported that exposure of newborn rats to ghrelin for 7 or 14 days causes a reduction in pancreatic weight, attenuated pancreatic DNA synthesis and reduced DNA content, consistent with the idea that ghrelin might act directly on the pancreas to influence its development [50].

The present study supports the emerging concept that neuroendocrine defects during critical periods of development can lead to lifelong metabolic perturbations. Based on the observation that SL mice display early ghrelin resistance, future work will be required to address methods to improve ghrelin sensitivity in neonatally overfed pups. Interestingly, weight loss induced by caloric restriction has been reported to reverse the central ghrelin resistance observed in adult mice with diet-induced obesity [26, 51]. However, for metabolic programming, the timing of the intervention will be important. For example, food restriction can either have beneficial or detrimental long-term metabolic effects, depending on whether nutritional intervention occurs at an earlier or later stage of development. Neonatal caloric restriction in obesity-prone rats is associated with ameliorated hypothalamic development and improved metabolism [52]. If caloric restriction occurs after weaning, however, the opposite phenotype is observed, *i.e.*, obesity-prone rats increase their food intake and become even more obese once as they are allowed to eat *ad libitum* [53].

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Appendix A

Supplementary data

Conflict of interest

The authors declare no conflict of interest

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Figure Legends

Figure 1. Small litter rearing accelerates postnatal weight gain and causes long-term metabolic alterations. (A) Pre- and (B) post-weaning body weight curves in C57BL6 male mice raised in small (SL) and normal (NL) litters and fed a chow diet after weaning (n = 15-16 per group). (C) Cumulative food intake of adult NL and SL mice on chow diet (n = 8 per group). (D) Respiratory exchange ratio of adult NL and SL mice (n = 8 per group). (E) Body composition of adult SL and NL mice (n = 9-11 per group). (F) Weight gain, (H) change in total fat mass, and (I) fasting glycemia of adult SL and NL mice fed a high-fat/high-sucrose diet (HFHS) for 8 weeks (n = 10-15 per group). Values are shown as the mean ± SEM. *P < 0.05 versus NL.

Figure 2. Neonatal overfeeding attenuates ghrelin levels during postnatal life. Serum (A) total and (B) acyl ghrelin levels of P8, P12, P14, P16, P22, and P60 (adult) mice raised in normal litters (NL) and small (SL) litters (n = 4-10 per group). Relative expression of (C) *Ghrelin* and (D) *Goat* mRNA in the stomach of P14, P16, P22, and P60 (adult) mice raised in SL and NL (n = 4-6 per group). Values are shown as the mean ± SEM. *P < 0.05 versus NL.

Figure 3. Neonatal ghrelin injections have moderate effects on the metabolic phenotype of neonatally overfed mice. (A) Serum acyl ghrelin levels of P16 NL pups injected with saline and SL pups injected with ghrelin (5, 10, and 50 ug/kg i.p.) or saline (n = 2-3 per group). (B) Pre- and (C) post-weaning growth curves (body weights) of SL and NL mice neonatally injected with ghrelin (10 ug/kg i.p.) or saline (n = 7-26 per group); the black bar represents the injection period. (D) Lean mass, (E) fat mass, and (F) fasting glycemia of adult SL and NL mice neonatally injected with ghrelin (10 ug/kg i.p.) or saline (n = 7-11 per group). Values are shown as the mean ± SEM. *P < 0.05 versus NL saline; †P < 0.05 versus NL ghrelin; $^{\#}$P < 0.05 versus SL saline.

Figure 4. Attenuated central response to peripheral ghrelin in neonatally overfed pups. (A) Relative expression of *GHSR* mRNA in the arcuate nucleus/median emimence of P14, P16, P22, and P60 (adult) mice raised in SLs and NLs (n = 4-5 per group). (B) Representative images and quantification of cFos-immunoreactive cells in the arcuate nucleus (ARH) 2 hours after intraperitoneal (ip) administration of ghrelin (2 mg/kg) or saline alone in P14, P16, P22, and P60 mice raised in SLs and NLs (n = 4-7 per group). (C) *GHSR*, (D) *Npy*, (E) *Agrp*, and (F) *Pomc* mRNA expression in the arcuate nucleus/median eminence of P14 SL and NL pups intraperitoneally injected with ghrelin (2 mg/kg i.p.) or saline alone (n = 3-5 per group). Values are shown as the mean ± SEM. (A) *P < 0.05 versus NL. (B) *P < 0.05 versus NL ghrelin; (C-E) *P < 0.05 versus saline (C-E). Scale bar, 200 um.

Figure 5. Peripheral ghrelin does not induce neuronal activation in the dorsomedial hypothalamic nucleus (DMH) during neonatal life. (A) Relative expression of *GHSR* mRNA in the dorsomedial nucleus of P14, P16, P22, and P60 (adult) mice raised in SLs and NLs (n = 4-5 per group). (B) Representative images and quantification of cFos-immunoreactive cells in the dorsomedial nucleus (DMH) nucleus 2 hours after intraperitopenal (ip) administration of ghrelin (2 mg/kg) or saline alone in P14, P16, P22, and P60 mice raised in SLs and NLs (n = 4-8 per group). Values are shown as the mean ± SEM. *P < 0.05 versus NL. Scale bar, 400 um.

Figure 6. Normal central response to central ghrelin in neonatally overfed pups. Representative images and quantification of cFos-immunoreactive cells in the arcuate nucleus (ARH) 90 minutes after intracerebroventricular (icv) administration of ghrelin (240 ug/ml) or saline alone in P14 mice raised in SLs and NLs (n = 3-6 per group). Values are shown as the mean ± SEM. *P < 0.05 versus saline. Scale bar, 200 um.

Figure 7. Altered ghrelin transport in the mediobasal hypothalamus of overfed pups. (A) Representative images showing tanycytic processes and cell bodies labeled by fluorescent ghrelin (25 nmoles per animal; *green* on left panel, *white* on right panel) 5 minutes after intravenous injection. Left panel, PECAM-immunoreactive pituitary portal blood vessels are shown in red. Right panel, GFAP-immunoreactive astrocytes are shown in red. (B) Representative images of clathrin immunoreactivity (*green*) in tanycytes treated *in vitro* for 15 minutes with fluorescent ghrelin (50 nM, *red*). (D) Representative western blots and quantification of ghrelin and clathrin in immunoprecipitated (IPP) clathrin-coated vesicles from tanycytes treated *in vitro* for 15 minutes with vehicle or ghrelin (1µg/ml). (D) Representative western blots and quantification of ghrelin in mediobasal hypothalamic explants from P14 normal litter (NL) and small litter (SL) mice 45 minutes after intraperitoneal administration of ghrelin (2 mg/kg) or saline (n = 4 per group). Values are shown as the mean ± SEM. *P < 0.05 versus NL saline; $^{\#}$P < 0.05 versus NL ghrelin. Scale bar, 10 um.

Figure 1

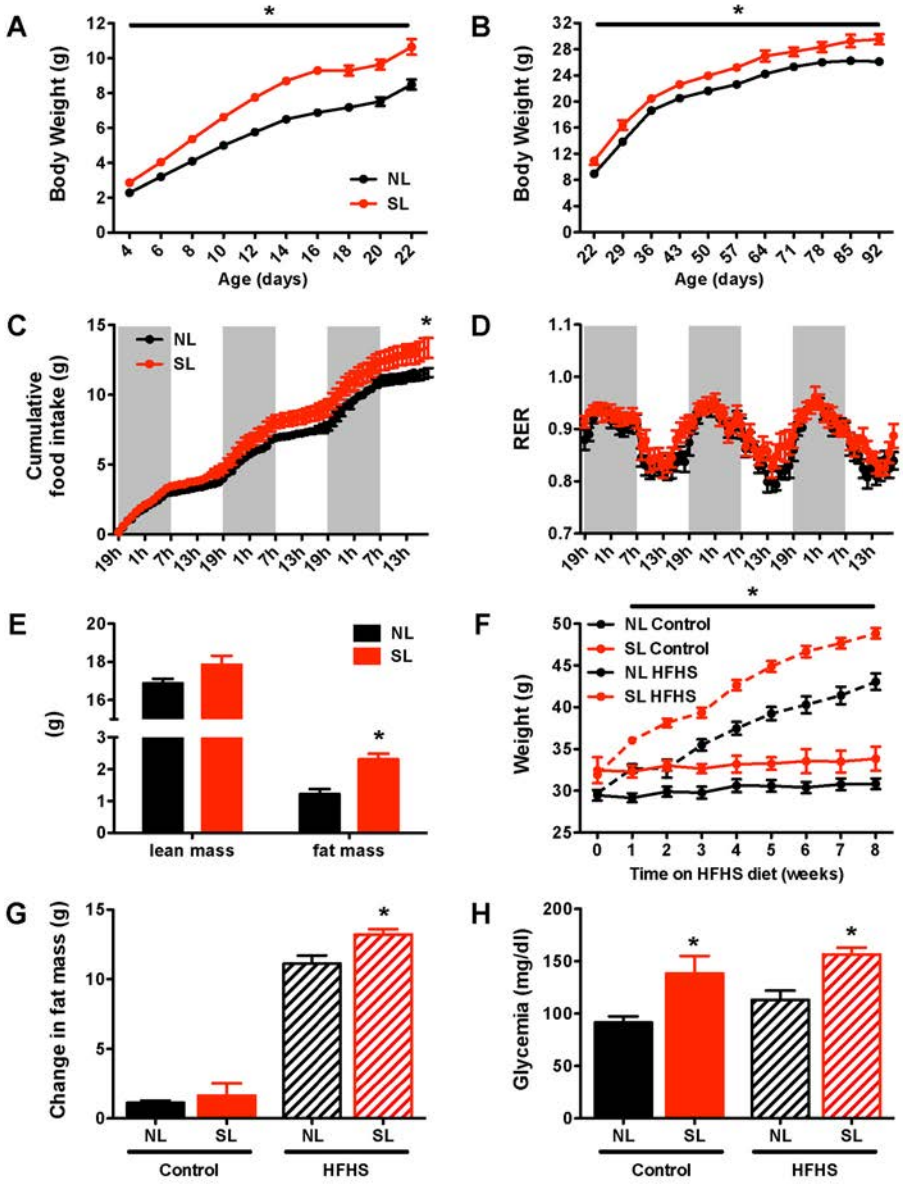

A
Body Weight (g)
Age (days)
NL
SL
B
Body Weight (g)
Age (days)
C
Cumulative food intake (g)
NL
SL
D
RER
E
(g)
lean mass
fat mass
NL
SL
F
Weight (g)
NL Control
SL Control
NL HFHS
SL HFHS
Time on HFHS diet (weeks)
G
Change in fat mass (g)
NL
SL
Control
HFHS
H
Glycemia (mg/dl)
Control
HFHS

Figure 2

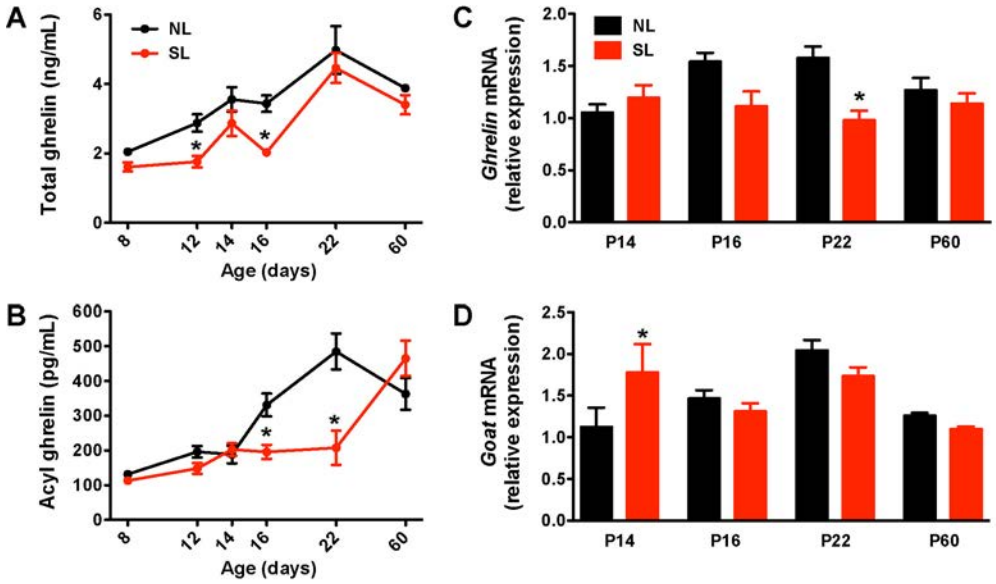

A
Total ghrelin (ng/mL)
NL
SL
Age (days)
B
Acyl ghrelin (pg/mL)
Age (days)
C
Ghrelin mRNA (relative expression)
P14
P16
P22
P60
D
Goat mRNA (relative expression)
P14
P16
P22
P60

Figure 3

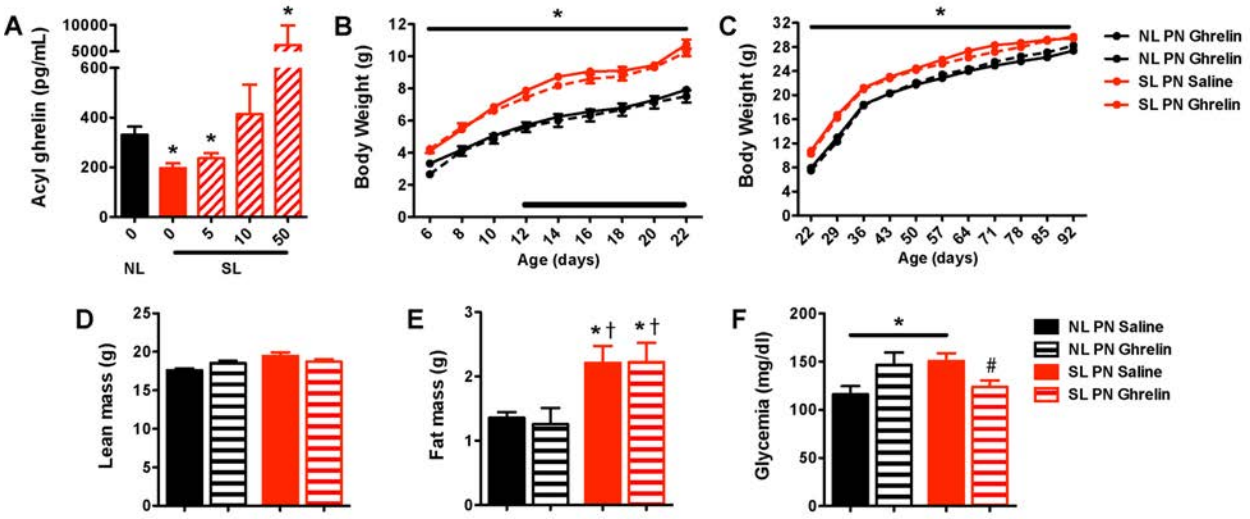

A
Acyl ghrelin (pg/mL)
10000
5000
600
400
200
0
0
0
5
10
50
NL
SL
B
Body Weight (g)
Age (days)
C
Body Weight (g)
Age (days)
NL PN Ghrelin
NL PN Ghrelin
SL PN Saline
SL PN Ghrelin
D
Lean mass (g)
E
Fat mass (g)
F
Glycemia (mg/dl)
NL PN Saline
NL PN Ghrelin
SL PN Saline
SL PN Ghrelin

Figure 4

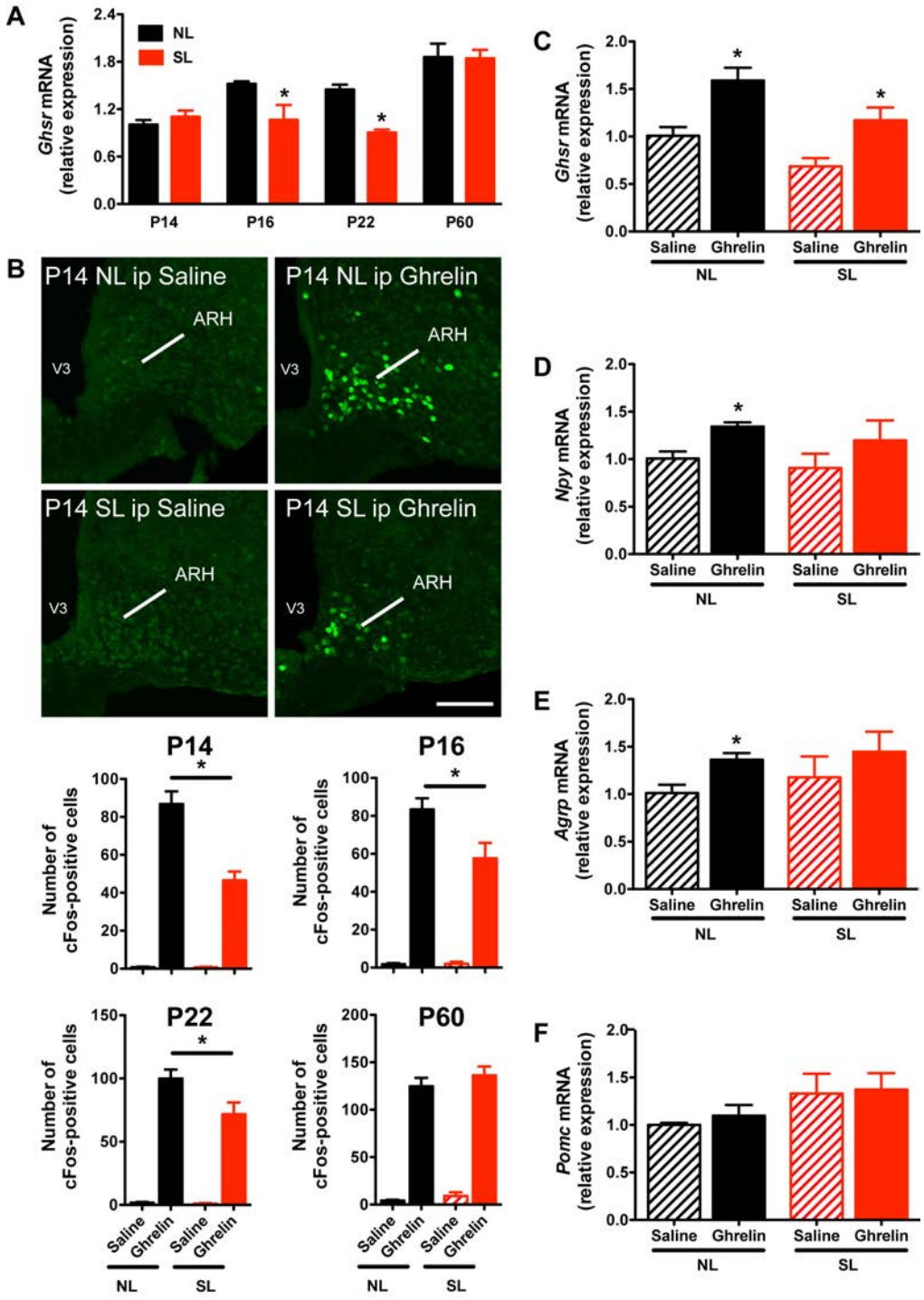

A
Ghsr mRNA (relative expression)
NL
SL
P14
P16
P22
P60
B
P14 NL ip Saline
P14 NL ip Ghrelin
P14 SL ip Saline
P14 SL ip Ghrelin
ARH
V3
P14
P16
P22
P60
Number of cFos-positive cells
Saline
Ghrelin
NL
SL
C
Ghsr mRNA (relative expression)
D
Npy mRNA (relative expression)
E
Agrp mRNA (relative expression)
F
Pomc mRNA (relative expression)

Figure 5

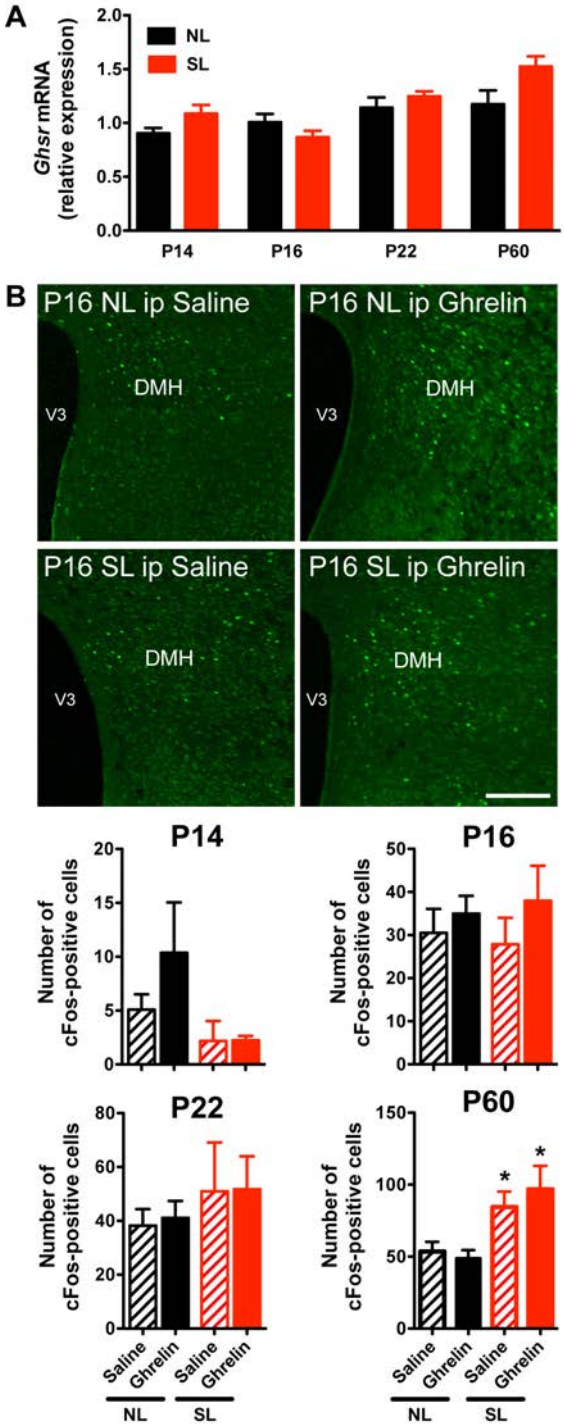

A
Ghsr mRNA (relative expression)
2.0
1.5
1.0
0.5
0.0
NL
SL
P14
P16
P22
P60
B
P16 NL ip Saline
P16 NL ip Ghrelin
P16 SL ip Saline
P16 SL ip Ghrelin
DMH
V3
P14
P16
P22
P60
Number of cFos-positive cells
Saline
Ghrelin
NL
SL
*

Figure 6

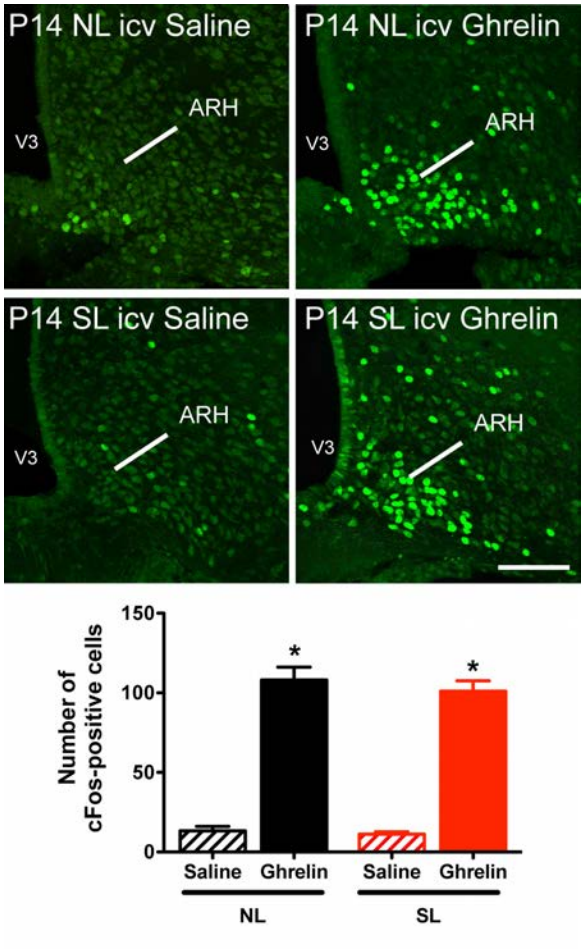
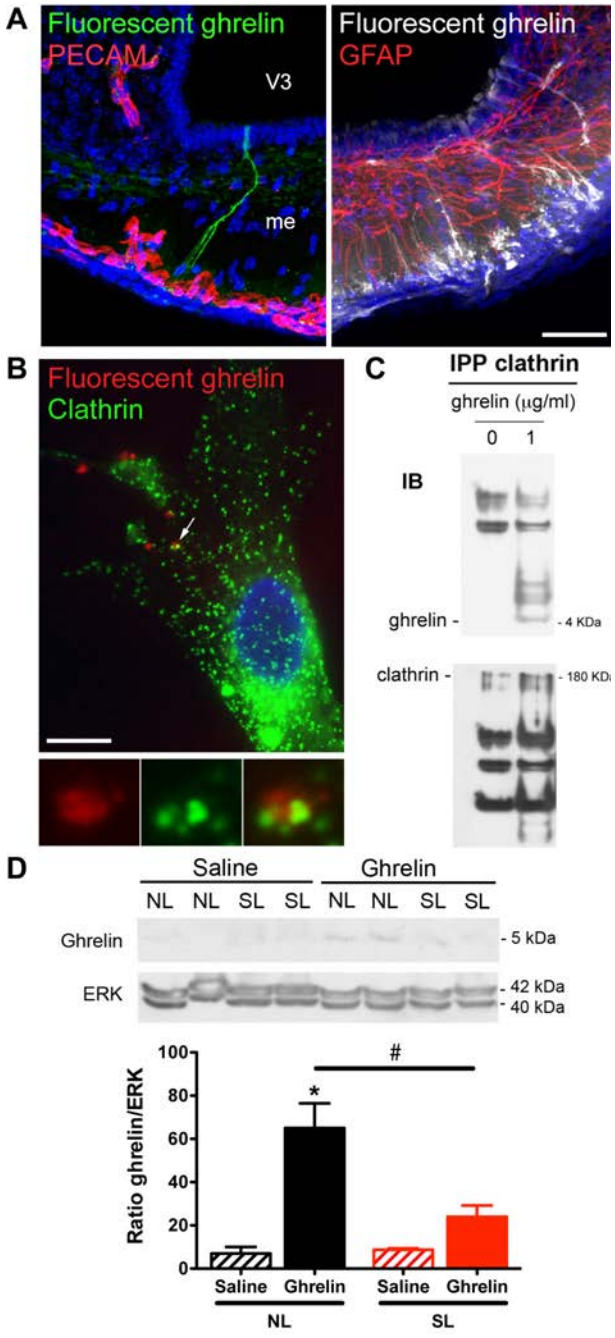

P14 NL icv Saline
ARH
V3
P14 NL icv Ghrelin
ARH
V3
P14 SL icv Saline
ARH
V3
P14 SL icv Ghrelin
ARH
V3
Number of cFos-positive cells
150
100
50
0
*
*
Saline
Ghrelin
Saline
Ghrelin
NL
SL

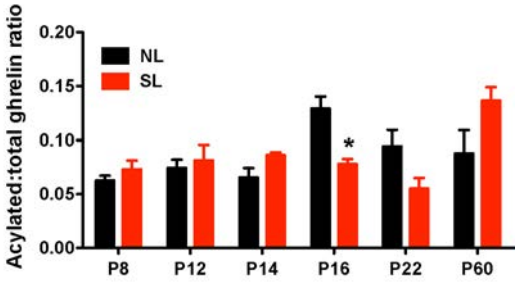
Figure 7


A
Fluorescent ghrelin
PECAM
V3
me
Fluorescent ghrelin
GFAP
B
Fluorescent ghrelin
Clathrin
C
IPP clathrin
ghrelin (μg/ml)
0 1
IB
ghrelin -
- 4 KDa
clathrin -
- 180 KDa
D
Saline
Ghrelin
NL NL SL SL NL NL SL SL
Ghrelin
- 5 kDa
ERK
- 42 kDa
- 40 kDa
Ratio ghrelin/ERK
100
80
60
40
20
0
*
#
Saline Ghrelin Saline Ghrelin
NL
SL

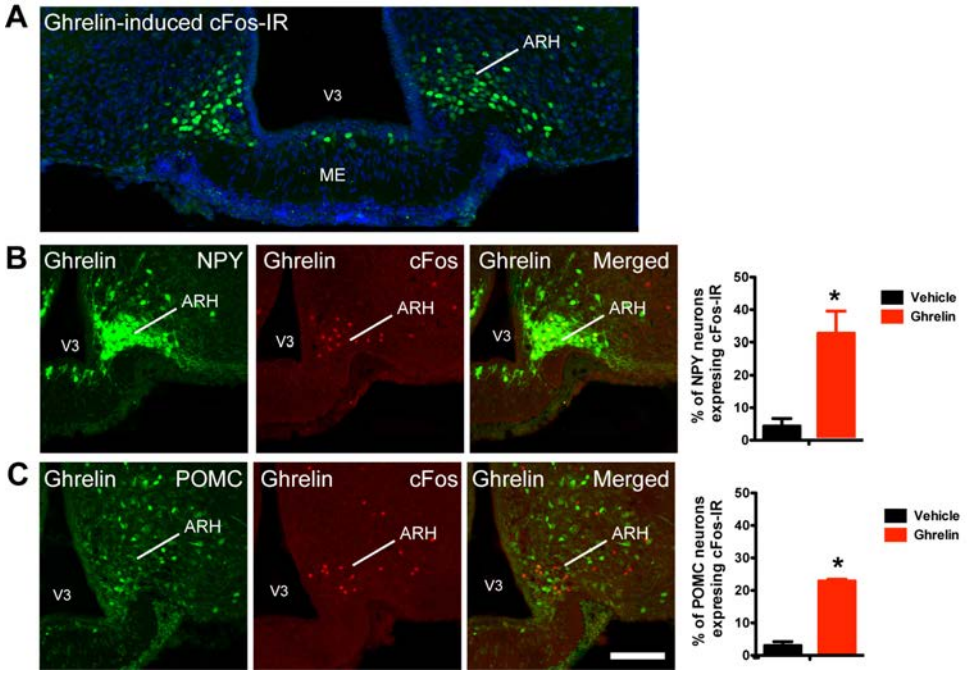
Neonatal overnutrition causes early alterations in the central response to peripheral ghrelin

Gustav Collden, Eglantine Balland, Jyoti Parkash, Emilie Caron, Fanny Langlet, Vincent Prevot, Sebastien G. Bouret

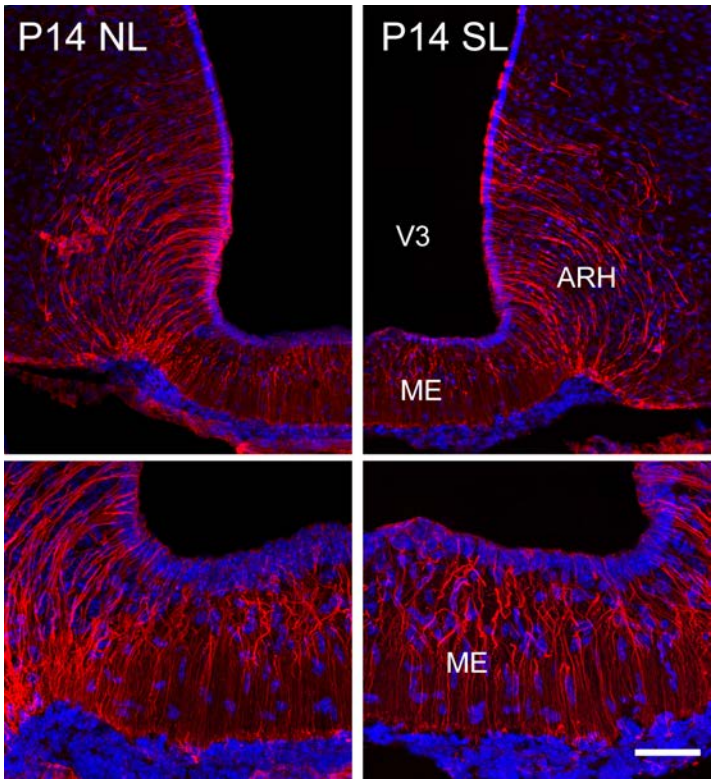
Supplementary Figures 1 to 3



Supplementary Figure 1. Lower acyl:total ratio in neonatally overourished pups. Ratio of acyl to total ghrelin of P8, P12, P14, P16, P22, and P60 (adult) mice raised in normal litters (NL) and small (SL) litters (n = 4-10 per group).



Supplementary Figure 2. Identification of ghrelin-responsive cells. (A) Representative images of cFos-immunoreactive cells in the median eminence (ME) and arcuate nucleus (ARH) 2 hours after intraperitoneal administration of ghrelin (2 mg/kg) in P14 mice. Confocal images and quantitative comparisons of cFos-immunoreactive cells (red fluorescence) 2 hrs after intraperitoneal administration of ghrelin (2 mg/kg) or vehicle alone in P14 SL and NL pups that had (B) NPY- and (C) POMC-containing neurons labeled with the green fluorescent protein (GFP) (n = 4 per group). V3, third ventricle. Scale bar, 120 um (A); 170 um (B-C).



Supplementary Figure 3. Organization of tanycyte processes in the ME/ARH of NL and SL mice. Representative images of vimentin immunoreactivity (a marker of tanycytes; red fluorescence) in the median eminence (ME) and arcuate nucleus (ARH) of SL and NL mice at P14 mice. V3, third ventricle. Scale bar, 120 um (upper panels), 60 um (lower panels).

Results: Article 2

Article 2: Neonatal leptin antagonism ameliorates metabolic programming in neonatally overnourished mice

Colldén G, Bouret SG

In preparation

Postnatal overnutrition in rodents is known to cause persistent alterations in the adult metabolic phenotype, such as increased susceptibility to obesity, insulin resistance and hypertension (Plagemann et al., 1999e). This may be related to postnatal hyperleptinemia (Schmidt et al., 2001) which has known effects on both peripheral and central development and metabolic programming (Bouret et al., 2004b; Attig et al., 2013b), and has been proposed to contribute to the development of leptin resistance (Knight et al., 2010).

To investigate the importance of postnatal hyperleptinemia for the adverse metabolic programming observed in postnatally overfed mice, we studied the long-term metabolic outcomes of partial leptin antagonism during early postnatal development, in addition to assessing the acute effects of neutralizing hyperleptinemia on central leptin sensitivity in overfed neonates.

Neonatal Leptin Antagonism Ameliorates Metabolic Programming In Neonatally Overnourished Mice

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Abstract

It is increasingly accepted that alterations of the perinatal nutritional environment may predispose individuals to development of metabolic disease. However, relatively little is known about the underlying biological processes mediating this metabolic imprinting. We previously reported that neonatal leptin promotes development of hypothalamic circuits that control body weight and glucose homeostasis in later life. Here, we examined how nutritional manipulation of leptin secretion during neonatal life impacts lifelong metabolic regulation. To achieve this aim, we used a divergent litter size mouse model of postnatal overnutrition. Neonatal nutritional manipiulation was associated with marked changes in leptin levels during the pre-weaning preiod as revealed by a 3- to 7-fold increase in leptin levels in neonatally overfed pups compared to control pups. Neonatally overnourished pups also exhibited central leptin resistance as demonstrated by reduced leptin-induced activation of pSTAT3 in the arcuate nucleus of the hypothalamus. Physiologically, neonatally overnourished mice displayed rapid weight gain during lactation and remained overweight as adults. These mice also showed increased adiposity and perturbations in glucose homeostasis in adulthood. Remarkably, administation of a leptin antagonist when endogenous leptin levels are high normalized fat mass and reversed insulin intolerance in neonatally overnourished mice. These metabolic improvements appears to be the result of improved responsiveness of hypothalamic neurons to leptin. In conclusion, early postnatal overnutrition causes metabolic alterations that appear to be the result of markedly elevated leptin levels and central leptin resistance during important periods of growth and development.

Keywords : Leptin, postnatal overnutrition, hypothalamus, metabolic programming

Introduction

As the prevalence of obesity and metabolic syndrome has dramatically increased throughout the western world, there is an urgent need to understand the biological mechanisms driving this epidemic. Abnormal nutrition occuring specifically during fetal and/or postnatal life is known to increase the risk of obesity and metabolic syndrome in adulthood in rodents and humans (Taylor and Poston, 2007). How such temporally restricted nutritional insults can have persistent effects on metabolic regulation is not known, but likely involve abnormal neuroendocrine dysregulations during critical periods of growth and development (Levin, 2000).

The central regulation of metabolism is controlled primarily by a network of distinct nuclei located in the hypothalamus. Key among these is the arcuate nucleus of the hypothalamus (ARH). The ARH contains neuronal populations devoted to metabolic regulation, including neurons co-expressing the orexigenic neuropeptides Neuropeptide Y (NPY) and agouti-related protein (AgRP) and neurons containing anorexigenic pro-opiomelanocortin (POMC)-derived peptides. Both POMC and NPY/AgRP neurons are highly responsive to circulating metabolic signals such as leptin, ghrelin and insulin (Williams and Elmquist, 2012)

The concept that metabolic signals that act through these hypothalamic circuits to regulate metabolism in adult animals may also be involved in the neonatal development of the same hypothalamic circuits, stems from the discovery that the adipocyte-derived hormone leptin directly stimulates the axonal growth of neurons located in the ARH during postnatal life (Bouret et al. 2004). Leptin primarily mediates its influence on hypothalamic development by binding to the long-form leptin receptor LepRb that is expressed in several hypothalamic nuclei, including the PVH, VMH, and the ARH (Caron et al., 2010; Bouret et al., 2012). Upon binding to the LepR-Rb, leptin activates several intracellular signaling cascades including MAPK/ERK, PI3K/AKT and JAK/STAT, where the leptin-induced phosphorylation of STAT3 and ERK is thought to be particularly important for the neurotrophic actions of leptin

(Bouret et al., 2012). Notably, neonatatal leptin exposure in leptin-deficient mice attenuates body weight gain, hyperphagia and impairments of sympathetic outflow in leptin-deficient mice (Bouyer and Simerly, 2013).

Although results from leptin- and leptin receptor-deficient mice pointed out the importance of neonatal leptin in the development of metabolic-related pathways, monogenic defects that cause severe obesity, such as single mutation in the *ob* gene, remain relatively rare (Farooqi and O'Rahilly, 2005). A more physiologically- and clinically-relevant approach to understand the importance of neonatal leptin in life-long metabolic regulation is to use nutritional manipulations that alter leptin levels specifically during postnatal life. In the present study, we used a divergent litter size mouse model of postnatal overnutrition to determine if changes in nutrition during this developmental critical period influence changes in circulating levels of leptin that may result in lifelong metabolic dysregulations.

Methods

Animals

Offspring of C57BL6 mice (Charles River Laboratory), produced in our mouse colony, were used in these studies. Litters were normalized to 7 pups per litter on postnatal day 1 (P1), with 4 male and 3 female pups per litter. On P3, some litters were culled to 3 pups per litter (2 male + 1 female pups, SL = small litter) to induce postnatal overnutrition, whereas the control litters were left at 7 pups/litter (NL = normal litter). Animals were fed standard chow from weaning unless specified otherwise. Only male pups were used for the studies. Each experimental group in all experiments consisted of offspring from at least 3 litters. Animal usage was in compliance with and approved by the Institutional Animal Care and Use Committee of the University of Lille and the Saban Research Institute of the Children's Hospital of Los Angeles.

Neonatal injections of the leptin antagonist

Starting at P6, pups raised in normal or small litters were treated once daily with intraperitoneal injections of a pegylated leptin antagonist (PESLAN-1, Protein Laboratories Rehovot, 2mg/kg) for a total of 10 days. The controls received equivolume injections of vehicle (sterile water).

Physiological measurements

Pups were weighed once every two days from P4 to P22, and once weekly after weaning using an analytical balance. Body composition analysis (fat/lean mass) was performed at P120 using a LaTheta 100 X-ray Computed Tomography scanner. Glucose tolerance in P120 mice by an intraperitoneal injection of glucose (75mg/kg) after an overnight fast, and then the blood glucose levels were measured at 0, 15, 30, 45, 60, 90, 120 and 150min following glucose challenge injection using a OneTouch glucometer. Insulin tolerance was

assessed in P120 mice by an intraperitoneal injection of human insulin (0.75U/kg) after a 6 hour fast, and then the blood glucose levels were measured at 0, 15, 30, 45, 60, 90, and 120 min following insulin challenge injection using a OneTouch glucometer.

Leptin assays

Pups were decapitated on P4, P6, P8, P10, P12, P16, P18, P20 and P22 and trunk blood was collected. For the adult samples, blood was collected from the tail vein. Serum leptin was measured using the multiplex technology (Millipore) for the pups samples and a commercial ELISA kit (Millipore Mouse Leptin ELISA kit, Cat. # EZML-82K) for the adult samples.

pSTAT3 immunohistochemistry

A pegylated leptin antagonist (PESLAN-1, Protein Laboratories Rehovot, 2mg/kg) was injected once daily in pups raised in normal or small litters on P8 and P9. The controls received equivolume injections of vehicle (sterile water). On P10, mice received an injection of the leptin antagonist, followed 4 hours laters by leptin administration (1 mg/kg, Peprotech). Animals were perfused 45 min later with a solution of 2% paraformaldehyde. Frozen coronal sections were cut at 25 um and pretreated for 20 min in 0.5% NaOH and 0.5%H2O2 in KPBS, followed by immersion in 0.3% glycine for 10 min. Sections were then placed in 0.03% SDS for 10 min and placed in 4% normal serum + 0.4% Triton X-100 + 1% BSA (fraction V) for 20 min before incubation for 48h with a rabbit anti-pSTAT3 antibody (1:1,000, Cell Signaling). The primary antibody was localized with Alexa Fluor 568 Goat anti-Rabbit IgGs (Invitrogen; 1:200). Sections were counterstained using bis-benzamide (Invitrogen; 1:10,000) to visualize cell nuclei, and coverslipped with buffered glycerol (pH 8.5).

Two sections through the ARH from animals of each experimental group were acquired using a Zeiss AxioImager Z1 microscope equipped with a 20X objective. pSTAT3-labeled cells in the ARH were manually counted using Image J analysis software (NIH). The

average number of cells counted in two ARH hemisections from each mouse was used for statistical comparisons.

Statistical analyses

All values were expressed as means ± SEM. Statistical analyses were conducted using GraphPad PRISM (version 5.0d). Statistical significance was determined using unpaired two-tailed Student's t-tests, and a two-way ANOVA followed by the Bonferroni multiple comparisons post-hoc test when appropriate. *P*-values less than 0.05 were considered to be statistically significant.

Results

Postnatal overnutrition causes early hyperleptinemia

To examine the effect of neonatal nutrition on leptin levels, we used a mouse model of divergent litter size. To this aim, litter size was manipulated beginning on postnatal day 3 (P3) by randomly distributing pups among mothers such that normal litters (normal fed) had 7 pups and small litters (overfed) had 3 pups. The plasma leptin profile, which typically shows a distinct surge during the neonatal period in normally nourished mice (Ahima et al., 1998) (Fig. 1A), was greatly amplified and prolonged in SL mice (Fig. 1B). In NL mice, the leptin surge showed one sharp peak at postnatal day 8 (Figs. 1A). Remarkably, the leptin surge in SL pups remained elevated throughout the latter period of lactation, being significantly higher than in NL mice on postnatal days 8, 10, 12, and 16 (Fig. 1B).

Neonatal leptin antagonism enhances the response to peripheral leptin in overnourished pups

To determine the physiological importance of neonatal hyperleptinemia in overnourished pups, we selectively blocked leptin action in SL mice using a leptin antagonist. The antagonist was administered daily from P6 to P16, *i.e.*, when endogenous leptin levels are abnormally high in SL mice. Based on previous studies showing that neonatal overnutrition causes central leptin resistance (Glavas et al., 2010b), we first investigated the effects of the leptin antagonist on the central sensitivity to leptin. We used pSTAT3 as a surrogate marker of leptin receptor activation. As expected, peripheral leptin injection caused a marked increase in the number of pSTAT3-immunoreactive cells in the ARH of control (NL) mice at P10 (Fig. 2). However, this effect was markedly attenuated in SL mice (Fig. 2). However, SL pups treated with the leptin antagonist showed a significant increase in the number of pSTAT3 cells compared to vehicle treated SL pups (Fig. 2). In contrast, NL pups treated with the leptin antagonist displayed attenuated induction of pSTAT3 following leptin administration (Fig. 2).

Neonatal leptin antagonism improves metabolic outcomes in SL mice

Neonatal overnutrition has been shown to be associated with metabolic perturbations (Plagemann et al., 1999e; Glavas et al., 2010b; Colldén et al., 2014) In good agreeement with these findings, we found that small litter rearing was associated with changes in growth rates as revealed by a significant increase in pre-weaning body weight curve in SL animals compared to NL mice (Fig. 3A). As early as P6, overfed pups displayed heavier body weights compared with control animals (Fig. 3A), and this elevated body weight persisted into adulthood (Fig. 3B). The elevated body weight in SL mice comprised both an increase in fat mass (Fig. 3F) characterized by higher subcutaneous (Fig. 3G) and visceral (Fig. 3H) fat accumulation, as well as increased lean mass (Fig. 3C) and anonasal length (Fig. 3D). This increase in adiposity was associated with impaired insulin sensitivity (Fig. 4B), however SL mice were comparable to controls with regards to glucose tolerance (Fig. 4 A).

Neonatal exposure with the leptin antagonist had no effect on the pre- and post-weaning growth trajectories in either NL or SL mice (Fig. 3A-B), and there were no significant differences in the adult length (Fig. 3C) or lean mass (Fig. 3D). However, neonatal leptin antagonist treatment improved glucose tolerance in both NL and SL pups (Fig. 4A), and normalized fat mass (Fig. 3F-H) and insulin sensitivity (Fig. 4B) only in SL mice. Antagonist-treated NL and SL mice also tended to display lower serum leptin levels as adults compared to vehicle-treated mice, although it did not reach difference (Fig. 3E).

Discussion

Circulating hormones are well established as important environmental signals that can modulate growth and development (Martin et al., 1984; Steculorum and Bouret, 2011a; Attig et al., 2013a). However, an association between leptin and neonatal overnutrition in regard to lifelong metabolic regulation has never been examined. The experiments presented here represent the first systematic characterization of letin secretion pattern in neonatally

overnourished pups. We report that pups raised in SL display an exaggerated leptin surge between P6-P16 and that attenuation of leptin action in SL pups results in lifelong metabolic benefits.

The present study is in line with previous studies showing that changes in nutriton during the pre- and/or post-natal life causes marked changes in circulating leptin levels during early postnatal life. Maternal obesity/diabetes and postnatal overnutrition can increase leptin levels throughout postnatal life and cause leptin resistance during critical periods of hypothalamic development (Kirk et al., 2009; Glavas et al., 2010b; Steculorum and Bouret, 2011b). In contrast, maternal malnutrition (including calorie restriction and protein restriction) during pregnancy and lactation or postnatal malnutrition blunts the naturally occurring postnatal leptin surge (Delahaye et al., 2008; Coupé et al., 2010).

It becoming clear that perturbations in perinatal nutrition cause a wide range of hormonal changes in both the dams and their offspring, and our data indicate that changes in perinatal leptin action during critical periods of development may represent a likely cause for the perinatal nutrition-induced alterations in metabolic function. SL pups display elevated levels of leptin and central leptin resistance and these hormonal changes are associated with weight gain, increased adiposity and insulin intolerance. Our observation that adult SL mice do not display glucose intolerance but do exhibit insulin resistance mirrors previous results by Rodrigues and colleagues who showed that in spite of impaired insulin-stimulated cellular glucose uptake, neonatally overfed mice displayed normal glucose tolerance (Rodrigues et al., 2007). It further mirrors a common observation that postnatally overfed pups often exhibit hyperinsulinemia and exacerbated glucose-induced insulin secretion (de Souza Rodrigues Cunha et al., 2009) even in the absence of abnormal glycemia (Plagemann et al., 1999e; Boullu-Ciocca et al., 2008). Remarkably, blockade of leptin action during phases of hyperleptinemia in SL mice ameliorates central leptin sensitvity, normalizes adiposity and restores insulin tolerance, showing the importance of neonatal leptin in nutritionally-induced metabolic imprinting. Consistent with the idea that neonatal leptin contributes to the adult phenotype of the organism, previous studies also reported that daily leptin treatment during

early postnatal life in pups born to malnourished dams normalizes their metabolic abnormalities (Vickers et al., 2008).

The site of action of neonatal leptin to influence metabolic function remains to be determined. The leptin receptor exists in multiple alternatively spliced isoforms, of which only the long form (LepRb) associates with Janus kinase 2 (JAK2) to mediate intracellular signaling. Upon leptin binding, LepRb initiates multiple intracellular signal transduction pathways that result in the activation of STAT family transcription factors, extracellular signal-regulated kinases (ERK), and phosphoinositol-3 kinase. LepRb mRNA is abundant in various parts of the hypothalamus during postnatal life, including in the ARH, VMH, DMH, and LHA (Caron et al., 2010). LepRb mRNA is also transiently elevated in other regions of the postnatal mouse brain, such as the cortex, hippocampus, and laterodorsal nucleus of the thalamus (Caron et al., 2010) However, in contrast to hypothalamic neurons, neurons located in these brain structures do not exhibit pSTAT3 immunoreactivity after peripheral leptin administration (Caron et al., 2010), implicating that leptin may not reach these brain regions at early ages. Based on previous data showing a neurotrophic action of leptin on ARH neurons during postnatal development (Bouret et al., 2004b), it is likely that some of the effects of neonatal leptin on metabolic regulation are mediated through the ARH. Leptin has also been suggested to be involved in the postnatal development of other organs involved in metabolic regulation, such as the pancreas (Vickers et al. 2005). Fetal pancreatic islets express functional leptin receptors and can stimulate proliferation of fetal islet cells (Islam and Sjöholm, 2000). Consistent with this idea, Attig and colleagues showed that leptin is crucial for normal postnatal development of the pancreas (Attig et al., 2011) and that neonatal leptin administration normalizes pancreatic development in piglets (Attig et al., 2013a). In addition, Kieffer and colleagues described a reciprocal regulatory relationship between leptin and insulin termed the « adipoinsular axis » wherein insulin stimulates adipogenesis and leptin production and leptin acts directly at the pancreas to inhibit insulin secretion (Kieffer and Habener, 2000). There is also the possiblity that leptin antagonism may have directly influenced the development of adipose tissue. Fetal adipose tissue

expresses the long form of the leptin receptor and it has been suggested that leptin acts as an autocrine/paracrine factor in fetal adipose tissue development (Chen et al., 2000c). In addition there is some evidence that leptin can stimulate adipocyte differentiation (Aprath-Husmann et al., 2001). One curious observation in our study was that leptin antagonism selectively reduced subcutaneous fat stores in overfed mice, and had a more moderate effect on visceral fat stores. Although little is known about the differential regulation of subcutaneous and visceral fat tissue by leptin, studies in lean human subjects show that the leptin receptor expression as well as expression of two negative regulators of leptin signaling, OB-RGRP and SOCS3, are predominantly located to subcutaneous, but not visceral adipose tissue (Séron et al., 2006), supporting the idea that subcutaneous fat may be particularly responsive to the paracrine actions of leptin.

Neonatal leptin antagonist treatment did not influence the growth trajectory or the adult weight or length of either NL or SL mice, suggesting that factors other than leptin, mediate the effects of postnatal overnutrition on somatic growth. Given our recent study showing that SL mice display reduced levels of circulating ghrelin and that neonatal overnutrition attenuates ghrelin uptake from the periphery to the brain causing central ghrelin resistance (Colldén et al., 2014), it is unlikely that ghrelin contribute to the overgrowth of SL mice. Nevertheless, because insulin, GH and IGF-1 levels are known to be influence somatotic growth and because these hormones are increased in the context of neonatal overnutrition (Kappeler et al., 2009; Habbout et al., 2013a), it is possible that insulin, GH and/or IGF-1 may play a role in mediating the effect of postnatal nutrition on somatotropic function.

Acknowledgements

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Figure Legends

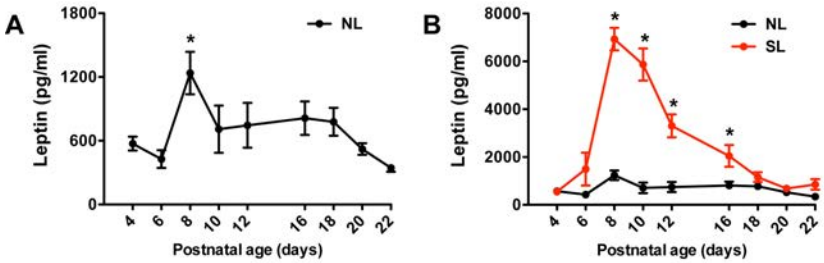
Figure 1. Small litter rearing causes exaggerated neonatal leptin surge. Serum leptin levels in of P4, P6, P8, P10, P12, P16, P18, P20 and P22 mice raised in nomral (NL) (A, B) and small (SL) (B) litters (n = 4-8 per group). Values are shown as mean ± SEM. *P < 0.05 versus NL.

Figure 2. Neonatal overnutrition causes central leptin resistance. Representative images and quantification of pSTAT3-immunoreactive cells in the arcuate nucleus (ARH) 45 minutes after intraperitoneal administration of leptin (1mg/kg) in P10 NL and SL mice treated with the leptin antagonist (PESLAN-1) or control for three consecutive days (n = 9 per group). Values are shown as mean ± SEM. *P < 0.05 versus control.

Figure 3. Neonatal leptin antagonism improves body composition without affecting growth in neonatally overnourished mice. (A) pre- and (B) postweaning growth curves in NL and SL mice treated daily with intraperitoneal injections of vehicle or PESLAN-1 (2mg/kg) (n = 9-12 per group). The black bar represents the injection period. (C) Weight, (D) length (n = 9-12 per group), (E) plasma leptin (n = 3-5 per group), and (F-H) body composition (n = 9-12 per group) of adult NL and SL mice treated neonatally with vehicle or leptin antagonist. Values are shown as mean ± SEM. *P < 0.05 versus vehicle.

Figure 4. Neonatal leptin antagonism reverses alterations in glucose metabolism in neonatally overfed mice. (A) Glucose and (B) insulin tolerance tests and area under the curves of adult NL and SL mice treated neonatally with vehicle or leptin antagonist (n = 6-11). Values are shown as mean ± SEM. *P < 0.05 versus vehicle.

Figure 1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Postnatal age (days)
B
8000
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Leptin (pg/ml)
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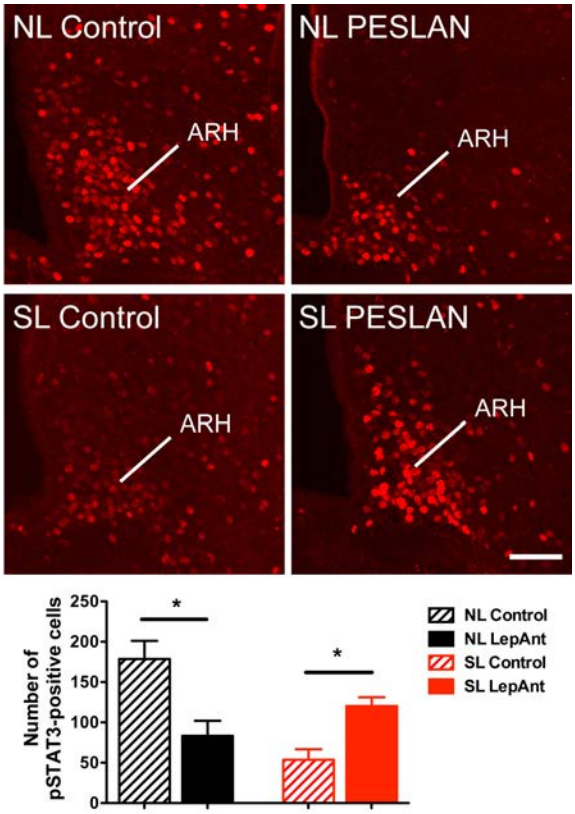
Postnatal age (days)

Figure 2


NL Control
ARH
NL PESLAN
ARH
SL Control
ARH
SL PESLAN
ARH
Number of pSTAT3-positive cells
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150
100
50
0
*
*
NL Control
NL LepAnt
SL Control
SL LepAnt

Figure 3

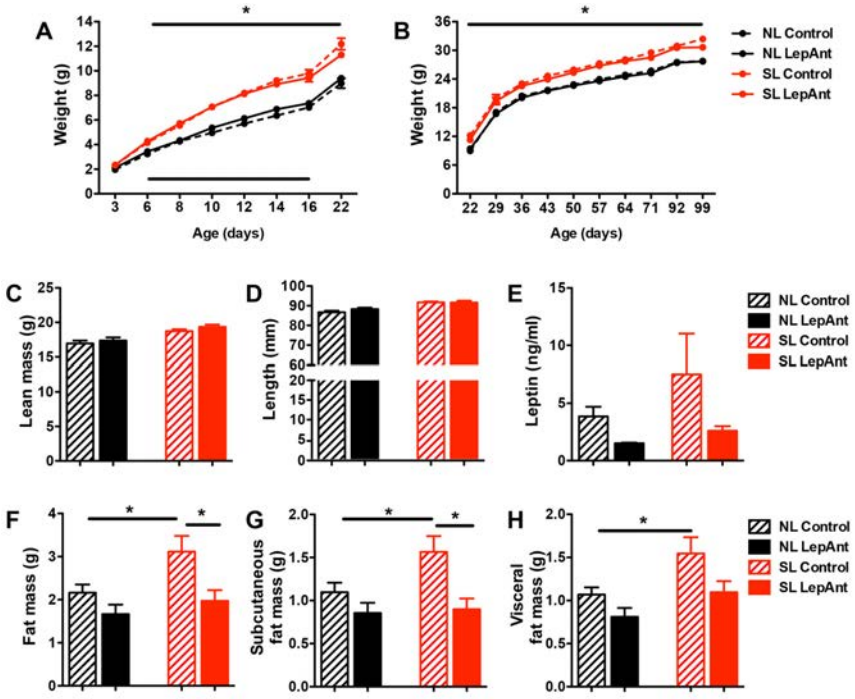
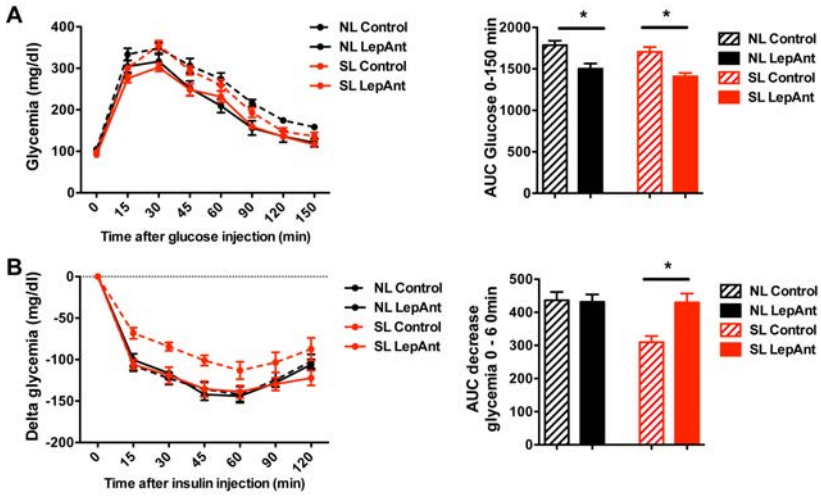

A
Weight (g)
Age (days)
B
Weight (g)
Age (days)
NL Control
NL LepAnt
SL Control
SL LepAnt
C
Lean mass (g)
D
Length (mm)
E
Leptin (ng/ml)
F
Fat mass (g)
G
Subcutaneous fat mass (g)
H
Visceral fat mass (g)

Figure 4


A
Glycemia (mg/dl)
Time after glucose injection (min)
NL Control
NL LepAnt
SL Control
SL LepAnt
AUC Glucose 0-150 min
B
Delta glycemia (mg/dl)
Time after insulin injection (min)
AUC decrease glycemia 0 - 60min

Discussion

Discussion

Altered postnatal secretion pattern of ghrelin and leptin by overnutrition and its significance

The early work from our lab on the effects of leptin on the postnatal development of the hypothalamus (Bouret et al., 2004b) established a new paradigm in the science of metabolic programming, showing that a hormone implicated in the regulation of energy homeostasis could shape the early development of the same neural circuits that it acts upon in adulthood. This mirrored findings made almost 30 years earlier that the sex steroid estrogen could stimulate neurite growth from hypothalamic explants (Toran-Allerand, 1976), which has since then grown into the recognition that both estrogen and other sex hormones like testosterone have a fundamental impact on the perinatal development of sexually dimorphic brain regions and can influence several aspects of brain maturation such as cell survival, cell migration, axonal growth and synaptogenesis (Simerly, 2002). Since leptin is known to be regulated by both acute and chronic changes in nutrition (Kolaczynski et al., 1996; Weigle et al., 1997), this discovery provided a plausible hormonal mechanism linking perinatal changes in nutrition with the alterations in metabolic programming that had been previously documented.

Like leptin, ghrelin is known to be regulated by both acute and chronic changes in energy balance in a manner opposite to leptin (Klok et al., 2007). Interestingly, leptin and ghrelin have been found to exert marked but opposite effects on postnatal hypothalamic development. Whereas leptin stimulates the growth of axonal projections from ARH neurons (Bouret et al., 2004b), ghrelin inhibits the development of axonal projections emanating from the ARH (Steculorum et al., Under revision), suggesting that these two hormones may act in concert to regulate different phases of development, and indeed may even reciprocally regulate each others signalling, since ghrelin was shown to directly inhibit both leptin-induced pSTAT3 activation and leptin-induced axon growth (Steculorum et al., Under revision). Steculorum and colleagues further showed that neonatal ghrelin treatment or neonatal ghrelin blockade not only impacted

hypothalamic development but also cause persistent metabolic changes characterized by increased body weight, fat mass, hyperglycemia and leptin resistance, which again mirrored earlier findings on the long-term impact of postnatal leptin treatment on the metabolic phenotype (Pico et al., 2011). In light of these findings, characterizing how the secretion patterns of ghrelin and leptin are changed during postnatal development in response to overnutrition may be crucial to understand how postnatal overnutrition can induce such lasting changes on metabolic regulation.

The work presented in this thesis provides the first detailed description of how the postnatal secretion pattern of ghrelin and leptin are influenced by early life overnutrition. Consistent with previous findings (Steculorum et al., Under revision; Sakata et al., 2002), we show that although normally fed pups exhibit a gradual increase in ghrelin levels throughout the lactation period, with a particularly marked increase in circulating acyl ghrelin levels during the third postnatal week, serum ghrelin levels remain at a low level during this period in the overfed pups.

Earlier studies have reported on the effects of SL rearing on serum ghrelin levels in various conditions. Lacerda-Miranda and Soares and colleagues reported that SL pups displayed decreased acyl ghrelin levels following a 4 hour fast at weaning (Soares et al., 2012) and at 6 months (Lacerda-Miranda et al., 2012), whereas there are differing reports on serum ghrelin levels in fed young adult SL rodents, some reporting no change (Stofkova et al., 2009; Fuente-Martín et al., 2012). However, to our knowledge no report on how the pattern of ghrelin secretion is affected by overnutrition during early postnatal development has previously been published. Our results also indicate that the plasma leptin profile, which typically shows a distinct surge during the neonatal period in normally nourished mice (Ahima et al., 1998), was greatly amplified and prolonged in SL mice. Separate studies have reported that SL rearing causes hyperleptinemia at P7, P14 or P21, respectively (Schmidt et al., 2000, 2001; Rodrigues et al., 2009b; Stefanidis and Spencer, 2012), but a detailed integrated mapping of the pattern of postnatal leptin secretion in neonatally overfed pups is still lacking.

To test whether normalization of the hypoghrelinemic state of the overfed pups observed in the late postnatal period could have a beneficial effect on their metabolic

programming, we injected overfed pups with low doses of ghrelin during the second half of lactation. Surprinsingly, neonatal ghrelin treatment did not alter long-term metabolic outcomes in SL pups with the exception of a modest reduction in fasting glucose. The fact that normalization of postnatal ghrelin levels did not cause any long-term effects led us to investigate whether postnatal overnutrition alters the ability of hypothalamic neurons to respond to ghrelin during postnatal development.

We found that although overfed mice displayed an impaired central response to peripheral ghrelin, these animals had a response similar to control pups when ghrelin was administered centrally. These observations suggest that hypothalamic neurons of SL mice retain the ability to respond to ghrelin, when exposed to the hormone directly but that ghrelin transport from the periphery to the brain might be altered in overfed mice. This hypothesis was confirmed with our western blot experiment that showed that the amount of ghrelin present in the mediobasal hypothalamus following a peripheral ghrelin injection was much lower in overfed mice than in control mice. We also found that this altered ghrelin transport to the hypothalamus might be mediated through abnormal ghrelin uptake by tanycytes located in the median eminence.

A better understanding of the mechanisms underlying ghrelin transport to the hypothalamus under physiological and pathological conditions will be crucial as we seek to develop interventional studies to ameliorate and hopefully reverse the altered ghrelin transport in neoanatally overfed mice. Interestingly, Langlet and colleagues recently demonstrated that fasting-induced improvements in the central accessibility of peripheral hormones such as leptin and ghrelin can be mimicked by VEGF treatment which induces vascular plasticity at the ME (Langlet et al., 2013b). VEGF combined with ghrelin might thus provide a better model for the normalization of neonatal ghrelin signalling in SL pups. Since hyperleptinemia itself has been implicated in the development of central ghrelin resistance, although only in adult diet-induced obese mice (Briggs et al., 2014), another intervention that may more completely normalize endocrine signalling in postnatally overnourished pups could be combined leptin antagonism and ghrelin treatment.

Our finding that neonatal ghrelin treatment did have an impact on adult fasting glycemia in both NL and SL mice may be related to an effect of neonatal ghrelin on pancreatic development. Previous works have shown that neonatal nutrition can influence GHSR expression in peripheral organs. For example, GHSR expression in peripheral organs such as the white adipose and heart muscle is upregulated in SL weanling mice, (Lacerda-Miranda et al., 2012; Soares et al., 2012). In addition, Dembinski and Warzecha and colleagues showed that neonatal ghrelin treatment inhibits pancreatic growth in 7-14 old day pups, whereas in contrast it had a stimulatory effect on pancreatic growth in weanling and adolescent rats. They related this age-dependent effect to an interaction with IGF-1 in postweaning but not preweaning rats (Dembiński et al., 2005; Warzecha et al., 2006). Because SL mice display elevated IGF-1 levels during postnatal development (Kappeler et al., 2009), we can speculate that neonatal ghrelin treatment has a stimulatory effect on pancreatic growth in overfed mice although future studies will be required to specifically test this hypothesis.

In addition to the defects in ghrelin signalling, we found that neonatally overnourished mice exhibit a severely exaggerated postnatal leptin surge. To investigate the contribution of this exaggerated leptin surge to the altered metabolic programming exhibited by overfed pups, we examined the long-term effects of partial leptin antagonism during the leptin surge (P6-P16) in neonatally overfed mice. Strikingly, neonatal leptin antagonism completely normalized fat mass and insulin sensitivity to control levels compared with vehicle-treated SL pups, in spite of having no effect on growth curves or final weight.

Postnatal leptin is known to stimulate ARH axonal growth, but small litter mice have paradoxically been shown to have impaired development of ARH axonal fibers (Bouret et al., 2007). Hyperleptinemia and excessive leptin signalling is known to induce central leptin resistance, probably due to induction of negative feedback molecules such as SOCS3, PTP1B or TCPTP by phosphorylated STAT3, and indeed hyperleptinemia may be essential to the development of leptin resistance (Knight et al., 2010). Consistent with this idea we and others have found that the hyperleptinemia in SL mice is associated with attenuated leptin-induced pSTAT3 in the ARH of SL pups (Glavas et al., 2010b). Remarkably, we were able to significantly enhance leptin-induced pSTAT3 following

treatment with the leptin antagonist. Importantly, STAT3 is one of the main leptin receptor signalling pathway that mediate the neurotrophic effect of leptin during postnatal development (Bouret et al., 2012).

One plausible mechanism whereby leptin antagonism improved the long-term metabolic phenotype could therefore through enhanced central leptin signalling during this critical period of hypothalamic development. It is also possible that leptin antagonism may have altered the phenotype of the overfed pups by modulating inflammatory processes in the hypothalamus, adipose tissue, or other peripheral organs. Hyperleptinemia is strongly associated with markers of inflammation (Leon-Cabrera et al., 2013) and leptin antagonism has been shown to exert beneficial effects in various inflammatory conditions (Elinav and Gertler, 2009; Singh et al., 2013). Hypothalamic inflammation has in turn been suggested to be involved in the long-term changes of metabolic control by causing hypothalamic degeneration (Thaler et al., 2012b) and inflammation in adipose tissue is highly implicated in re-modelling and expansion of adipose tissue (Wernstedt Asterholm et al., 2014).

Rapid weight gain during critical periods of development and leptin

- **A unifying factor in the perinatal programming of disease?**

Although perinatal undernutrition and overnutrition are both well recognized to increase the risk of long-term ill health and have been argued to act via distinctly different disturbances in endocrine secretion, most literature is consistent with the idea that undernutrition by itself is not a causative factor in adverse metabolic programming, but rather indirectly causes adverse metabolic programming by inducing accelerated growth during critical periods of development.

In the Dutch winter famine cohort, one remarkable finding was that fetuses exposed to famine only during early gestation were at a particularly elevated risk of adverse effects such as obesity (Ravelli et al. 1976), cardiovascular disease, dyslipidemia and “general poor health” in adulthood, whereas foetuses exposed during mid- and particularly late gestation, were generally not at increased risk of elevated BMI or metabolic disease compared to unexposed foetuses. Even though these foetuses were malnourished during

early gestation, their birth weights were slightly above the birth weights of unexposed foetuses, whereas foetuses exposed during mid/late gestation had lower birth weights, suggesting that early-exposed fetuses underwent accelerated gestational growth in mid/late gestation following the period of malnutrition (Roseboom et al., 2001). Although there is little data on the direct association of human fetal growth with later metabolic outcomes, there is an abundance of studies linking maternal obesity and maternal gestational weight gain with both early and late-onset obesity in offspring (Oken, 2009), and one study linked fetal weight gain between the 2nd trimester and birth with obesity at 3 years of age (Parker et al., 2012).

Early gestation in humans correspond roughly to the second half of gestation in rodents, whereas late gestation in humans correspond to the early postnatal period in rodents. These findings are thus consistent with the data on perinatal nutrition in rodents, where it has been found that malnutrition during gestation but not lactation is associated with increased fat mass (Desai et al., 2005; Krechowec et al., 2006; Bautista et al., 2008; Sutton et al., 2010; Palou et al., 2012), whereas malnutrition during both gestation and lactation, or lactation alone, is associated with lower fat mass and resistance to diet-induced obesity and its associated adverse effects (Desai et al., 2005; Bieswal et al., 2006; Fagundes et al., 2007; Velkoska et al., 2008b; Pinheiro et al., 2008; Palou et al., 2010, 2011; Patterson et al., 2010; Coupe et al., 2012; Garg et al., 2012; Sun et al., 2014). That is, whereas postnatal undernutrition leads to a phenotype distinctly different from that of postnatal overnutrition with lower risk of obesity and metabolic disease (Jimenez-Chillaron et al., 2006)., prenatal undernutrition with early postnatal catch-up growth causes obesity, mirroring the effects seen in models of postnatal overnutrition.

Consistent with the idea that the adverse metabolic programmng induced by both prenatal undernutrition and postnatal overnutrition share a similar aetiology, prenatal undernutrition followed by normal postnatal nutrition has actually been observed to cause a postnatal increase in leptin levels that is both magnified (Yura et al., 2005; Coupé et al., 2010) and/or more sustained (Bautista et al., 2008; Coupé et al., 2010) compared control mice, mirroring the secretion pattern of leptin observed in postnatally overnourished mice.

Thus it could be that both prenatal undernutrition and postnatal overnutrition induce their adverse metabolic effects by a shared pathway involving accelerated growth and hyperleptinemia during the critical postnatal period of hypothalamic maturation. The critical role of overnutrition and hyperleptinemia specifically during early postnatal development in rodents is further supported by the observation that offspring of dams fed a high-fat diet during postnatal development have increased weight and fat mass and exhibit early hyperleptinemia, whereas offspring exposed to HF-diet during gestation but not postnatal development have normal weight, fat mass and postnatal leptin levels (Mitra et al., 2009; Sun et al., 2012b; Guberman et al., 2013; Vogt et al., 2014), and in fact may even be less susceptible to diet-induced obesity than offspring that were normally nourished during gestation (Vogt et al., 2014). Conversely models of postnatal undernutrition such as maternal restriction or large litter rearing may program an obesity-resistant phenotype through hypoleptinemia during the period of postnatal hypothalamic development (Bautista et al., 2008; Delahaye et al., 2008; Patterson et al., 2010). This is consistent with observations that antagonising leptin signalling during postnatal life has been found to reduce weight gain and fat gain both on standard and high-fat diets (Beltrand et al., 2012).

Our data on the effects of neonatal leptin antagonism on metabolic programming in male mouse pups show that whereas only SL mice responded to antagonist treatment with significant reductions in fat mass and enhanced insulin sensitivity, there was nonetheless a tendency towards lower fat mass and increased lean mass, as well as significantly enhanced glucose tolerance also in the normally fed mice. These findings are thus in line with the aforementioned literature suggesting that relative hypoleptinemia during early postnatal development may have beneficial effects on metabolic programming in normally fed male pups, but that the beneficial effects of neonatal leptin antagonism are magnified when used to correct an existing hyperleptinemic state.

Earlier studies on the long-term effects of postnatal manipulation of leptin signalling have produced inconsistent results. Although a majority of studies report obesogenic or other deleterious effects of postnatal leptin treatment treatment (Passos et al., 2009b; Marques et al., 2010; Samuelsson et al., 2013) (see Pico et al., 2011 for review of studies

up to 2008), most studies reporting negative effects of leptin treatment were conducted with intraperitoneal injections in normal male rodent pups, similar to the set-up used in our study (Pico et al., 2011). In contrast, studies reporting beneficial effects have used other routes of administration such as oral, maternal or ICV, or else been conducted in female or undernourished pups. This suggests that the effects of neonatal leptin manipulation depend on several factors such as sex, administration route and interactions with the nutritional environment. Leptin treatment is probably more likely to not cause adverse effects in situations of existing hypoleptinemia, whereas leptin antagonism is more likely to be beneficial in overnutrition and other situations that associate with hyperleptinemia.

It is likely that sex is an important consideration, since authors have reported divergent effects of both postnatal leptin antagonism (Attig et al., 2008b; Beltrand et al., 2012) and postnatal leptin treatment (Vickers et al. 2005; Vickers et al. 2008) in prenatally underfed males and females, including diverging effects of leptin antagonism on hypothalamic neuropeptide and neurotrophic factor levels in males and females (Mela et al., 2012).

However, male and female pups both exhibit similar differential responses to the pre- or postnatal timing of undernutrition with regards to adult weight and body composition. (Desai et al., 2005; Zambrano et al., 2006). Since over- or undernutrition causes multiple coordinated endocrine changes involving thyroid, ghrelin, insulin and IGF-1 among other hormones, which are all involved in growth and development and/or metabolic programming, this may underlie the robustness of the different phenotypes caused by postnatal under- or overnutrition, and explain why manipulations of leptin signalling alone produce less consistent results, and may further explain why leptin antagonism did not alter somatic growth or final body weight in our overnourished mice.

The consistent effect of accelerated/retarded growth during the postnatal period in rodents on fat mass may also be related to the programming of adipogenesis and expansion of fat mass that primarily occurs during the formation of hypothalamic projection pathways during postnatal development (Cryer and Jones, 1979; Dugail et al., 1986).

From rodents to human

As discussed previously, the human data is consistent with rodent data in that developing organisms are probably particularly sensitive to nutritional disturbances during the period of development of functional circuits in the hypothalamus, which in humans is proposed to be relatively mature at birth. Future studies will be required to examine the relationship between perinatal endocrine disturbances and metabolic programming in humans. Human foetuses have been reported to exhibit a 5-10 fold elevation of serum leptin from the second to third trimester (Ogueh et al., 2000). This coincides with the fetal growth of adipose tissue (Lepercq et al., 2001) which expands primarily by hyperplasia in the second trimester and expands by hypertrophy in the third trimester (Poissonnet et al., 1983). However others report little change in fetal leptin concentrations until a few weeks before birth (Jaquet et al., 1998; Cetin et al., 2000), when they increase sharply, suggesting that leptin may be more implicated in the late rather than early maturation of hypothalamic neuronal circuits in human.

Fetal leptin levels are positively correlated with maternal diabetes indices (Cetin et al., 2000; Vela-Huerta et al., 2012; Higgins et al., 2013), fetal birth weight (Wolf et al., 2000; Sooranna et al., 2001; Weyermann et al., 2006) and fetal hyperinsulinemia (Wolf et al., 2000). Fetal hyperleptinemia is thus associated with several risk factors known to predispose to later obesity. Although the effects of perinatal nutrition on ghrelin during fetal development is less well studied, ghrelin is present in fetal circulation between the 2nd trimester and birth, and is suggested to be primarily of fetal origin (Fuglsang, 2007). Fetal ghrelin levels have been reported to be increased in IUGR foetuses (Cortelazzi et al., 2003) and decreased in newborn macrosomic infants (Ding et al., 2012), thus suggesting that fetal ghrelin levels are nutritionally regulated in humans similar to rodents.

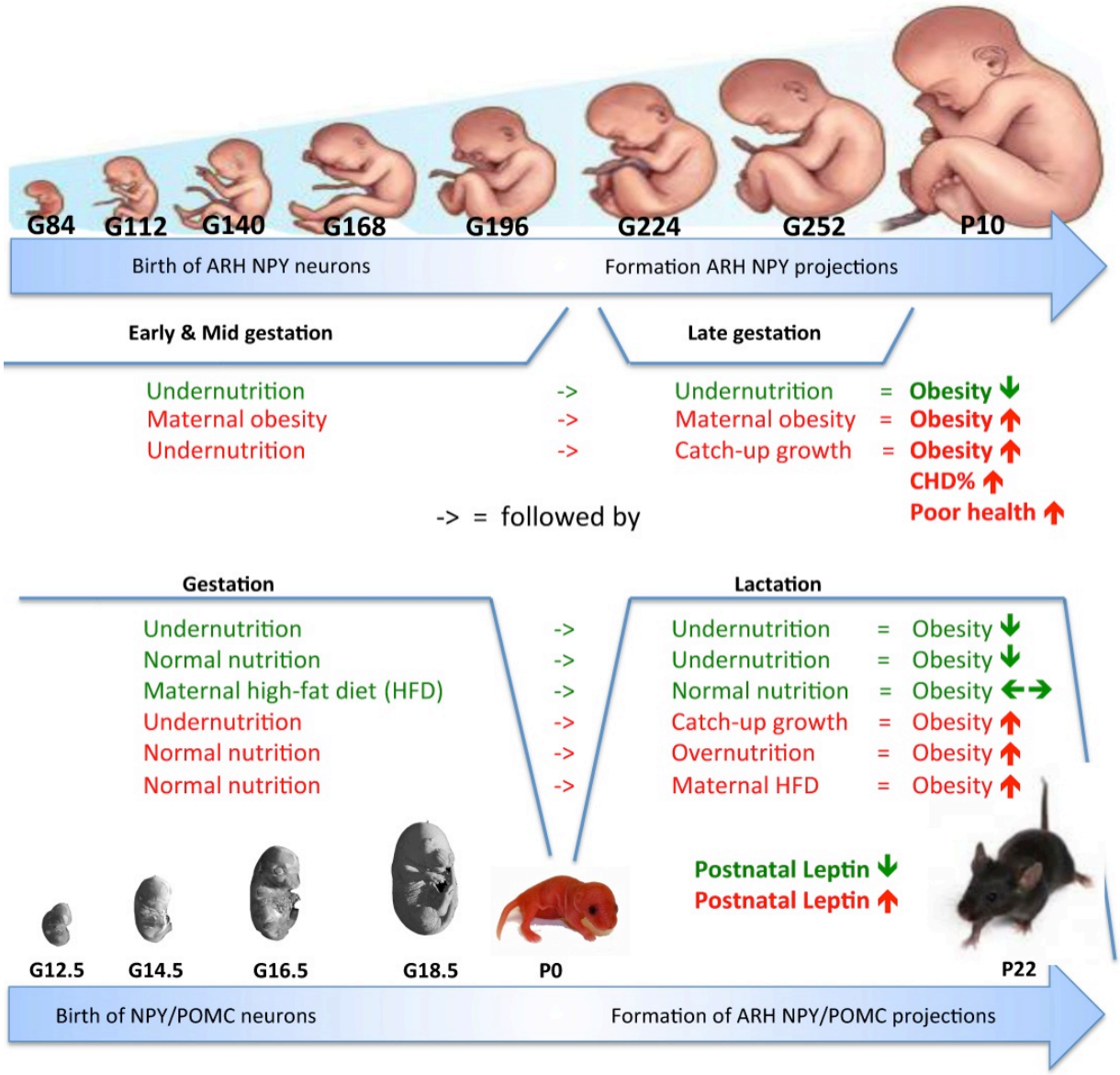


Figure 12: Converging evidence in humans and rodents suggest overnutrition coinciding with the formation of hypothalamic projection pathways involved in energy metabolism is of particular importance in the nutritional programming of disease. Undernutrition and overnutrition during the maturation of the hypothalamic feeding circuits have generally been observed to exert opposite effects on metabolic programming, whereas undernutrition or overnutrition prior to this developmental phase often do not cause adverse effects unless followed by rapid catch-up growth or continued overnutrition, suggesting that a common link between the often observed adverse effects of perinatal undernutrition and overnutrition may be accelerated growth during the maturation of hypothalamic feeding circuits, with leptin as an important mediator of the effects. Arrows signify that the nutritional environment on the left is followed by the environment on the right, with its final consequences on later obesity risk on the right, green/red = decreased/increased risk.

Although much of the early literature identified low birth weight as a risk factor for later obesity and metabolic disease, more recent analyses have concluded that there exists a more or less linear positive relation between birth weight and subsequent obesity risk (Yu et al., 2011; Schellong et al., 2012). Lower birth weight babies actually have lower risk of obesity, but do in contrast have a higher risk of metabolic dysfunctions such as hypertension, dyslipidemia, type 2 diabetes and metabolic syndrome (Harder et al., 2007; Silveira and Horta, 2008; Mu et al., 2012). These findings are consistent with the idea that under- and overnutrition and their associated changes in leptin during the maturation of the hypothalamic feeding circuits during late gestation have divergent effects on the subsequent susceptibility to obesity. Although it also highlights the limitations of using simple obesity indices like BMI to estimate the risk of metabolic disease, since fetal nutrition is likely to affect both adipose tissue cellularity and growth of organs and skeletal muscle, which can all result in lowered weight but higher risk of metabolic dysfunction, or vice versa.

Such adverse metabolic effects have generally not been observed in rodents subjected to postnatal undernutrition. One factor contributing to these disparate results may be that while the development of the hypothalamic feeding circuits is considered to occur primarily before birth in humans, the human brain is still undergoing major development in the first years of life (Jernigan et al., 2011), particularly with respect to synaptogenesis, synaptic pruning and axonal myelination (Semple et al., 2013), and several brain regions, including the cortex, hippocampus and the hypothalamic-pituitary axes are susceptible to environmental conditions such as stress during early postnatal life (Charmandari et al., 2003), suggesting that adverse nutritional experiences after birth, such as stress, overnutrition or rapid catch-up growth, may still have an adverse impact on brain development and metabolic programming.

Conclusions

Conclusions

Abnormal nutrition during perinatal life has emerged as a crucial risk factor for the development of obesity and metabolic disease, with overnutrition or accelerated growth during the critical period of the formation of the hypothalamic neural circuits regulating metabolism perhaps representing a particularly important factor in the adverse early programming of metabolism. Leptin and ghrelin are two hormones acting in the hypothalamus to regulate energy homeostasis that have also been implicated in the early programming of metabolic disease through their effects on hypothalamic development. It is however unclear what is the role of dysregulated leptin and ghrelin signalling in the metabolic programming of disease caused by postnatal overnutrition.

We show here for the first time that overnutrition causes widespread alterations in the postnatal ghrelin system, including reduced secretion of acyl ghrelin, and impaired central sensitivity to ghrelin linked to defective transport of ghrelin from the periphery into the brain.

We further show that neonatal hyperleptinemia is crucially implicated in the adverse metabolic programming of postnatally overfed mice, since partial blockade of leptin signalling during neonatal development restores fat mass and insulin sensitivity to the level of normally fed mice, possibly by improving neonatal central leptin signaling. We thus demonstrate the importance of temporary nutritionally induced alterations in leptin signalling to the long-term development of metabolic disease.

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Appendix

Neonatal Ghrelin Programs Development of Hypothalamic Feeding Circuits

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Abstract

The gut-brain axis is an important component of vertebrate physiology. The stomach-derived hormone ghrelin is a key regulatory factor of this axis and promotes appetite through its effects on neurons that are located in the arcuate nucleus of the hypothalamus (ARH). To identify the physiological and neurobiological importance of ghrelin during development, we specifically blocked the action of ghrelin during neonatal life in mice. Neonatal ghrelin blockade resulted in enhanced ARH neural projections. These organizational effects of ghrelin were associated with long-term deleterious metabolic effects. In addition, chronic administration of ghrelin during postnatal life impaired the normal development of ARH projections. Consistent with these observations, direct exposure of ARH neurons to ghrelin blunted axonal growth and blocked the documented neurotrophic effect of leptin. Collectively, these data reveal an unexpected inhibitory role for ghrelin in the development of hypothalamic neural circuits, and they show the importance of neonatal ghrelin for lifelong metabolic regulation.

Introduction

In the midst of the present obesity epidemic, there is a need to better understand the mechanisms and factors involved in the development of this pathological condition. The physiological mechanisms that underlie hunger and satiety have only recently been determined (1, 2). The discovery of leptin and ghrelin led to a paradigm shift in our understanding with the realization that our subconscious motivation to eat can be powerfully and dynamically regulated by hormonal signals (3-5). Epidemiological studies and animal models have provided valuable information on the role of the perinatal environment in the susceptibility for diseases in adult life, such as obesity and type 2 diabetes. Previous studies showed that various environmental challenges, including deficient or excess nutrition, during development program organisms for metabolic diseases in later life (6) (7, 8).

Body weight and energy balance are dynamically regulated by a sophisticated network of neural circuits. At the center of this regulatory network is the arcuate nucleus of the hypothalamus (ARH), which contains sets of neurons devoted to metabolic regulation. The ARH comprises orexigenic neurons that co-produce neuropeptide Y (NPY) and agouti-related peptide (AgRP) as well as anorexigenic neurons that contain proopiomelanocortin (POMC)-derived peptides. These ARH neuronal populations are directly regulated by leptin and ghrelin (9, 10). NPY/AgRP and POMC neurons provide overlapping projections to other parts of the hypothalamus, including the paraventricular (PVH) and dorsomedial (DMH) nuclei of the hypothalamus and the lateral hypothalamic area (LHA), to exert their metabolic effects (11, 12).

It has been suggested that impaired hypothalamic development during perinatal life results in lifelong metabolic dysregulation because of the importance of the hypothalamus in the control of eating and energy balance (6). In mice, ARH neural projections primarily develop during the first three weeks of postnatal life under the influence of genetic and environmental factors, including endocrine signals (8, 13). Leptin represents a powerful neurotrophic agent that promotes the formation of ARH-derived circuits (14). However, the

exact nature of hormonal factors that influence the development of appetite-related neural projections remains elusive and mainly restricted to the well-described axonotrophic effect of leptin (14). For example, little is known about the biological function of ghrelin during neonatal life. However, despite the well documented orexigenic effect of ghrelin, the congenital deletion of ghrelin or its receptors results in normal body weight, growth rate and food intake (15-18), raising the possibility that lack of ghrelin during early life may cause compensatory developmental changes (19). The observation that maternal ghrelin injection causes high birth weight in the offspring, also supports a role for neonatal ghrelin in growth and development (20).

In the present study, we selectively inhibited the action of ghrelin during the pre-weaning period to determine the role of neonatal ghrelin in hypothalamic development and adult metabolic regulation. We also investigated whether abnormally elevated levels of ghrelin, during a developmental period when endogenous ghrelin levels are low, have enduring effects on hypothalamic development. The results indicate that neonatal ghrelin is important for normal maturation of hypothalamic axonal projections, and that the perturbation in ghrelin action during pre-weaning period results in lifelong metabolic disturbances. The data also underline the importance of the correct timing and amplitude in ghrelin's action for normal development of hypothalamic neural circuits.

Results

Ghrelin is developmentally regulated and acts on ARH neurons during early life

To assess the function of neonatal ghrelin, we first examined the levels of circulating ghrelin in neonatal mice. Relatively low levels of total and acylated ghrelin were found in the serum at P6; however, the levels increased after P10 and reached adult-like levels by P14 (Figure 1A). This increase in circulating ghrelin levels between P6 and P14 was also observed in fasted pups (Figure 1C), supporting the hypothesis that these changes in ghrelin levels are developmentally acquired versus the hypothesis that they are caused by changes in nutrition. Consistent with this idea, no correlation was found between stomach weight and acylated ghrelin levels at P14 (Figure 1D). To determine whether neonatal ghrelin acts on the developing hypothalamus, we next examined the mRNA expression of the ghrelin receptor *GHSR* in the ARH of neonatal mice. The ARH of P6 and P10 neonates contained levels of *GHSR* mRNA that were similar to those found in adult animals (Figure 1B). However, *GHSR* mRNA decreased at P14, *i.e.*, when neonatal ghrelin levels are elevated (Figure 1B). Ghrelin acts via multiple intracellular signaling pathways, including the MEK→ERK pathway, to modulate cellular and organismal physiology (21). Accordingly, we utilized the immunohistochemical detection of phosphorylated (activated) forms of ERK (pERK) to examine the ability of ghrelin to activate GHSR in neonatal ARH neurons. A peripheral injection of ghrelin in wild-type mice robustly induced pERK immunoreactivity (IR) as early as P6 (Figure 1E), indicating that ghrelin can act on ARH neurons when endogenous levels of this hormone are low. However, there was a 1.5-2 fold increase in the number of pERK-IR cells between P6 and P10-P90 animals (Figure 1E). To explore the potential cell-type specificity of ghrelin's action, we examined the ability of ghrelin to activate ERK in the NPY/AgRP and POMC neurons of neonatal animals. We found ghrelin-stimulated pERK IR colocalized with green fluorescent protein (GFP) in the ARH of P10 transgenic mice expressing GFP in NPY- and POMC-containing neurons (Figure 1F). This analysis revealed that neonatal ghrelin stimulated pERK-IR in approximately 60% and 10% of NPY and POMC

neurons, respectively. Also, 80% and 8% of pERK-IR cells were NPY+ and POMC+, respectively.

Blockade of ghrelin action during early life causes metabolic disturbances

To determine the physiological importance of neonatal ghrelin, we selectively inhibited the action of ghrelin during the pre-weaning period using NOX-B11-2, which is an anti- ghrelin compound based on a mirror-image oligonucleotide (so-called Spiegelmer) that specifically binds and inhibits the bioactive acylated form of ghrelin (22). Starting at P4, pups were treated daily with intraperitoneal injections of the anti-ghrelin compound or an inactive control, for a total of 18 days (Figure 3A). To confirm that the action of ghrelin was specifically disrupted during the injection period, we first examined ghrelin-induced cFos IR, which is a marker of neuronal activation. Neonates injected with the anti-ghrelin compound had an attenuated response to ghrelin as revealed by a reduced number of cFos-IR cells in the ARH following ghrelin injection (Figure 2A). This reduction in ghrelin-induced cFos IR was observed 2 h after the anti-ghrelin treatment and persisted for up to 24 h after the injection (Figure 2B). A significant reduction of ghrelin-induced cFos IR was also observed in pups treated with the anti-ghrelin compound for 18 days (Figure 2C). However, P36 animals treated with the anti-ghrelin compound neonatally displayed a normal central response to ghrelin, which shows that the effect is reversible (Figure 2A). Notably, the stomach content of the anti-ghrelin-treated neonates appeared similar to that of control animals, suggesting that the anti-ghrelin compound does not alter milk intake (Figure S1D). Stomach *Ghrelin* mRNA levels were similar between the anti-ghrelin and control neonates (Figure 2E) but, as previously reported (23), animals treated with the anti-ghrelin compound display elevated levels of ghrelin (Figure 2F). This increase in ghrelin levels is expected because the anti-ghrelin compound was modified with a 40 kDa-PEG, which delayed the elimination of the NOX-B11-2 and the ghrelin-NOX-B11-2 complex.

Physiologically, the anti-ghrelin-injected neonates survived to adulthood and had body weights undistinguishable from those of their control littermates until P14 (Figure 3B).

Starting at P14, the anti-ghrelin-treated neonates had significantly higher body weights than the control mice, and they remained overweight after weaning and until adulthood (Figure 3B). Notably, there was no difference in body length between the anti-ghrelin treated neonates and control mice (data not shown). The daily food intake was significantly higher in adult mice treated with the anti-ghrelin compound neonatally when compared with control mice (Figure 3D). Moreover, adult mice treated with the anti-ghrelin compound neonatally displayed an increase in body fat mass characterized with a higher visceral fat accumulation and an unchanged subcutaneous fat accumulation (Figure 3C). Consistent with these findings, anti-ghrelin treated mice have elevated levels of leptin (Figure 3E), and altered leptin sensitivity (Figure 3G), when compared with control mice. In addition, the fed glucose levels were significantly elevated in the adult mice treated with the anti-ghrelin compound neonatally (Figure 3F) and anti-ghrelin treated mice displayed elevated levels of glucose 90-120min after a glucose challenge, as compared to control mice (Figures 3H).

Alteration of ghrelin action during neonatal affects development of ARH neuronal projections

During development, axonal projections ascend from the ARH to reach their target nuclei, such as the PVH, during the second week of life (14). To investigate whether neonatal ghrelin blockade influences the pattern of ARH axonal projections involved in feeding regulation, we implanted the fluorescent axonal tracer DiI into the ARH of anti-ghrelin-treated neonates and control mice. Although the overall distribution of ARH DiI-labeled fibers was relatively similar between anti-ghrelin and control neonates, clear differences were apparent in the density of the labeled fibers. The density of DiI labeling in the PVH of P12 mice injected with anti-ghrelin was 1.5-fold higher than that observed in the control mice (Figure 4A). To determine whether the defect in ARH projections observed in the anti-ghrelin neonates was permanent, we performed immunohistochemical labeling of AgRP in brain sections from adult mice that were neonatally injected with the anti-ghrelin compound or the control. AgRP immunolabeling can be used as a marker of ARH projections because AgRP expression is restricted to the ARH in the adult mouse brain. The density of AgRP-IR fibers in

the PVH was 3-fold higher in adult mice that were neonatally treated with anti-ghrelin compared with control mice (Figure 4B). In addition, the density of α-MSH-IR fibers (another neuropeptidergic system expressed by ARH neurons) innervating the PVH was 2-fold increased in adult mice neonatally treated with anti-ghrelin (Figure 4C). A substantial increase in the density of AgRP and α-MSH labeled fibers was observed in the parvocellular and magnocellular parts of the PVH (data not shown). Similar increases in DiI, AgRP, and α-MSH fiber densities were also observed in the DMH and LHA (Figure S1A-D), which are other major terminal fields of ARH projections. These data indicate that neonatal ghrelin blockade causes the widespread disruption of ARH neural projections.

To address whether endogenous ghrelin controls hypothalamic development, we assessed ARH neural projections in ghrelin knockout (*Ghrl-/-*) mice (17). We detected significantly higher densities of DiI-labeled ARH axons in *Ghrl-/-* neonates compared to wild-type littermates at P12 and P21 (Figures 4A and 4F). However the density of DiI-labeled ARH axons in *Ghrl-/-* mice normalized at P35 (Figure 4F). Similarly, α-MSH and AgRP-containing axons were normal in adult *Ghrl-/-* mice (Figure 4B-C).

Many of the key events that occur during the development of functional neural systems, including the hypothalamus, are particularly sensitive to developmental cues during restricted ontogenic periods. These so-called “critical periods” tend to occur early in development and often coincide with the expression of signals that influence the formation of cellular nuclei and axonal connections. To test the structural effects of ghrelin in adults, we injected the anti-ghrelin compound from P100 to P107 and then evaluated AgRP and α-MSH fibers. In contrast to neonatal anti-ghrelin injections, adult injections with the anti-ghrelin did not affect the density of AgRP- and α-MSH-IR fibers (Figure 4D-E and Figure S1A-D). Consistent with these observations, adult anti-ghrelin injections do not cause marked metabolic alterations (Figure S2). These results suggest that the neurodevelopmental activity of ghrelin is essentially restricted to the neonatal period.

Neonatal ghrelin blockade does not affect development of DMH projections

To determine whether neonatal ghrelin blockade also affected the development of other non-ARH circuits, we examined neuronal projections from the DMH, which is another hypothalamic nuclei known to contain high densities of ghrelin receptors (24). Despite a marked attenuation in the density of ARH DiI-labeled fibers, neural projections from the DMH appeared to be unaltered in anti-ghrelin treated neonates (Figure S3A), which suggests that neonatal ghrelin specifically affects the development of ARH projections. Consistent with these observations, ghrelin receptors were expressed at very low levels in the DMH throughout postnatal life. Notably, *GHSR* mRNA levels were 4-5 times lower in the DMH compared to the ARH (Figure S3B). Similarly, the number of ghrelin-induced pERK-IR was 4-5 times lower in the DMH compared to the ARH (Figure S3C). Levels of *GHSR* mRNA and ghrelin-induced pERK-IR were also low in other hypothalamic and extra-hypothalamic regions during neonatal life, suggesting that the developmental action of ghrelin is primarily restricted to the ARH (Figure 1G-H).

Ghrelin acts directly on ARH neurons to block axon growth

If ghrelin is a critical regulator of arcuate axon development, the direct exposure of ARH explants to ghrelin should result in changes in neurite outgrowth. To test the effects of ghrelin on neurite outgrowth, we incubated isolated ARH explants derived from P4 mice with ghrelin or vehicle. After 48 hours, the addition of ghrelin to the culture medium produced a 2-fold reduction in the density of neurites extending from ARH explants when compared with the control (Figures 5A). In addition to decreasing the density of ARH axons, ghrelin significantly reduced the overall length of axon extensions from ARH explants (Figure 5A). A substantial disruption of both AgRP and α-MSH fiber outgrowth was observed in ghrelin-treated explants (Figure S1E). Together, these data provide direct evidence that ghrelin acts on ARH neurons to inhibit axonal growth and elongation. Notably, exposure of ARH explants to the anti-ghrelin

compound prevented the ghrelin-induced decrease in neurite outgrowth (Figure 5B), further validating the use of the anti-ghrelin compound to inhibit ghrelin's action.

Neonatal hyperghrelinemia causes abnormal maturation of arcuate neural projections and metabolic disturbances

Our in vitro findings indicate that ghrelin inhibits ARH axons growth. Based on these observations, we next investigated whether a premature increase in ghrelin levels during neonatal life can impact hypothalamic development. Ghrelin was administered daily in wild-type pups from P4 to P12, *i.e.*, when endogenous ghrelin levels are low (Figure 6A). Plasma levels of acylated ghrelin following exogenous i.p. ghrelin administration (2 mg/kg BW) in neonates were markedly increased 15 min after injection, remained high 1h after the injection and returned to levels similar to vehicle-treated pups by 12 h after injection (Figure 6B). The density of ARH DiI-labeled fibers was 1.5-fold reduced in the PVH of P12 ghrelin-injected mice (postnatal (PN) ghrelin) as compared to control mice (Figure 4A). To determine if the defects in ARH projections observed in ghrelin injected neonates were permanent, we also performed immunohistochemical labeling of AgRP and α-MSH in brain sections from adult mice that were neonatally injected with ghrelin or vehicle. Consistent with DiI labeling in neonates, the average density of AgRP-IR (Figure 4B) and α-MSH-IR (Figure 4C) fibers in the PVH was diminished in adult mice injected with ghrelin neonatally relative to that of control mice. However, α-MSH fibers were more markedly affected as compared to AgRP-IR fibers; mice injected with ghrelin neonatally had a reduction of 1.5 and 3 fold of AgRP- and α-MSH-IR fibers, respectively, as compared to control mice (Figure 4B-C). Consistent with previous studies indicating that that a reduced density of AgRP- and α-MSH-containing fibers is associated with of metabolic dysfunctions (6, 8, 13), adult animals neonatally injected with ghrelin also display metabolic disturbances (Figure 6C-F). These results indicate that chronic neonatal hyperghrelinemia causes structural alterations in ARH neural circuits and long-term metabolic defects. However, the effect of neonatal ghrelin on body weight regulation appears

sex specific because treatment with the anti-ghrelin or ghrelin in neonatal female mice and rats, respectively, does not result in changes in body weight [data not shown and (20)].

Neonatal hyperghrelinemia attenuates leptin-induced pSTAT3 in the ARH

To better understand how a premature hyperghrelinemia during neonatal life can impair hypothalamic development, we conducted a series of studies to investigate the interaction between ghrelin and leptin signaling. The rational for studying ghrelin-leptin interactions specifically is based on the observation that our ghrelin injection period coincides with a naturally occurring leptin surge that has been reported to promote ARH development (14). Because leptin's neurotrophic effects required intact ARH LepRb-pSTAT3 signaling (25), we first examined leptin-induced pSTAT3 in the ARH of neonates chronically injected with ghrelin. As expected, leptin treatment caused a marked increase in pSTAT3 staining in the ARH of control pups on P10 (Figure 5C). However, the same leptin treatment resulted in significantly fewer numbers of pSTAT3-IR cells in the ARH of P10 neonates chronically injected with ghrelin (Figure 5C). Ghrelin-injected neonates display a 2-fold reduction in pSTAT3-IR neurons following leptin administration as compared to control pups (Figure 5C). These results suggest that neonatal hyperghrelinemia impairs leptin signaling in ARH neurons during postnatal development in mice.

Ghrelin blunts the neurotrophic effect of leptin in vitro

Because neonates chronically injected with ghrelin exhibit impaired central leptin-induced pSTAT3 and because LepRb->pSTAT3 signaling is known to be critical for the regulation ARH neurite outgrowth (25), we assessed whether alteration of projection pathways from the ARH in ghrelin-injected neonates could also be due to reduced ability of leptin to promote ARH neurite extension during development. As reported previously (14), organotypic ARH explants exposed to leptin (100 ng/mL) exhibit elevated ARH axon growth as compared to control, as revealed by a higher density of β III tubulin-IR fibers extending from the edge of

ARH explants (Figure 5D). However, the neurotrophic effect of leptin on ARH axons was blunted when explants were also exposed to ghrelin (Figure 5D). Explants treated with both leptin and ghrelin had a reduction of 1.8-fold of axons extending from the edge of ARH explants as compared to explants treated with leptin alone (Figure 5D). These in vitro data show that ghrelin blunts the trophic effect of leptin on ARH neurons.

Neonatal ghrelin blockade promotes development of ARH projections in ob/ob *mice*

Previous studies showed that ARH projections are markedly reduced in leptin-deficient (*ob/ob*) mice. Because neonatal ghrelin blockade increases the density of ARH fibers in wild-type mice, we next evaluated the ability of the anti-ghrelin compound to promote ARH projections in *ob/ob* mice. Circulating acylated ghrelin levels were comparable between wild-type and *ob/ob* mice at P10 (Figure 5E) and no differences were found in the hypothalamic *GHSR* mRNA levels between wild-type and *ob/ob* pups (Figure 5F). Neonatal injections of the anti-ghrelin compound from P4 to P12 resulted in a 2.2- and 1.7-fold increase in the density of AgRP- and αMSH-IR fibers, respectively (Figure 5G-H).

Discussion

Ghrelin is one of the most potent orexigenic peptides identified so far. Both pharmacological and physiological evidence demonstrated that acute treatment of adult rats and mice with ghrelin stimulates food intake, increases body weight, and induces fat deposition (26-28). However, lifelong genetic deletion of ghrelin or its receptor results in normal growth and relatively unaltered food consumption, suggesting that lack of ghrelin during early life may cause compensatory developmental mechanisms (15-18). In the present study, we report that ghrelin exerts profound organizational effects on ARH neural projections during early postnatal life. We show that reduced ghrelin action results in enhanced densities of ARH neural projections during the pre-weaning period. In contrast, abnormally elevated levels of ghrelin permanently disrupt normal development of ARH neural projections.

Our data indicate that the ghrelin receptor GHSR-1a is expressed at relatively high levels in the ARH as early as P6. The functionality of neonatal GHSRs is demonstrated by the ability of ghrelin to induce intracellular signaling (as evidenced by the induction of pERK immunoreactivity). In adults, ghrelin acts primarily on NPY/AgRP neurons to regulate feeding (29, 30). Supporting these findings, the present study shows that, during neonatal life, ghrelin induces pERK immunoreactivity in the vast majority of neonatal NPY/AgRP neurons, whereas only a small subset of POMC neurons exhibits pERK immunoreactivity after ghrelin administration. Nevertheless, it is also known that ghrelin can also regulate the activity of POMC neurons indirectly, via trans-synaptic GABAergic inputs arising from NPY neurons (29, 31, 32). It is important to note in mind that, during early postnatal life, GABA is not an inhibitory transmitter but it provides an excitatory drive (33). Consistent with the idea of an indirect effect of ghrelin on neonatal POMC neurons, treatment of P10 mice with ghrelin induces cFos expression in more than 25% of POMC neurons, whereas less than 6% of POMC neurons express pERK immunoreactivity after ghrelin injections (data not shown).

This observation is particularly interesting because cFos labels neurons that are both directly and transsynaptically activated, whereas pERK is generally thought to be a marker of direct neuronal activation. These findings raise the possibility that the change in POMC axon growth following ghrelin and anti-ghrelin treatment may be the result of non-cell autonomous and/or post-synaptic mechanisms but also from cell-autonomous effects on a small portion of POMC neurons that express GHSR. Because the vast majority of NPY/AgRP neurons are known to express GHSR and respond to ghrelin directly, the regulation of axon growth in these neurons is likely to be cell autonomous. It also remains to be investigated the exact nature of the effects of ghrelin on POMC and NPY neuronal activity during neonatal life. In adults, ghrelin depolarizes (activates) NPY/AgRP neurons whereas it hyperpolarizes (inhibits) POMC neurons. However, based on our observations that NPY/AgRP and POMC projections are decreased in ghrelin-treated animals, it is likely that ghrelin exerts inhibitory effects on POMC but also NPY/AgRP neurons during early postnatal life. Consistent with the idea that metabolic hormones can exert different neurophysiological effects in neonates versus adults, a recent study from Baquero and colleagues reported that during the pre-weaning period leptin exerts stimulatory electrophysiological effects on NPY/AgRP neurons although it inhibits those neurons at weaning and in adult (34). Notably, we found that ghrelin influences development of projections to both the neuroendocrine and pre-autonomic compartments of the PVH. The developmental action of ghrelin differs from that of leptin and insulin, which selectively promote formation of ARH projections to the pre-autonomic sub-division of the PVH (35, 36). However, similar to leptin, the developmental action of ghrelin appears primarily restricted to the ARH and do not affect projections originating from the DMH [the present study and (14)]. It also remains possible that neonatal ghrelin could influence the development of non-feeding circuits. Supporting this hypothesis, neonatal ghrelin injections perturb sexual maturation (20), suggesting that ghrelin can also influence the development of neuronal systems involved in reproductive function.

The precise mechanisms responsible for the disruption of ARH projections caused ghrelin remain to be fully determined but likely involve an interaction with the well-documented trophic effect of leptin. In our animal model of chronic hyperghrelinemia, manipulation of ghrelin levels occurs when circulating ghrelin are physiologically low, *i.e.* from P4 to P12. Notably, this developmental window also coincides with a naturally occurring surge in circulating leptin that promotes formation of ARH projection pathways (14, 37). Whether there is a direct interaction between leptin and ghrelin signaling remains controversial. For example, although ARH neurons co-express GHSR-1a and leptin receptors (38), GHSR knockout mice display unaltered leptin sensitivity (38). However, adult transgenic mice overexpressing ghrelin are resistant to the anorexigenic effects of leptin (39). Our data also support the hypothesis that ghrelin signaling can affect leptin sensitivity during neonatal life. Chronic hyperghrelinemia impairs the ability of neonatal leptin to induce phosphorylation of STAT3 in the ARH. Importantly, LepRb→pSTAT3 signaling is essential to mediate the neurotrophic effects of leptin on ARH neural projections (25). Our in vitro experiments further showed that ghrelin blunts the documented neurotrophic effect of leptin on ARH neurons. Remarkably, neonatal injection of anti-ghrelin also increases the density of POMC and AgRP projections in *ob/ob* mice, which display abnormal development of ARH projections (40). In contrast, neonatal leptin treatment in *ob/ob* mice only increases the density of AgRP fibers and has no effect on POMC-derived projections (35). This discrepancy suggests that the ability of ghrelin to modulate ARH axonal outgrowth is not exclusively dependent on leptin action.

The ultimate architecture of hypothalamic pathways involved in appetite regulation requires the precise temporal action of specific sets of hormones. A neonatal leptin surge (37) is followed by gradual increases in ghrelin levels [the present study and (20)], and these hormones appear to initiate and terminate the development of ARH neural projections, respectively. Previous studies reported that a premature, delayed or abolished leptin surge causes disruption of ARH projections (41-43). In agreement with these findings, we found

that advanced or blunted/delayed ghrelin action also alters ARH axon growth. These findings highlight the importance of the correct timing of leptin and ghrelin actions to promote normal hypothalamic development. Similar to leptin, the developmental activity of ghrelin appears to be specific for ARH projections and is restricted to a neonatal window of maximum sensitivity that corresponds to a period when the ARH projections are established. Blockade of ghrelin during early postnatal life cause marked structural alterations; however, the treatment of adult mice with ghrelin does not affect the densities of ARH axons. Surprisingly, although ghrelin knockout neonates displayed a marked increase in the density of ARH projections, the density of both AgRP- and α-MSH-containing fibers become normal in adult ghrelin knockout mice. Our anatomical observations indicate that changes in ARH fibers projections in ghrelin knockout mice occur between P21 and P35. The precise biological substrates of these compensatory mechanisms remain to be investigated. Nevertheless, these data indicate that ARH peptidergic pathways continue to remodel and change not just early in development but even during the post-weaning period in response to environmental influences as well as genetically programmed events. These data are also consistent with the "mismatch" hypothesis that suggests that when the pre- and post-weaning environment is identical the phenotype is normal, whereas when there are changes between the environment during critical developmental periods and the adult life, phenotypic aberrations develop (44). Our findings in adult animals are also in agreement with the absence of metabolic phenotype in ghrelin knockout mice.

Our results also indicate that not only the correct timing, but also the correct amplitude of ghrelin is important for normal development of hypothalamic feeding pathways. Both a surfeit and paucity of ghrelin action during early life causes alterations in hypothalamic development and long-term metabolic perturbations. A surprising finding was that abnormally high and low densities in ARH projections caused by the ghrelin and anti-ghrelin treatment, respectively, are associated with to the same metabolic phenotype, *i.e.* elevated body weight and hyperglycemia. Also, leptin-deficient animals display an altered development of ARH

projections that are associated with obesity and hyperphagia (14). These findings are consistent with other neuroanatomical observations showing that both a reduction and an increase in neurogenesis and hypothalamic cell numbers are associated with obesity (45-48). These data indicate that appropriate patterns of hypothalamic connectivity must be established during development to accomplish an optimal control of energy metabolism and that both an excess and an insufficiency in hypothalamic feeding connections might affect the functionality of those circuits. The exact site of action of neonatal ghrelin to influence lifelong metabolic function remains to be determined but likely involves a direct action at the level of the ARH. Consistent with the idea that intact hypothalamic projections are required for normal metabolic regulation, disruption of hypothalamic neural projections secondary to deletion of insulin signaling in POMC neurons is associated with impairment of glucose homeostasis (36). In addition, perturbations in the development of ARH projections are a common feature of metabolic malprogramming (43, 49) (42, 50). Therefore, although future studies are needed to investigate the relative contribution of ARH neurons in the programming effects of ghrelin, it is it is likely that defective ARH projections contribute to the ultimate phenotype of mice treated with the anti-ghrelin and ghrelin. Nevertheless, it also remains possible that neonatal ghrelin acts on peripheral organs that are known to express *GHSR* mRNA during development, including the stomach, intestine and pituitary gland (51).

In conclusion, our study defines a crucial organizational role for neonatal ghrelin during development and provides evidence for an interaction between multiple hormonal signals (leptin, ghrelin) to shape the ultimate architecture of hypothalamic feeding circuits. Our data also underline the importance of the correct magnitude and timing in ghrelin's action in influencing the development of ARH projection. These findings highlight the importance of timing for the design of optimal interventional studies to ameliorate diseases, such as metabolic syndrome. The physiopathological relevance of the present findings is supported by several observations. First, hyperghrelinemia is the hallmark of patients suffering from Prader-Willy syndrome (PWS). Remarkably, PWS patients exhibit a premature

hyperghrelinemia that occurs before development of obesity and hyperphagia (52). Second, neonates exposed to undernutrition during the intrauterine and/or postnatal life display a marked increase in circulating ghrelin levels that are associated with higher risks to develop obesity and hyperphagia in later life (53). A better understanding of the relationship between neonatal ghrelin action and development of perinatally-acquired metabolic diseases will be crucial as we seek to develop interventional studies to ameliorate and hopefully reverse this metabolic malprogramming.

Methods

Animals

C57BL/6 mice were housed in individual cages under specific pathogen-free conditions, maintained in a temperature-controlled room with a 12 h light/dark cycle, and provided *ad libitum* access to water and standard laboratory chow (Special Diet Services). Homozygous transgenic mice that selectively express the enhanced green fluorescent protein (EGFP) in POMC-containing neurons and humanized renilla GFP (hrGFP) in NPY-containing neurons were kindly provided by Drs. M. Low (Oregon Health Science University) and B. Lowell (Harvard Medical School), respectively. For all experiments, the litter size was adjusted to 6 pups one day after birth to ensure adequate and standardized nutrition until weaning. Only male mice were studied.

Ghrelin knockout animals

Ghrl-/- mice (on a C57/B6 background) were obtained from Regeneron Pharmaceuticals (Tarrytown, New York) and bred in the Monash Animal Services facility. This genetic mouse line has been described previously (17).

Ghrelin assays

Male offspring of C57BL/6 mice were decapitated on P6 (n = 5), P10 (n = 5), P14 (n = 4), and P90 (Adult, n = 6) and trunk blood was collected in a chilled tube containing Pefabloc (AEBSF, Roche Diagnostics). Total and acylated ghrelin levels in the plasma were assayed using ELISA kits (Millipore). Acylated ghrelin levels were also characterized in mouse neonates injected with ghrelin. For this, P10 mice were injected with ghrelin (2 mg/kg) or vehicle (0.9% NaCl) and trunk blood was collected in a chilled tube containing Pefabloc (AEBSF, Roche Diagnostics) by decapitation 15 min, 1h, or 12 hr after injection (n = 4-6 per group).

For the fasting experiments, P6 and P14 mice were separated from their dams and placed on a heated pad for 4h before the experiment. Trunk blood was collected in a chilled

tube containing Pefabloc (AEBSF, Roche Diagnostics) and acylated ghrelin levels in the plasma were assayed using ELISA kits (Millipore).

Measurement of *GHSR* and *ghrelin* mRNA

ARH and DMH of P6, P10, P14, and P90 mice and (n = 4-5 per group; for *GHSR* analysis) and stomach of P14 mice (n = 7 per group; for *ghrelin* analysis) fed *ad libitum* were dissected. Hypothalami of P12 *ob/ob* and wild-type littermates (n = 6 per group) fed *ad libitum* were also dissected. Total RNA was isolated using the Arcturus PicoPure RNA isolation kit (Invitrogen). cDNA was generated with the high-capacity cDNA Reverse Transcription Kit (Applied Biosystem). Quantitative real-time PCR analysis was performed using TaqMan Fast universal PCR Mastermix. mRNA expression was calculated using the 2-ddCt method after normalization with GAPDH as a housekeeping gene. Inventoried TaqMan Gene expression assays *GHSR* (Mm00616415_m1), *ghrelin* (Mm00445450_m1), and *Gapdh* (Mm99999915_g1) were used. All assays were performed using an Applied Biosystems StepOnePlus real time PCR system.

pERK immunohistochemistry and analysis

On P6, P10, P14, or P90 wild-type mice were given an intraperitoneal injection of ghrelin (2 mg/kg, Phoenix Pharmaceuticals, n = 4-5 per group) or vehicle alone (0.9% NaCl, n = 4 per group) and were perfused 45 min later with a solution of 4% paraformaldehyde. POMC-EGFP, and NPY-hrGFP mice were also injected with ghrelin or vehicle alone on P10 (n = 4-5 per group). Frozen coronal sections were cut at 20 um and then processed for immunofluorescence. Briefly, sections were incubated for 48 h in a rabbit anti-pERK (1:1,000, Cell Signaling). The primary antibody was localized with Alexa Fluor 568 Goat anti-Rabbit IgGs (Invitrogen, 1:200). Sections were then counterstained using bis-benzamide (Invitrogen, 1:10,000) to visualize cell nuclei and coverslipped with buffered glycerol (pH 8.5).

Injections of the anti-ghrelin compound NOX-B11-2

Materials. Anti-ghrelin NOX-B11-2 and the non-functional control Spiegelmers were prepared by NOXXON Pharma AG, Berlin, as previously described (23).

Neonatal injections in wild-type mice. Offspring of C57BL/6 mice were used. Starting at P4, pups were treated daily with intraperitoneal injections of NOX-B11-2 (15 mg/kg) or an inactive control (23) for a total of 18 days (n = 8-10 per group). To ensure identical development of the pups within the same litter, all pups of the same dam received administration of similar substances. Each experimental group in all experiments consisted of offspring from at least three litters.

Adult injections in wild-type mice. P100 mice were treated daily with intraperitoneal injections of anti-ghrelin NOX-B11-2 (15 mg/kg) or an inactive control (23) for a total of 7 days (n = 3 per group).

Neonatal injections in ob/ob mice. Starting at P4, pups were treated daily with intraperitoneal injections of NOX-B11-2 (15 mg/kg) or an inactive control for a total of 8 days (n = 4 per group).

Physiological measurements

Mice (n = 8-10 per group) were weighed every two days from P4 to P22 (weaning) and weekly after weaning using an analytical balance (n = 8-10 per group). The naso-anal length was also measured at weaning. To measure food consumption, mice were housed individually in cages and, after one week of acclimation, food intake was measured every 24 h for 3 days from pre-weighed portions of food dispensed from the wire cage tops. The average daily food intake of each mouse (n = 6-8 per group) was used for statistical comparisons. Magnetic resonance imaging (MRI) scanning (micro-MRI Bruker-Pharmascan 7T) was performed on mice fed *ad libitum* to evaluate in vivo body fat (n=3-4 per group). Eighteen 0.75 mm-thick cross-sections covering the whole abdominal cavity were acquired.

Visceral, subcutaneous, and total fat was quantified using the ITKSnap 2.0.0 software (54). Fed glucose levels were assessed in adult mice (n = 6-8 per group) using a glucometer (One Touch Ultra, Johnson & Johnson). Glucose tolerance was performed in adult mice (n = 12 per group) by an i.p. administration of glucose (1.5 mg/g body weight) after overnight fasting, and then the blood glucose levels were measured 0, 15, 30, 45, 60, 90, 120, and 150 min following glucose challenge, as previously described (55).The leptin sensitivity test was performed in adult mice (n = 5 per group). Briefly, mice were injected i.p. at 6:30 pm with vehicle (5 mM sodium citrate buffer, pH 4.0) on day 1, followed by leptin on day 2 (1 mg/kg, PreproTech). Animals were weighed daily during the injection period. Serum leptin levels were assayed in the samples using a leptin ELISA kit (Millipore) (n = 6-8 per group).

Determination of stomach content in pups

The stomach of P14 mice fed *ad libitum* (n = 7-8 per group) was rapidly dissected and weighed. It was then cut open, emptied of its contents, and weighed again. The difference between the full and empty stomach weight was used as an estimate of milk intake.

Neonatal ghrelin injections

Offspring of C57BL/6 mice were injected daily with ghrelin (Phoenix Pharmaceutical, 2 mg/kg) from P4 to P12. Controls received equivolume injections of vehicle (0.9% NaCl).

DiI implants

Mice (n = 4–6 per group) were perfused with 4% paraformaldehyde. The brains were removed and numerically coded to insure unbiased processing and analysis. Crystals of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Santa Cruz) were implanted as previously described (14). Briefly, an insect pin was used to place a crystal of DiI (15 um in diameter) into the ARH of each brain under visual guidance. After incubation in the dark for 2 weeks at 37 C, hypothalamic sections were collected from each brain and evaluated by confocal microscopy as described below.

AgRP and α-MSH immunohistochemistry

Mice (n = 3-7 per group) were perfused transcardially with 4% paraformaldehyde. The brains were then frozen and sectioned at a 30-um thickness and processed for immunofluorescence using standard procedures (14). The primary antibodies used for IHC included rabbit anti-AgRP (1:4,000, Phoenix Pharmaceuticals) and sheep anti-α-MSH (1:40,000, Millipore). The primary antibodies were visualized with Alexa Fluor 488 goat anti-rabbit IgGs or Alexa Fluor 568 donkey anti-sheep IgGs (1:200, Invitrogen). Sections were counterstained using bis-benzamide (1:10,000, Invitrogen) to visualize cell nuclei and coverslipped with buffered glycerol (pH 8.5).

cFos immunohistochemistry

On P12 mice were injected intraperitoneally with ghrelin (Phoenix Pharmaceutical, 2 mg/kg) or vehicle alone (0.9% NaCl) and perfused 2 h, 6 h, 12 h, or 24 h later. Injections occured 12 h after the last injection of anti-ghrelin or control solution. P21 and P36 mice neonatally injected with control or anti-ghrelin compound were also injected intraperitoneally with ghrelin (Phoenix Pharmaceutical, 2 mg/kg) or vehicle alone (0.9% NaCl) and perfused 2h later. The brains were then frozen, sectioned at a 25-um thickness, and incubated in a rabbit primary antiserum directed against the N-terminal domain of Fos (Ab-5, Oncogene; 1:2,000). The primary antibody was localized with affinity-purified IgGs conjugated with Alexa 488 (Invitrogen; 1:200). Sections were counterstained using bis-benzamide (1:10,000) to visualize cell nuclei and coverslipped with buffered glycerol (pH 8.5).

pSTAT3 immunohistochemistry

Ghrelin (Phoenix Pharmaceutical, 2 mg/kg) was injected intraperitoneally in pups daily from P4 to P9. Controls received equivolume injections of vehicle (0.9% NaCl). On P10, mice received an injection of ghrelin (2 mg/kg, i.p.), followed two days later by leptin administration

(3 mg/kg, i.p.) (n = 4-6 per group). Animals were perfused 45 min later with a solution of 2% paraformaldehyde. Frozen coronal sections were cut at 25 um and pretreated for 20 min in 0.5% NaOH and 0.5%H2O2 in KPBS, followed by immersion in 0.3% glycine for 10 min. Sections were then placed in 0.03% SDS for 10 min and placed in 4% normal serum + 0.4% Triton X-100 + 1% BSA (fraction V) for 20 min before incubation for 48h with a rabbit anti-pSTAT3 antibody (1:1,000, Cell Signaling). The primary antibody was localized with Alexa Fluor 568 Goat anti-Rabbit IgGs (Invitrogen; 1:200). Sections were counterstained using bis-benzamide (Invitrogen; 1:10,000) to visualize cell nuclei, and coverslipped with buffered glycerol (pH 8.5).

Isolated ARH explant cultures

Brains were collected from P4 mice and sectioned at a 200-um thickness with a vibroslicer as previously described (14). The ARH was then carefully dissected out of each section under a stereomicroscope. Explants (n = 6-19 cultures per group) were cultured onto a rat tail collagen matrix (BD Bioscience). Beginning on the first day in vitro, each explant was transferred to fresh modified Basal Medium Eagle (Invitrogen) containing either ghrelin (100 ng/ml, Phoenix Pharmaceuticals), leptin (100 ng/ml, Peprotech), leptin + ghrelin (100 ng/ml each), or vehicle alone. To further validate the blocking effects of the anti-ghrelin compound on ghrelin's actions, ARH explants were also treated with ghrelin and NOX-B11-2 (100 ng/ml), or ghrelin and an inactive control (100 ng/ml). After 48 h, the explants were fixed in paraformaldehyde and neurites extending from the explants were stained with β III tubulin (rabbit, 1:5,000, Covance) as described previously (56).

Quantitative analysis of cell numbers and fiber density

For the histological experiments, two sections through the ARH (for pERK, and pSTAT3 staining) and the PVH, DMH, and LHA (for α-MSH and AgRP staining and DiI labeling) from animals of each experimental group (n = 3-7 animals per group) were acquired using a Zeiss LSM 710 confocal system equipped with a 20X objective. For the in vitro experiments,

sections through 5 different regions of interest (100 x100 um) spaced at 100, 200, 300, 400, and 500 um extending radially from the edge of the ARH explants (n = 6-19 per group) were acquired using a Zeiss LSM 710 confocal system equipped with a 10X objective. Slides were numerically coded to obscure the treatment group. Image analysis was performed using ImageJ analysis software (NIH).

For the quantitative analysis of fiber density (for α-MSH, AgRP, and DiI), each image plane was binarized to isolate labeled fibers from the background and to compensate for differences in fluorescence intensity. The integrated intensity, which reflects the total number of pixels in the binarized image, was then calculated for each image. This procedure was conducted for each image plane in the stack, and the values for all of the image planes in a stack were summed. The resulting value is an accurate index of the density of the processes in the volume sampled (14).

For the quantitative analysis of cell number, the numbers of pERK-, pSTAT3-, POMC-, NPY-, POMC+pERK-, and NPY+pERK-labeled cells in the ARH were manually counted using Image J analysis software (NIH). Only GFP-positive cells that had a corresponding bis-benzamide-stained nucleus were included in our counts. The average number of cells counted in two ARH hemisections from each mouse was used for statistical comparisons.

Statistical analysis

All values were expressed as the means ± SEM. Statistical analyses were conducted using GraphPad PRISM (version 5.0a). Statistical significance was determined using unpaired two-tailed Student's t-tests and a two-way ANOVA followed by the Bonferroni post-hoc test when appropriate. $P < 0.05$ was considered to be statistically significant.

Study approval

Animal usage was in compliance with and approved by the Institutional Animal Care and Use Committee of the Saban Research Institute of the Children's Hospital of Los Angeles.

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Figures

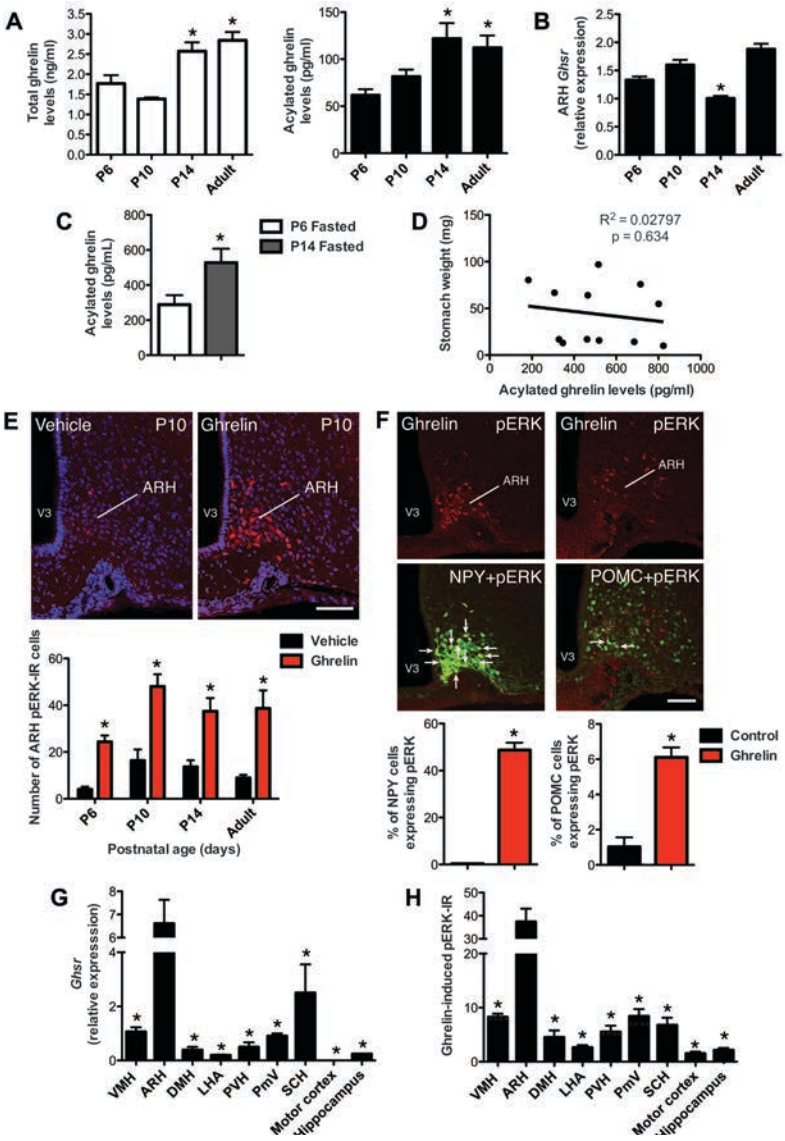

Figure 1. Ghrelin signaling in neonatal ARH neurons. (A) Total plasma ghrelin levels and acylated ghrelin levels of P6, P10, P14 and adult mice (n = 6 for P10; n = 5 for P6 and P14; n = 4 for adult). (B) Relative expression of *GHSR* mRNA in the ARH of P6, P10, P14, and adult mice (n = 6 for P10; n = 5 for P6 and P14; n = 4 for adult). (C) Circulating acylated ghrelin levels of P6 and P14 mice after a 4h fasting (n = 6 per group). (D) Correlation between stomach weight and circulating acylated ghrelin levels in P14 mice (n = 12 per group). (E) Confocal images and quantitative comparisons of pERK+ cells after administration of ghrelin or vehicle alone in P6, P10, P14, and adult mice (n = 5 for P10 and P14; n = 4 for P6 and adult). (F) Confocal images and quantitative comparisons of pERK+ cells after administration of ghrelin or vehicle alone in NPY- and POMC-GFP pups on P10 (n = 4 for vehicle; n = 5 for ghrelin). Arrows point to double-labeled cells. (G) Relative expression of *GHSR* mRNA in the brain of P14 mice (n = 4 per group). (H) Quantitative comparisons of pERK+ cells in the brain of P14 mice after administration of ghrelin (n = 4). ARH, arcuate nucleus; VMH, ventromedial nucleus; LHA, lateral hypothalamic area; PmV, ventral premammillary nucleus; SCH, suprachiasmatic nucleus. V3, third ventricle. Values are shown as the mean ± SEM. (A) **P* < 0.05 versus P6 and P10; (B) **P* < 0.05 versus P6, P10, and adult; (C), **P* < 0.05 versus P6 fasted. (E-F), **P* < 0.05 versus vehicle; (G-H), **P* < 0.05 versus ARH. Scale bars, 120 um.

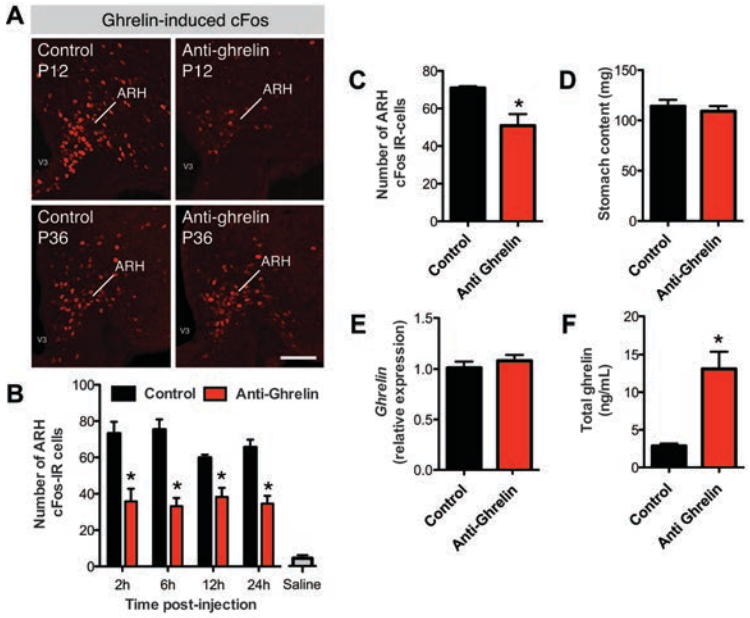

Figure 2. Effects of the anti-ghrelin compound. (A) Representative images of ghrelin-induced cFos immunoreactivity (marker of cellular activation) from P12 and P36 mice neonatally injected with control or anti-ghrelin. (B) Quantitative comparisons of ghrelin-induced-cFos immunoreactivity in the arcuate nucleus (ARH) of P12 mice 2 h, 6 h, 12 h, and 24 h after intraperitoneal administration of control or anti-ghrelin (n = 3 for control; n = 4 for saline and anti-ghrelin). Grey bar shows number of cFos-IR cells in saline-treated animals. (C) Number of cFos-immunoreactive cells of P21 mice neonatally injected with control or anti-ghrelin 2 h after intraperitoneal administration of ghrelin (2 mg/kg) (n = 3 per group). (D) Stomach content of P14 pups injected with the control of anti-ghrelin compound (n = 7 for control; n = 8 for anti-ghrelin). (E) Relative expression of *Ghrelin* mRNA in the stomach of P14 pups injected with control of anti-ghrelin (n= 7 per group). (F) Total plasma ghrelin levels of P14 pups injected with control or anti-ghrelin (n= 7 per group). Values are shown as the mean ± SEM. **P* < 0.05 versus control and ghrelin + NOX-B-11-2. Scale bar, 120 um.

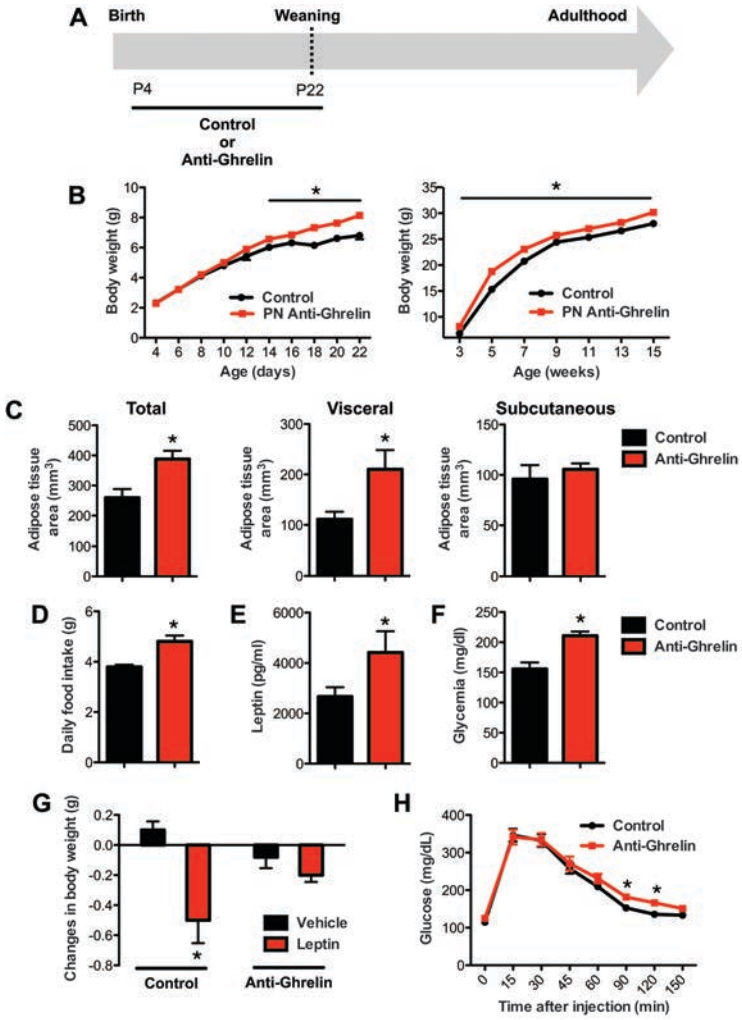

Figure 3. Neonatal ghrelin blockade causes metabolic disturbances. (A) Schematic representation of the experimental design used to specifically block ghrelin action during neonatal life. Starting at P4, pups were treated daily with intraperitoneal injections of the anti-ghrelin compound NOX-B11-2 (15 mg/kg) or an inactive control, for a total of 18 days. (B) Pre- and post-weaning growth curves (body weights) of mice neonatally injected with control or anti-ghrelin compound (n = 8 for control; n = 10 for anti-ghrelin). (C) Body adiposity assessed by MRI at 120 days of age in animals neonatally injected with control or anti-ghrelin (n = 3 for control; n = 4 for anti-ghrelin). (D) The daily food intake of P90 mice neonatally injected with control or anti-ghrelin (n = 6 for control; n = 8 for anti-ghrelin). (E) Plasma leptin and (F) blood glucose levels at 70 days of age in mice neonatally injected with control or anti-ghrelin (n = 6 for control; n = 8 fr anti-ghrelin). (G) Leptin sensitivity at 100 days of age in mice neonatally injected with control or anti-ghrelin (n = 5 per group). (H) Glucose tolerance test (GTT) of P80-P100 mice neonatally injected with control or anti-ghrelin (n = 12 per group). Values are shown as the mean ± SEM. *P < 0.05 versus control or vehicle.

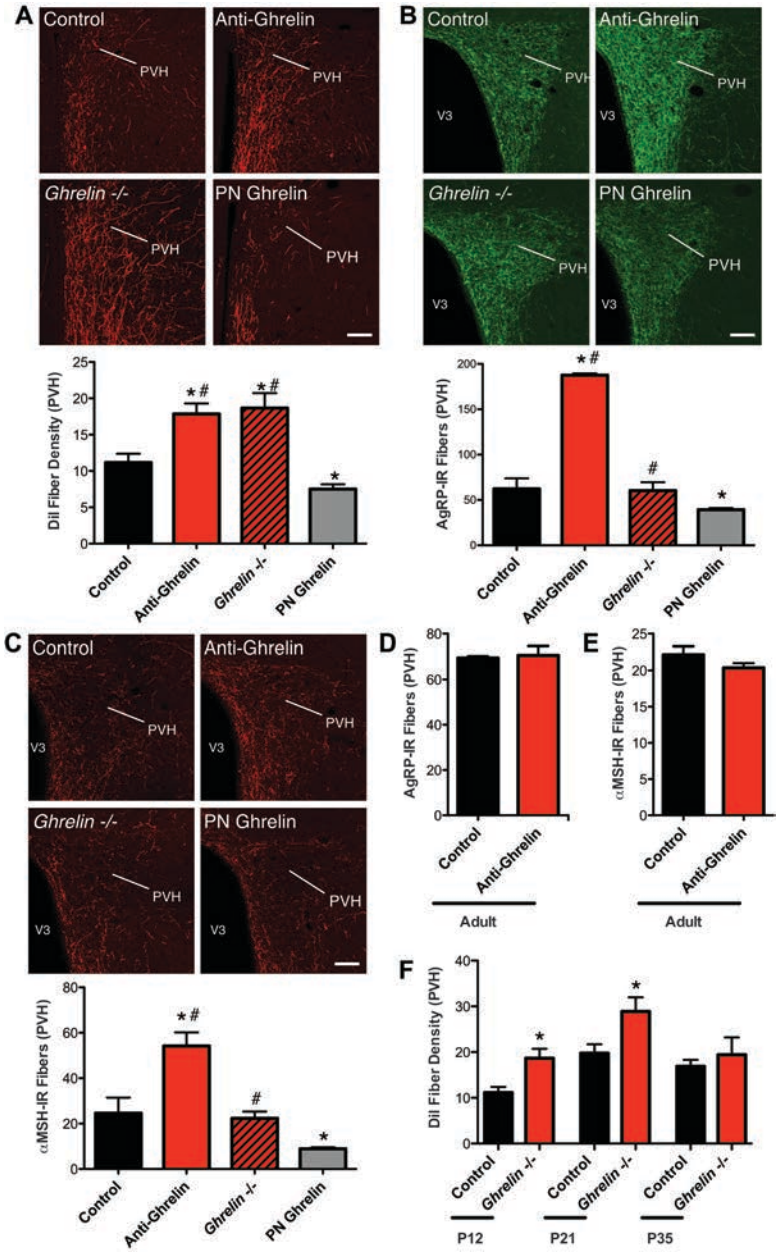

Figure 4. Neonatal ghrelin influences the normal developmental pattern of ARH neural projections. (A) Confocal images and quantification of the density of arcuate DiI-labeled fibers innervating the paraventricular nucleus (PVH) in P12 mouse pups injected with the control, anti-ghrelin compound, or ghrelin, and ghrelin knockout (*Ghrelin -/-*) pups (n = 5 for control; n = 4 for ghrelin -/-; n = 6 for anti-ghrelin and PN ghrelin). Confocal images and quantification of (B) AgRP-IR fibers and (C) α-MSH-IR fibers at 100-120 days of age in the PVH of mice neonatally injected with the control or anti-ghrelin compound, mice neonatally injected with ghrelin, and *Ghrelin -/-* mice (n = 6 for control, ghrelin -/-, and anti-ghrelin; n = 7 PN ghrelin). Quantification of AgRP- (D) and α-MSH-IR (E) fibers at 100-120 days of age in the PVH of mice injected with control or anti-ghrelin during adult life (n = 3 per group). (F) Quantification of the density of arcuate DiI-labeled fibers innervating the paraventricular nucleus (PVH) in P12, P21, and P35 control and ghrelin knockout (*Ghrelin -/-*) mice (n = 4 for P12; n = 6 for P21 and P35). V3, third ventricle. Values are shown as the mean ± SEM. **P* < 0.05 versus control; $^{\#}P$ < 0.05 versus PN ghrelin. Scale bars, 150 um.

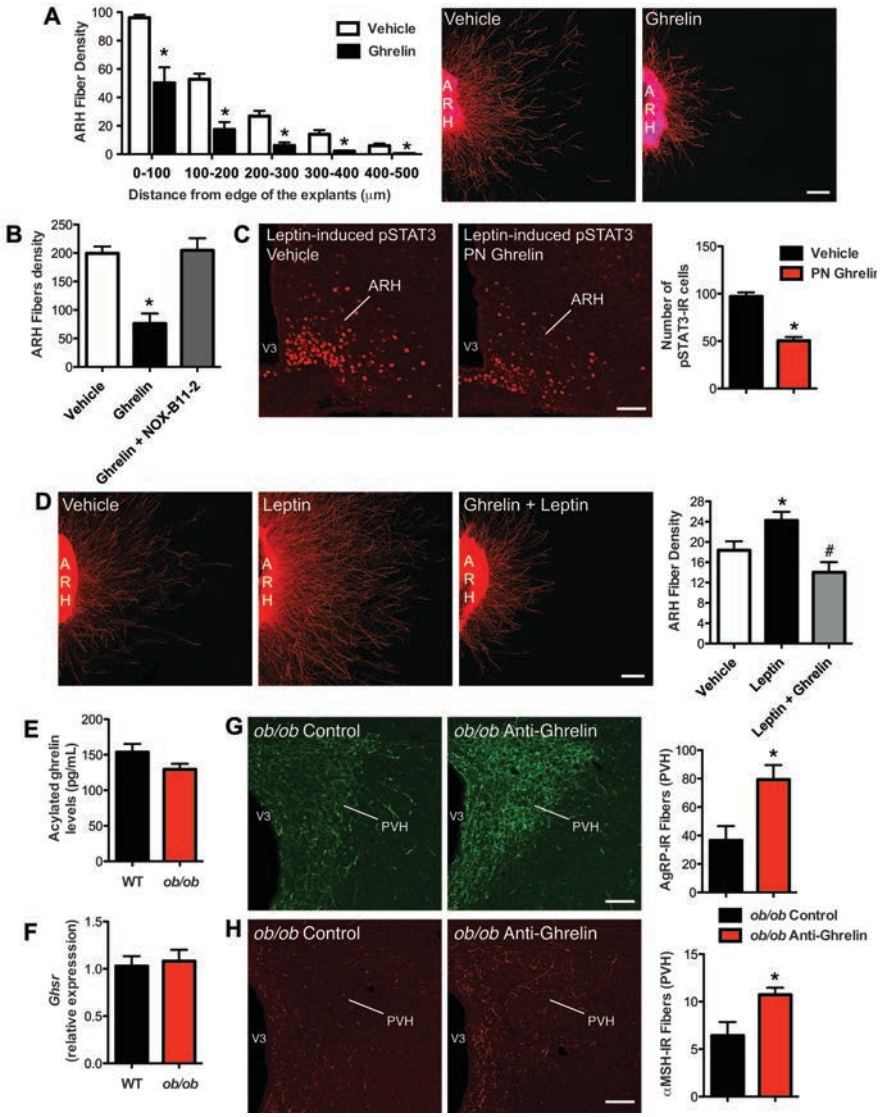

Figure 5. Ghrelin blocks axonal growth from neonatal ARH neurons. (A) Quantification and photomicrographs of β III tubulin-immunopositive fibers, a marker of neurites, from isolated organotypic cultures of neonatal ARH incubated for 48 h with vehicle or ghrelin (100 ng/ml) (n = 6 cases per group). (B) Quantification of β III tubulin-immunopositive fibers from isolated cultures of neonatal ARH incubated for 48 h with vehicle, ghrelin, or ghrelin and NOX-B11-2 (n = 8 for ghrelin, and ghrelin + NOX-B-11-2; n = 11 for vehicle). (C) Photomicrographs and quantification of the number of leptin-induced pSTAT3 immunoreactive cells in the arcuate nucleus (ARH) of P10 pups injected with ghrelin (2 mg/kg) or vehicle from P4 to P10 (n = 4 for vehicle; n = 6 for PN ghrelin). (D) Images and quantification of the overall density of β III tubulin-immunopositive fibers from isolated organotypic cultures of neonatal ARH incubated for 48 h with vehicle, leptin (100 ng/ml), or leptin + ghrelin (n = 9 for leptin; n = 15 for leptin + ghrelin; n = 19 for vehicle). (E) Circulating acylated ghrelin level of P10 wild-type (WT) and leptin deficient (*ob/ob*) mice (n = 9 for WT; n = 12 for *ob/ob*). (F) Relative expression of *GHSR* mRNA in the hypothalamus of P12 WT and *ob/ob* mice (n = 6 per group). Confocal images and quantification of (G) AgRP-IR fibers and (H) α-MSH-IR fibers in the paraventricular nucleus (PVH) of P12 *ob/ob* mice neonatally injected with the control or anti-ghrelin compound (n = 4 per group). V3, third ventricle. Values are shown as the mean ± SEM. **P* < 0.05 versus vehicle; #*P* < 0.05 versus leptin. Scale bars, 100 um (A and D); 150 um (C, G, and H)

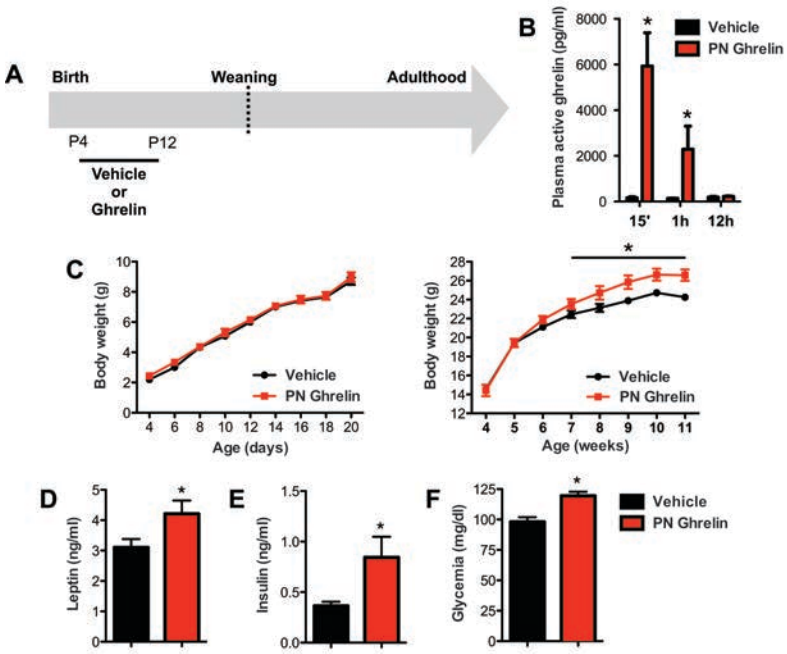

Figure 6. Chronic neonatal hyperghrelinemia causes metabolic disturbances. (A) Schematic representation of the experimental design used to increase ghrelin levels during neonatal life. Starting at P4, pups were treated daily with intraperitoneal injections of ghrelin (2 mg/kg) (post-natal ghrelin; PN ghrelin) or vehicle (control), for a total of 8 days. (B) Acylated ghrelin levels in P10 mice injected with vehicle (0.9% NaCl) or ghrelin (2 mg/kg) (n = 4 for vehicle; n = 6 for PN ghrelin). Values are shown as the mean ± SEM. **P* < 0.05 versus control. (C) Pre- and post-weaning growth curves (body weights) of mice neonatally injected with control or ghrelin (n = 5 for vehicle; n = 7 for PN ghrelin). (D) Plasma leptin and (E) insulin levels at 90 days of age in mice neonatally injected with control or ghrelin (n = 4 for control; n = 5 for PN ghrelin). (F) Fasting glucose levels in P80 mice neonatally injected with control or ghrelin (n =5 for control; n = 7 for PN ghrelin). Values are shown as the mean ± SEM. **P* < 0.05 versus vehicle.

Supplemental Data

Neonatal Ghrelin Programs Development of Hypothalamic Feeding Circuits

Sophie M. Steculorum, Gustav Collden, Berengere Coupe, Sophie Croizier, Sarah Lockie, Zane B Andrew, Florian Jarosch, Sven Klussmann, Sebastien G. Bouret

Supplemental Figure 1

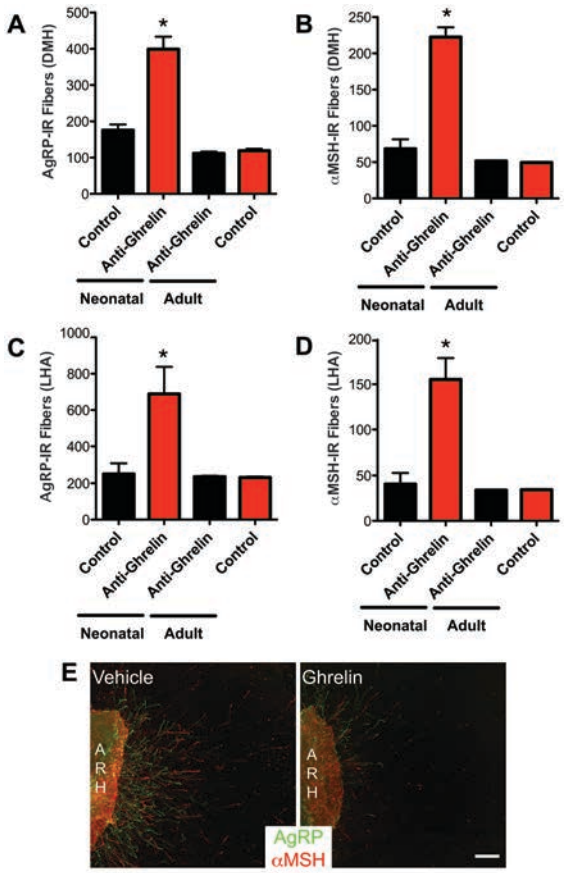

Figure S1. Neonatal ghrelin influences the normal developmental pattern of arcuate projections. Quantification of AgRP- (A, C) and α-MSH-IR (B, D) fibers at 100-120 days of age in the DMH (A-B), LHA (C-D) of mice injected with control or anti-ghrelin during neonatal (n = 6 for control; n = 7 for anti-ghrelin) or adult (n = 3 per group) life. (E) Photomicrographs of AgRP- (green fluorescence) and α-MSH-IR (red fluorescence) fibers from isolated organotypic cultures of neonatal ARH incubated for 48 h with vehicle or ghrelin (100 ng/ml). ARH, arcuate nucleus, DMH, dorsomedial nucleus, LHA, lateral hypothalamic area. Values are shown as the mean ± SEM. *P < 0.05 versus control. Scale bar, 100 um

Supplemental Figure 2

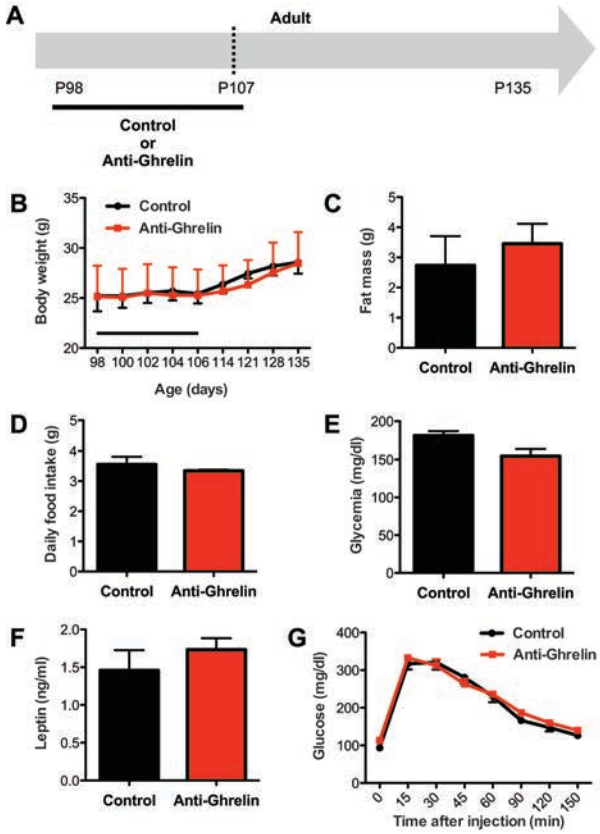

Figure S2. Adult ghrelin blockade does not cause marked metabolic alterations. (A) Schematic representation of the experimental design used to block ghrelin action during adult life. Starting at P98, mice were treated daily with intraperitoneal injections of the anti-ghrelin compound NOX-B11-2 (15 mg/kg) or an inactive control, for a total of 10 days. (B) Body weights of mice injected with control or anti-ghrelin compound (n = 5 per group); the black bar represents the injection period. (C) Fat mass of P135 animals injected with control or anti-ghrelin (n = 5 per group). (D) The daily food intake in adult mice injected with control or anti-ghrelin (n = 5 per group). (E) Blood glucose levels of P105 mice injected with control or anti-ghrelin (n = 5 per group). (F) Plasma leptin levels at 105 days of age in adult mice injected with control or anti-ghrelin (n = 5 per group). (G) Glucose tolerance test (GTT) of P108 mice injected with control or anti-ghrelin (n = 5 per group). Values are shown as the mean ± SEM.

Supplemental Figure 3

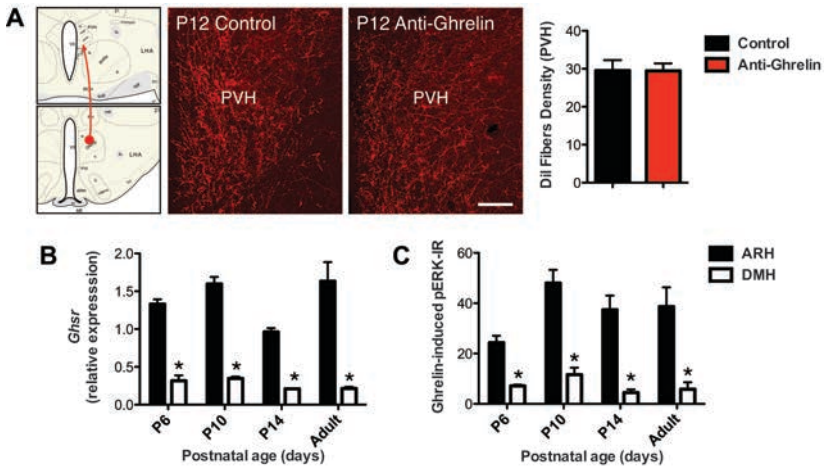

Figure S3. Normal development of DMH neural projections in mice neonatally treated with anti-ghrelin. (A) DiI crystals were implanted into the dorsomedial nucleus (DMH) of P12 mice treated with control or anti-ghrelin (n = 4 for control; n = 6 for anti-ghrelin) and neural projections to the paraventricular nucleus (PVH) were examined. The quantification revealed no difference in the density of DMH DiI-labeled fibers innervating the PVH between control-injected and anti-ghrelin injected neonates. (B) Relative expression of *GHSR* mRNA in the arcuate nucleus (ARH) and DMH of P6, P10, P14, and adult (P90) mice (n = 6 for P10; n = 5 for P6 and P14; n = 4 for adult). (C) Quantitative comparisons of pERK-immunoreactive cells in the ARH and DMH 45 min after intraperitoneal administration of ghrelin (2 mg/kg) in P6, P10, P14, and adult (P90) mice (n = 5 for P10 and P14; n = 4 for P6 and adult). *$P < 0.05$ versus ARH. Scale bar, 150 um. Schematic illustrations are based on Brain Maps: Structure of the Rat Brain (Swanson, 1998).

Resumé en francais

Resumé

Il est désormais clairement établi que la vélocité de croissance dans les premiers mois de la vie influence le risque d'apparition de maladies métaboliques, telles que l'obésité à l'âge adulte. L'un des mécanismes sous-jacents à cette programmation néonatale pourrait mettre en jeu des perturbations de l'environnement hormonal périnatal pendant des périodes critiques du développement. Cette hypothèse repose sur des travaux réalisés au laboratoire montrant que pendant le développement postnatal, des hormones telles que la ghréline et la leptine peuvent influencer le développement des circuits neuronaux impliqués dans la régulation de la prise alimentaire à l'âge adulte.

Dans ce contexte, l'objectif général de ce travail de thèse a été de définir les médiateurs hormonaux par lesquels l'environnement nutritionnel périnatal peut influencer le développement de maladies métaboliques à l'âge adulte. Une attention particulière a été portée à la contribution de la ghréline et de la leptine.

Pour induire une surnutrition postnatale, nous avons utilisé un modèle murin de surnutrition postnatale induit par la réduction de la taille de portées. Au troisième jour de vie post-natale, les portées ont été réduites à 3 petits (SL) pour induire une suralimentation et les portées contrôles ont conservé 7 petits. Comparées aux souris NL, les souris SL présentent un gain de poids rapide pendant la lactation, et présentent un surpoids à l'âge adulte même sous régime de nourriture standard. A l'âge adulte, les souris SL ont une adiposité et une glycémie à jeun plus élevée. Sous régime « obésogène » les souris SL présentent également une prise de poids et de graisse plus importante que les souris NL.

La première partie de ces travaux a été dédiée à l'étude de la nutrition périnatale sur le développement du système ghrélinergique. Les souris SL présentent des taux réduits de ghréline total et active pendant la troisième semaine de vie post-natale. Ces diminutions des taux de ghréline sont associées à une diminution de l'expression de ghreline dans l'estomac. La normalisation de la ghrélinémie ne parvient pas à restaurer un phénotype métabolique normale chez les souris SL ce qui suggère que les souriceaux SL présenteraient une résistance a la ghréline. En accord avec cette hypothèse, les

souriceaux SL présente une atténuation de la réponse centrale suite à l'injection périphérique de ghréline. Les mécanismes sous-jacents à cette résistance à la ghréline semblent mettre en jeu un défaut de transport de l'hormone via les tanycytes de l'éminence médiane.

La seconde partie de mon travail de thèse a consisté à étudier l'importance de la leptine dans la programmation nutritionnelle. Etant connu que les souris SL présent des taux anormalement élevés de leptine pendant la deuxième semaine de vie postnatale, nous avons émis l'hypothèse que le blocage partiel de la leptine chez les souris SL pourrait avoir des effets bénéfiques sur le métabolisme de ces souris. Les animaux injectés néonatalement avec l'antagoniste de la leptine ne présentent pas de différences de poids par rapport aux animaux contrôles. En revanche, l'injection de l'antagoniste de la leptine chez les souriceaux SL induit une amélioration de leur masse grasse et une normalisation de leur glycémie. Cette amélioration du phénotype métabolique des souris SL est associée à un rétablissement de la sensibilité centrale à l'hormone leptine.

Ainsi, les travaux réalisés au cours de ce doctorat confortent l'importance de l'environnement hormonal périnatal, et en particulier de la ghéline et de la leptine, dans la programmation nutritionnelle.

Introduction

Le modèle de petite portée (SL) est une variété de la technique de modification de la taille des portées qui a également été utilisée pour étudier la dénutrition. En bref, il consiste à réduire le nombre de souriceaux dans une portée nouveau-né à augmenter l'accès des petits restants au lait maternel. La procédure typique est d'abord normaliser le nombre de petits par portée à la taille moyenne de la portée de cette espèce / souche (généralement 7-10 petits) un ou deux jours après la naissance, puis sur le troisième ou quatrième jour après la naissance sacrifier un certain nombre de souriceaux pour porter la taille de la portée vers le bas (en général 3-4 soriceaux en fonction du protocole).

Comme les petits rongeurs ne d'auto-régulent pas leur consommation de lait, leur consommation peut être considérablement modifiée en fonction principalement sur le

taux de production de lait par la mère et la concurrence des autres soriceaux dans la portée. Ce sont des variables interdépendantes cependant; la production de lait maternel est fonction de la dite impulsion de lait - le produit du nombre de petits dans la portée ou de la masse totale de la portée, et la durée de l'allaitement (Russell, 1980), de telle sorte que la mère va augmenter ou diminuer sa consommation de nourriture et la production de lait à la suite de l'adapter à la taille de la portée. Dans les cas extrêmes, cela peut conduire à une baisse sévère de la production de lait si la portée est trop petite, ce qui conduit à un retard de croissance (Russell, 1980), ce qui explique pourquoi, aux fins de ce modèle, la taille de la portée minimum est généralement de 3.

Jamais le moins, dans la gamme de plus normal entre 3 à 20 petits / portée, la production totale de lait varie moins avec la taille de la portée de telle sorte que chez les souris qui avaient la taille de leur portée manipulé à la naissance pour créer portées comprises entre 3 à 18 soriceaux, il avait une relation inverse presque parfaite entre la taille de la portée et du poids des petits au sevrage (Johnson et al., 2001), alors que dans un modèle de rat le lait disponible par soriceau a presque doublé lorsque la taille de la portée a été manipulé de 12 jusqu'à 2 (Kumaresan et Anderson, 1967). Pour une configuration comme dans le présent projet, par exemple, avec une taille de la portée de la souris normale fixée à 7 soriceaux / portée et la taille de la portée réduite à 3 soriceaux / portée, les soriceaux dans la portée réduite pourrait s'attendre à gagner environ 30-40% plus de poids que les soriceaux de contrôle entre le jour postnatal 3 et le sevrage à 22 constitue le modèle de petite portée ainsi un modèle simple et fiable pour étudier les effets de la prise de poids accélérée au cours de la période de lactation.

Mis à part les effets désormais bien établis de de l'elevage en petits portées (SL) sur l'adiposité, la pression artérielle, l'homéostasie du glucose et le métabolisme des lipides (voir Habbout et al. 2013 pour l'examen), il a été associé à de nombreux autres changements pathologiques. Souris SL afficher des marqueurs de stress oxydatif infarctus augmenté, montrent des signes de développement rénal altéré et adultes insuffisance rénale (Habbout et al, 2012, 2013b). (Boubred et al, 2007, 2009;.. Yim et al, 2013), ainsi hypertrophie cardiaque plus tôt, cardiaque fibrose et dysfonctionnement microvasculaire d'apparition tardive (Velkoska et al, 2008b;.. Moreira et al, 2009;. Leite et al, 2012) et par conséquent sont plus vulnérables aux blessures de perfusion de

l'ischémie myocardique (Habbout et al. , 2012). Comme les adultes, ils présentent plusieurs signes d'altérations de la régulation des processus inflammatoires, telles que l'augmentation de l'hyperréactivité et l'inflammation du poumon (Ye et al., 2012) ainsi que d'une fébrile exacerbé et la réponse inflammatoire à l'endotoxine bactérienne (Clarke et al., 2012). Ils sont plus sensibles à la maladie du foie gras non-alcoolique (Ji et al., 2014) et riche en graisses obésité induite par l'alimentation (Glavas et al., 2010a).

Associée à ces effets physiopathologiques sont nombreuses modifications endocriniennes et neuroendocriniennes. Plusieurs études ont noté des glucocorticoïdes modifié la signalisation avec les niveaux de corticostérone et l'aggravation des tissus adipeux expression du récepteur des glucocorticoïdes dans les deux en développement et des rats adultes de SL (Boullu-Ciocca et al, 2005;. Hou et al., 2011).

Signalisation de la thyroïde a également été rapportée comme étant augmenté dans le développement de rats SL, avec des taux circulants élevés de TSH, T4 et l'hormone T3 active (Rodrigues et al., 2009a), tandis que les adultes 3-6 mois chez des rats âgés de SL de contraste affichées niveaux inférieurs de T3 actif, qui a été associée à une baisse des dépenses d'énergie par masse (Aust et al, 1986;.. Rodrigues et al, 2009a). Les niveaux de GH ont également été signalés à être élevée dans les deux souris en développement et adulte SL, alors que l'IGF-1 est élevée uniquement pendant le développement postnatal (Kappeler et al., 2009).

La plupart des études ont toutefois mis l'accent sur les effets sur l'axe neuro-endocrines régulation de l'équilibre énergétique. Dans une série d'expériences électrophysiologiques sur des tranches de cerveau issues de rats adultes élevés dans les portées normales ou petites, Plagemann, Davidowa et Li ont examiné les effets de la faible portée de l'élevage sur les réponses hypothalamiques à une variété de neuropeptides impliqués dans le métabolisme de l'énergie. Comme la plupart des autres modèles de la suralimentation, petit élevage de portée a été montré pour induire une résistance à la leptine centrale (Davidowa et Plagemann, 2000a;. Glavas et al, 2010a). Considérant que la leptine est connue pour stimuler les neurones liés à la satiété-VMH des rats normaux, les rats SL l'effet de la leptine dans le VMH était plutôt inhibitrice (Davidowa et Plagemann, 2000b). Petit portée élevage en outre induit une résistance à

l'insuline central, la réduction de la capacité de l'insuline à inhiber les neurones ARH et activer les neurones VMH (Davidowa et Plagemann, 2001, 2007).

Petit élevage de portée a également modifié la réponse centrale au Trésor peptides de la famille dérivé CRF et SRP, ce qui provoque le passage d'effets principalement stimulants dans le VMH dorsomédial et le noyau paraventriculaire de l'hypothalamus pour un effet inhibiteur chez les rats de SL (Davidowa et Plagemann, 2004) . Un bouton similaire chez les rats SL a été vu dans les effets de l'orexine sur les neurones PVH, de stimulation principalement à l'activité inhibitrice (Davidowa et Plagemann, 2005) .Le réponse hypothalamique à MCH a été modifié pour produire un stimulus plus cohérente orexigénique chez les rats SL par rapport à des contrôles (Davidowa et al., 2002b), Petit élevage de portée émoussé l'effet inhibiteur de l'amyline sur les neurones ARH orexigènes (Davidowa et al., 2004) et modifiée de même la réponse dans les neurones du PVH et DMH (Davidowa et al., 2006; Davidowa, 2007) par rapport aux témoins. Des changements semblables ont été observés dans la signalisation des neuropeptides liés à l'alimentation-hypothalamiques AgRP, NPY, MCH, CART et un MSH chez les rats de SL. Considérant que CART, l'a-MSH et NPY tous les neurones stimulés de la PVH chez les rats de contrôle, ces peptides place exercent des effets inhibiteurs dans le PVH de rats SL (Davidowa et al, 2003). Dans le VMH, le profil d'activation de AgRP a été modifiée à partir d'une combinaison de l'activité de stimulation et d'inhibition chez les rats témoins, d'une activité inhibitrice essentiellement chez les rats SL (Li et al., 2002). Petit élevage de portée dopaminergique accrue suppression de neurones HVM qui a été associée à une augmentation de l'expression VMH D1 récepteurs de la dopamine (Davidowa et al., 2002a).

Rats adultes SL affichent également la synthèse de base réduit de pro-thyroïde de libération de l'hormone (pro-TRH) dans le PVH antérieure, et sont moins sensibles aux changements induits par le jeûne, à la fois pro-TRH synthèse et de la corticostérone sécrétion (Arechiga-Ceballos et al ., 2014). Pour résumer ces résultats, il semble que plusieurs composantes du système neuroendocrinien de régulation du comportement alimentaire chez les rats de SL sont décalées vers un profil plus orexigénique qui pourrait expliquer l'hyperphagie couramment observée chez les rats de SL.

des hormones telles que la leptine sont connus pour avoir des effets directs sur l'hypothalamus en développement. Comme décrit précédemment, SL rongeurs présentent plusieurs anomalies dans leurs réponses à diverses hormones hypothalamiques locaux et circulant que l'on croit être à la base de leur phénotype au moins en partie. Pour récapituler brièvement, ils font preuve d'un commutateur à partir d'un stimulateur de la réponse inhibitrice de neurones VMH à la leptine (Davidowa et Plagemann, 2000b), un commutateur d'effets stimulateurs principalement de CRF dans le VMH et noyau dorso paraventriculaire de l'hypothalamus d'un effet inhibiteur dans rats SL (Davidowa et Plagemann, 2004), un commutateur similaire dans les effets de l'orexine sur les neurones PVH, de principalement de stimulation de l'activité inhibitrice (Davidowa et Plagemann 2005), et enfin que PANIER, l'a-MSH et NPY tous les neurones stimulés de le PVH chez les rats témoins, à la place de ces peptides a exercé des effets inhibiteurs dans le PVH de rats SL (Davidowa et al, 2003). Ces réponses altérées était en outre observé également dans les préparations hypothalamiques dissociées, ce qui suggère que petit élevage de portée provoque des changements structurels dans les circuits neuronaux hypothalamiques qui contrôlent l'équilibre énergétique. Petit portée élevage est en outre connu pour provoquer des modifications importantes dans les taux circulants de plusieurs hormones, comme la leptine et l'insuline (Schmidt et al., 2001), qui agissent dans l'hypothalamus et sont connus pour être impliqués dans le développement post-natal de l'hypothalamus (voir la section 4). Modifications temporaires dans les niveaux d'hormones induites par la suralimentation postnatale circulation peuvent donc être un facteur contribuant au développement de l'hypothalamus modifiée, et par conséquent impliqués dans les perturbations métaboliques à long terme causés par SL élevage.

Avant d'explorer cette hypothèse plus en détail, nous allons maintenant passer à l'hypothalamus, décrivant sa structure et le rôle central dans le contrôle de l'équilibre énergétique.

Le noyau arqué (ARH)

Le noyau arqué est un petit noyau triangulaire situé à la base du troisième ventricule. Il est situé à côté de l'éminence médiane (ME) - un organe circumventriculaires qui

contient un réseau dense de vaisseaux sanguins fenêtrées (voir la section 3.2.1 pour plus de détails). L'ARH est donc stratégiquement situé pour être en mesure de répondre à la circulation des signaux métaboliques qui ont un accès facilité à l'ARH par le ME. Conformément à une touche de fonction d'intégrateur de signaux périphériques de l'équilibre énergétique, l'ARH a une concentration unique élevée de récepteurs d'hormones circulantes plusieurs dérivés d'organes périphériques, tels que la leptine (Huang et al., 1996), la ghréline (Zigman et al. 2006) et l'insuline (Marks et al., 1990). L'ARH contient plusieurs populations distinctes de neurones. Il existe pour les cellules exemple neuroendocriniennes qui sécrètent une ou l'autre de la dopamine ou de la croissance de l'hormone de libération de l'hormone (GHRH) dans le système porte hypophysaire pour réguler la prolactine (Blum et al., 1987) et de signalisation de l'hormone de croissance (Bluet-Pajot et al., 1998) par l'intermédiaire de l'hypophyse. Les deux autres grandes populations de neurones dans l'ARH régulent réciproquement métabolisme énergétique et du comportement alimentaire dans des directions opposées. Neurones d'admission stimulant alimentaires produisent les peptides orexigènes neuropeptide Y (NPY) et Agouti-related protein (AgRP), tandis que les neurones qui inhibent l'alimentation lorsqu'ils sont stimulés à produire les peptides anorexigènes alpha-mélanocyte stimulating hormone (a-MSH) et de la cocaïne et l'amphétamine transcription Connexes (CART).

les deux NPY distincte / AgRP et POMC / CART exprimant populations, avec leurs projections étendues à d'autres noyaux hypothalamiques avec des fonctions importantes dans la régulation de l'alimentation et le métabolisme, sont considérés comme les neurones qui sous-tendent principalement le rôle de l'ARH dans le contrôle de l'homéostasie énergétique.

Le noyau paraventriculaire

Le noyau paraventriculaire est un site crucial pour le contrôle central de l'homéostasie énergétique en raison en grande partie grâce à sa régulation de la LPD et HPT axes par CRH et les neurones sécrétant la TRH. Les nombreuses connexions neuronales entre l'ARH et le PVH peuvent ainsi tenir une importance particulière pour l'intégration des signaux périphériques dans la régulation du métabolisme.

L'accès des molécules à des circuits périphériques d'alimentation hypothalic

Pour efficacement à affiner l'équilibre des dépenses d'énergie et la consommation d'énergie en fonction de l'état métabolique en constante évolution de l'organisme, les régions hypothalamiques régulant ces fonctions ont évolué de recevoir une rétroaction continue de nombreux facteurs circulants qui proviennent de différents organes périphériques. Cependant, le cerveau est séparé de la circulation générale par la barrière sang-cerveau-barrière (BBB). La BBB est composé d'une couche de cellules endothéliales qui tapissent les vaisseaux sanguins du cerveau, qui forment des complexes de jonctions serrées, ce qui rend le cerveau pratiquement imperméable à la circulation des molécules, à l'exception de l'eau, des gaz et des petites substances hydrophiles (Mitchell et Hatch, 2011) . Le cerveau reprend les substrats énergétiques circulant dont il a besoin à travers les différents systèmes de transport qui sont spécifiques pour le glucose, les acides aminés, le pyruvate, etc (Pardridge et Oldendorf, 1975; Cremer et al, 1979;. Pardridge et al, 1990;. Boado et al ., 1999), et certaines hormones comme l'insuline et la leptine peut également passer la BHE par des mécanismes de transport saturable similaires (Banks et al, 1997; Hileman et al 2000;. Hileman et al, 2002)..

Cependant, de tels systèmes de transport saturable peuvent ne pas être approprié pour les régions du cerveau qui dépendent de la rétroaction rapide et sensible sur les concentrations de facteurs de circulation, et en effet il a longtemps été soupçonné qu'il y ait une voie privilégiée contourner la BHE par lequel certaines hormones peut plus accéder rapidement à l'hypothalamus. Il ya plusieurs régions du cerveau connue sous le nom organes circumventriculaires qui sont densément peuplées par des vaisseaux sanguins fenêtrés, et en tant que tels sont considérés comme un manque BBB intact. Dans l'hypothalamus, l'ARH est adjacent à l'organe circumventriculaires connu comme l'éminence médiane (ME). La ME est connu pour être un élément central du système de portail hypophysaire qui relie l'hypothalamus à l'hypophyse, autant de cellules neuroendocrines hypothalamiques projettent sur le ME à sécréter l'hormone facteurs tels que la TRH, CRH, GHRH et la GnRH qui puis entrez le portail libérant système pour agir sur l'hypophyse antérieure.

Plus récemment, il a été démontré que le ME représente un candidat plausible comme une voie d'accès rapide pour les hormones métaboliques à l'ARH, car il a été montré pour contenir capillaires fenêtres qui s'étendent dans l'ARH de ventromédian (Ciofi et al, 2009;. Schaeffer et al ., 2013), qui contient d'ailleurs la concentration la plus dense de cellules exprimant des récepteurs de la leptine, l'insuline, la ghréline. Schaeffer et ses collègues ont aussi démontré chez la souris que les petites molécules <20 kDa peuvent diffuser passivement dans l'ARH ventromédian à travers les capillaires fenêtrés de la ME, y compris les hormones qui ciblent l'ARH, comme la ghréline. Fait intéressant, la capacité de la ghréline périphérique administré à diffuser dans l'ARH par cette voie a été montré à dépendre de l'état d'alimentation, que la diffusion a été sensiblement augmentée par le jeûne de la souris pendant 24 heures, puis réduite à des niveaux de base de réalimenter la souris (Schaeffer et . al, 2013). Langlet et ses collègues ont en outre montré que 24 heures de jeûne est associé à de réorganisation structurelle du réseau capillaire ME, ce qui provoque une augmentation des fenestrations des vaisseaux ME s'étendant dans l'ARH. Cette plasticité structurale semble être médiée par une réduction de la glycémie, comme le ME contient spécialisées des cellules endothéliales appelées tanycytes qui peuvent agir comme glucosensors et localement sécréter le facteur de croissance endothélial vasculaire (VEGF) en réponse à l'hypoglycémie, VEGF à son tour, entraîner le vasculaire réorganisation (Langlet et al., 2013a).

Le ME représente donc une voie probable pour l'accès rapide des hormones périphériques à l'ARH, et cohérent avec l'observation de la résistance hypothalamique à l'action des hormones métaboliques tels que la ghréline dans l'obésité (Briggs et Enriori, 2010), l'efficacité de cette voie a été montré pour être modulé par l'état d'alimentation.
résultats

Resultats

1) Validation du modèle - conséquences physiologiques de la suralimentation postnatale chez la souris C57BL6 / J

Souris de type sauvage C57BL6 / J ont été accouplés et la taille de leurs portées normalisées à 7 petits par portée le jour postnatal 1 (P1). Le P3, quelques portées ont été réduits à 3 petits par portée pour induire la suralimentation postnatale, alors que les portées témoins ont été laissés avec 7 petits par portée. Les soriceaux ont été pesés tous les jours à partir de P4 au sevrage au P22 et une fois par semaine après le sevrage à P90.

Souris soulevée dans de petites portées avait accéléré la prise de poids avant le sevrage, et est resté nettement en surpoids (10-15%) à l'âge adulte sur l'alimentation régulière de chow. Les mesures effectuées à l'aide des cages métaboliques indiquent également que les souris suralimentées après la naissance présentent une hyperphagie modérée à l'âge adulte et passent plus de temps engagé dans le comportement alimentaire que les animaux de contrôle. Analyse de la composition corporelle du corps entier en utilisant un scanner a montré 50% plus de graisse corporelle à 4 mois d'âge chez la souris après la naissance suralimentées par rapport à des souris témoins. En outre, une fois mis sur une alimentation riche en graisse haute-saccharose à l'âge adulte, la souris après la naissance suralimentés gagné significativement plus de poids et de graisse et affiché élevée de la glycémie à jeun et de l'insuline par rapport aux souris témoins sur le même régime alimentaire.

2) Les effets de la suralimentation postnatale sur la signalisation de la ghréline

Neonatally suralimentés et des souris témoins ont été sacrifiés à différents âges post-natal (P8, P12, P14, P16, P22, P60) Quantification de la ghréline et de gène de chèvre expression a été effectuée dans l'estomac, et la quantification de l'expression GHSR ARNm a été réalisée à l'ARH et l'noyau hypothalamique dorso (DMH). Sang ont également été recueillis pour les mesures de ghréline sérum.

Après le sacrifice, le sang a été immédiatement récupérés et traités avec un inhibiteur de protéase (Péfabloc SC) et ensuite acidifié à minimiser la dégradation de la ghréline acylé. Taux de ghréline totale et acyle sériques ont ensuite été mesurées avec un sandwich commercial ELISA (Millipore). Les souris témoins ont montré une augmentation progressive du taux de ghréline totale et active de P8 à P22 sérum. En revanche, les

souris après la naissance suralimentés affichée taux de ghréline totale réduite à P16, et les niveaux d'acyle ghréline réduit à P16 et P22, par rapport aux souris témoins.

analyse qRT-PCR a également été utilisé pour mesurer la ghréline de l'estomac et les niveaux d'ARNm de chèvre. Semblable à des taux sériques, de l'estomac la ghréline expression augmente progressivement à partir de la deuxième à la troisième semaine de vie postnatale chez les animaux témoins. Dans les petits suralimentées en revanche, l'expression de la ghréline est resté inchangé tout au long de la vie postnatale.

analyse qRT-PCR sur l'ARNm extrait de microdissection ARH a révélé que l'expression de l'ARNm dans le GHSR ARH a été réduite à P16 et P22 chez les souris suralimentées par rapport aux souris témoins. En revanche, dans le DMH l'expression de l'ARNm GHSR n'a pas changé avec le temps et n'est pas affectée par la suralimentation.

3) Les effets du traitement de la ghréline sur le phénotype métabolique néonatale

Parce que les souris après la naissance suralimentées afficher hypoghrelinemia pendant les périodes critiques du développement hypothalamique, nous avons injecté soriceaux suralimentées deux fois par jour avec la ghréline de P12 à P22. La dose appropriée a été déterminée par une étude pilote qui a montré que 10 ug / kg de ghréline normalise les niveaux de ghréline chez les petits suralimentées. Étonnamment, le traitement de la ghréline néonatale chez les petits suralimentées n'a entraîné aucune amélioration au poids corporel des adultes ou de la masse grasse à un régime de bouffe, pas plus qu'il n'a d'améliorer la réponse exagérée à un régime riche en graisses / saccharose affichée par les petites souris de portée.

4) Les effets de la suralimentation postnatale sur la sensibilité de la ghréline centrale

Sur la base des observations précédentes, nous avons supposé que les petits neonatally suralimentées sont résistants à la ghréline. Par conséquent, nous avons ensuite étudié la capacité des neurones hypothalamiques à répondre à la ghréline périphérique cFos en utilisant comme marqueur de l'activation neuronale.

Chez les souris de contrôle entre les âges P14-P22, une injection périphérique de la ghréline provoque une forte induction cFos dans les ARH, tandis que quelques cellules immunoréactives cFos sont détectables à l'ARH après une injection de solution saline. Par rapport aux souris témoins, des souris après la naissance a eu une suralimentés 1,4 à 2 fois plus faible nombre de cFos-IR cellules positives dans l'ARH en réponse à la ghréline à P14, P16 et P22. En outre, la capacité de la ghréline d'induire une augmentation de l'expression de l'ARNm AgRP est également atténuée chez les petits suralimentées par rapport aux souris témoins. En revanche, les injections intracérébroventriculaires de ghréline provoquent une activation neuronale similaire chez les souris normales et suralimentés. Pour étudier comment suralimentation affecte le transport de la ghréline, le montant de la ghréline présente dans l'hypothalamus médiobasal suivant une injection de ghréline périphérique a été mesurée par western blot, les résultats ont révélé que seulement chez les souris normales étaient des quantités significatives de la ghréline présente dans l'hypothalamus après l'injection, ce qui suggère que les souris suralimentées ont entravé le transport de la ghréline périphérique dans le cerveau.

Ensemble, ces données indiquent que la suralimentation postnatale est associée à une réduction significative des taux de ghréline actifs dans le sérum, ainsi que la réponse centrale sensiblement émoussée de la ghréline, au cours de la troisième semaine de vie post-natale, laquelle est lorsque la ghréline a été postulé pour exercer son effet maximal sur le développement hypothalamique postnatale. Ce défaut central est probablement due à une altération de transport de la ghréline périphérique dans l'hypothalamus.

5) Les effets à long terme de la leptine néonatale antagonisme chez la souris neonatally suralimentés

Parce que les souris suralimentés n'a pas répondu à un traitement ghréline malgré hypoghrelinemia marquée, notre attention s'est déplacée à la leptine. Similaire à la ghréline, la leptine est connu pour être un signal important pour le développement de l'hypothalamus pendant la vie postnatale, et après la naissance des souris suralimentées afficher hyperleptinémie sévère au cours de la période de lactation.

Sur la base de ces observations, nous avons injecté des soriceaux suralimentées avec un antagoniste de leptine nouvellement générée pendant les deux premières semaines de développement post-natal. Nos résultats montrent que les souris neonatally suralimentés soumis à l'affichage de l'antagonisme de la leptine néonatale considérablement réduit la masse grasse et une meilleure sensibilité à l'insuline à l'âge adulte. Cet effet peut être liée à l'amélioration de la signalisation de la leptine de souris postnatal suralimentés affichée depuis l'activation du pSTAT3 induite par la leptine dans l'ARH améliorée après un traitement aigu avec l'antagoniste de la leptine.

Ensemble, ces données suggèrent que hyperleptinémie néonatale est un facteur important qui contribue à la programmation métabolique indésirable observé chez les souris neonatally suralimentés.

Discussion

Les premiers travaux de notre laboratoire sur les effets de la leptine sur le développement post-natal de l'hypothalamus (Bouret et al., 2004b) a établi un nouveau paradigme dans la science de la programmation métabolique, montrant que une hormone impliquée dans la régulation de l'homéostasie énergétique pourrait façonner le développement précoce des mêmes circuits neuronaux qui agit sur l'âge adulte. Ce miroir constatations faites près de 30 ans plus tôt que l'oestrogène de stéroïdes sexuels pourrait stimuler la croissance des neurites à partir d'explants hypothalamiques (Toran-Allerand, 1976), qui a depuis lors devenu la reconnaissance que l'oestrogène et d'autres hormones sexuelles comme la testostérone ont un impact fondamental sur le développement périnatal de régions du cerveau dimorphisme sexuel et peuvent intervenir sur plusieurs aspects de la maturation du cerveau comme la survie cellulaire, la migration cellulaire, la croissance axonale et la synaptogenèse (Simerly, 2002). Depuis la leptine est connue pour être régulée par les deux changements aigus et chroniques sur la nutrition (Kolaczynski et al, 1996;.. Weigle et al, 1997), cette découverte a fourni un mécanisme hormonal plausible reliant changements périnatale nutrition avec les modifications dans la programmation métabolique qui a été préalablement documenté.

Comme la leptine, la ghréline est connue pour être régulée par les deux changements aigus et chroniques dans le bilan énergétique d'une manière contraire à la leptine (Klok et al., 2007). Fait intéressant, la leptine et la ghréline ont été trouvés à avoir des effets marqués mais opposés sur le développement hypothalamique postnatale. Considérant que la leptine stimule la croissance des projections axonales des neurones ARH (Bouret et al., 2004b), la ghréline inhibe le développement des projections axonales émanant de l'ARH (Steculorum et al., Cours de révision), ce qui suggère que ces deux hormones peuvent agir de concert de réglementer les différentes phases de développement, et puissent même réciproquement réglementer les uns des autres de signalisation, depuis la ghréline a été montré pour inhiber directement à la fois l'activation de pSTAT3 induite leptine et la croissance axonale induite leptine (Steculorum et al., cours de révision). Steculorum et ses collègues ont en outre montré que le traitement de la ghréline néonatale ou néonatale ghréline blocus non seulement touchés développement hypothalamique mais provoquent aussi des changements métaboliques persistants caractérisés par une augmentation du poids du corps, la masse grasse, l'hyperglycémie et résistance à la leptine, qui encore une fois reflété les conclusions antérieures sur l'impact à long terme de traitement post-natal de la leptine sur le phénotype métabolique (Pico et al., 2011). À la lumière de ces résultats, la caractérisation de la façon dont les modèles de sécrétion de ghréline et la leptine sont modifiés pendant le développement postnatal en réponse à la suralimentation peut être crucial de comprendre comment la suralimentation postnatale peut induire de tels changements durables sur la régulation métabolique.

Le travail présenté dans cette thèse fournit la première description détaillée de la façon dont le modèle de sécrétion postnatal de la ghréline et la leptine sont influencés par la vie du début suralimentation. Conformément aux résultats précédents (Steculorum et al, cours de révision;. Sakata et al, 2002.), Nous montrons que, bien que les petits nourris normalement présentent une augmentation progressive des taux de ghréline tout au long de la période de lactation, avec une augmentation particulièrement marquée des niveaux d'acyl ghréline circulation au cours de la troisième semaine après la naissance, les taux de ghréline sériques restent à un niveau faible pendant cette période dans les petits suralimentés.

Des études antérieures ont rapporté sur les effets de la SL élevage sur les niveaux de ghréline sérum dans diverses conditions. Lacerda-Miranda et Soares et ses collègues ont rapporté que SL soriceaux affichés diminution des niveaux de ghréline acyle suivants à 4 heures de jeûne au moment du sevrage (Soares et al., 2012) et à 6 mois (Lacerda-Miranda et al., 2012), tandis que prévoit différentes rapports sur les niveaux de ghréline sérique chez les jeunes adultes nourris SL rongeurs, certains rapports aucun changement (Stofkova et al, 2009;.. Fuente-Martin et al, 2012). Cependant, à notre connaissance, aucun rapport sur la façon dont le modèle de la ghréline sécrétion est affectée par la suralimentation au cours du développement postnatal précoce a déjà été publié. Nos résultats indiquent également que le profil de la leptine plasmatique, qui montre généralement une hausse nette au cours de la période néonatale chez les souris nourries normalement (Ahima et al., 1998), a été considérablement amplifiée et prolongée chez les souris de SL. Des études distinctes ont rapporté que SL élevage provoque hyperleptinémie à P7, P14 ou P21, respectivement (Schmidt et al, 2000, 2001;. Rodrigues et al, 2009b;. Stefanidis et Spencer, 2012), mais une cartographie détaillée de la structure intégrée de postnatale la sécrétion de la leptine chez les petits neonatally suralimentés fait encore défaut.

Pour tester si la normalisation de l'état hypoghrelinemic des soriceaux suralimentés observés dans la période postnatale tardive pourrait avoir un effet bénéfique sur leur programmation métabolique, nous avons injecté soriceaux gavés avec de faibles doses de ghréline au cours de la seconde moitié de la lactation. Surprinsingly, le traitement de la ghréline néonatale n'a pas modifié les résultats métaboliques à long terme dans SL soriceaux à l'exception d'une modeste réduction de la glycémie à jeun. Le fait que la normalisation des niveaux de ghréline postnatale n'a pas eu d'effets à long terme nous a conduit à examiner si la suralimentation postnatale altère la capacité des neurones hypothalamiques de répondre à la ghréline pendant le développement postnatal.

Nous avons constaté que bien que les souris suralimentées affiché une réponse centrale réduite de ghréline périphérique, ces animaux ont une réponse similaire à contrôler soriceaux quand la ghréline a été administrée de manière centralisée. Ces observations suggèrent que les neurones de l'hypothalamus de souris SL conservent la capacité à répondre à la ghréline, lorsqu'il est exposé à l'hormone directement, mais que le

transport de la ghréline de la périphérie vers le cerveau peut être modifiée dans des souris suralimentés. Cette hypothèse a été confirmée par Western blot notre expérience a montré que que la quantité de la ghréline présente dans l'hypothalamus médiobasal la suite d'une injection de ghréline périphérique est beaucoup plus faible chez les souris suralimentés que chez les souris témoins. Nous avons également constaté que ce transport de la ghréline modifié à l'hypothalamus pourrait être médiée par absorption de la ghréline anormale par tanycytes situés dans l'éminence médiane.

Une meilleure compréhension des mécanismes sous-jacents de transport de la ghréline à l'hypothalamus dans des conditions physiologiques et pathologiques sera crucial que nous cherchons à développer des études interventionnelles à améliorer et j'espère inverser le transport de la ghréline modifiée chez les souris neoanatally suralimentés. Fait intéressant, Langlet et ses collaborateurs ont récemment démontré que les améliorations apportées par le jeûne, l'accessibilité à la centrale d'hormones périphériques telles que la leptine et la ghréline peuvent être imitées par traitement vasculaire VEGF qui induit la plasticité à la ME (Langlet et al., 2013b). VEGF combiné avec la ghréline pourrait ainsi fournir un meilleur modèle pour la normalisation de la signalisation de la ghréline néonatale dans SL soriceaux. Depuis hyperleptinémie lui-même a été impliqué dans le développement de la résistance de la ghréline centrale, mais seulement chez les souris obèses induite par l'alimentation des adultes (Briggs et al., 2014), une autre intervention qui peuvent plus complètement normaliser la signalisation du système endocrinien chez les petits après la naissance suralimentées pourrait être combinée leptine antagonisme et le traitement de la ghréline.

Notre conclusion que le traitement de la ghréline néonatale a eu un impact sur la glycémie à jeun adulte chez les souris NL et SL peut être lié à un effet de la ghréline néonatale sur le développement du pancréas. Des travaux antérieurs ont montré que la nutrition néonatale peut influencer l'expression GHSR dans les organes périphériques. Par exemple, l'expression GHSR dans les organes périphériques tels que le tissu adipeux et le muscle cardiaque blanc est régulée à la hausse chez les souris sevrées SL, (Lacerda-Miranda et al, 2012;.. Soares et al, 2012). En outre, Dembinski et Warzecha et ses collègues ont montré que le traitement de la ghréline inhibe la croissance néonatale du pancréas en 7-14 ratons jour, alors que par contraste, il a eu un effet stimulant sur la

croissance du pancréas en sevrage et les rats adolescents. Ils ont raconté cet effet dépendant de l'âge d'une interaction avec l'IGF-1 dans sevrage mais pas avant le sevrage rats (Dembiński et al, 2005;. Warzecha et al, 2006.). Parce que les souris SL afficher élevés d'IGF-1 au cours du développement postnatal (Kappeler et al., 2009), nous pouvons supposer que le traitement de la ghréline néonatale a un effet sur la croissance du pancréas stimulatry bien que les études futures seront nécessaires pour tester spécifiquement cette hypothèse.

En plus des défauts de signalisation de la ghréline, nous avons constaté que la période néonatale souris suralimentées présentent une leptine postnatale poussée fortement exagérée. Pour étudier la contribution de ce leptine poussée exagérée à la programmation métabolique modifié présenté par les petits suralimentés, nous avons examiné les effets à long terme de l'antagonisme partielle de la leptine au cours de la poussée de la leptine (P6-P16) chez la souris neonatally suralimentés. Il est frappant, néonatale leptine antagonisme complètement normalisé masse grasse et sensibilité à l'insuline à des niveaux de contrôle par rapport à SL bébés traités avec le véhicule, en dépit de ne pas avoir d'effet sur les courbes de croissance ou le poids final.

Leptine postnatale est connu pour stimuler la croissance axonale ARH, mais les souris de petites portées ont paradoxalement été montré pour avoir altération du développement de fibres axonales ARH (Bouret et al., 2007). Hyperleptinémie et signalisation de la leptine excessive est connue pour induire une résistance à la leptine centrale, probablement due à l'induction de molécules de rétroaction négatives telles que SOCS3, PTP1B ou TCPTP par STAT3 phosphorylée, et hyperleptinémie effet peuvent être essentiels pour le développement de la résistance à la leptine (Knight et al., 2010) .Consistent avec cette idée que nous et d'autres avons constaté que le hyperleptinémie chez la souris SL est associée à atténué pSTAT3 induite leptine dans l'ARH de SL soriceaux (Glavas et al., 2010b) .Remarkably, nous étions en mesure d'améliorer de manière significative la leptine pSTAT3 induite après un traitement par la leptine antagonist.Importantly, STAT3 est une signalisation de la voie principale du récepteur de la leptine qui assurent la médiation de l'effet neurotrophique de la leptine au cours du développement postnatal (Bouret et al., 2012).

Un mécanisme plausible selon laquelle la leptine antagonisme améliorée à long terme phénotype métabolique pourrait donc par une meilleure signalisation de la leptine centrale au cours de cette période critique du développement hypothalamique. Il est également possible que l'antagonisme de la leptine peut avoir modifié le phénotype des soriceaux suralimentés par la modulation des processus inflammatoires dans l'hypothalamus, le tissu adipeux, ou d'autres organes périphériques. Hyperleptinémie est fortement associée à des marqueurs de l'inflammation et de la leptine antagonisme a été démontré à exercer des effets bénéfiques dans diverses conditions inflammatoires (Leon-Cabrera et al, 2013.) (Elinav et Gertler, 2009; Singh et al, 2013.). Inflammation hypothalamique a à son tour été suggéré d'être impliqués dans les changements à long terme de contrôle métabolique en provoquant la dégénérescence de l'hypothalamus (Thaler et al., 2012b) et de l'inflammation dans le tissu adipeux est fortement impliqué dans re-modélisation et l'expansion du tissu adipeux (Wernstedt Asterholm et al., 2014).

Conclusions

Nutrition anormale pendant la vie périnatale est apparue comme un facteur de risque crucial pour le développement de l'obésité et des maladies métaboliques, à la surnutrition ou accélération de la croissance au cours de la période critique de la formation des circuits neuronaux du métabolisme de régulation hypothalamique représentant peut-être un facteur particulièrement important dans le négatif au début programmation du métabolisme. La leptine et la ghréline sont deux hormones qui agissent dans l'hypothalamus pour réguler l'homéostasie énergétique qui ont également été mis en cause dans la programmation du début de la maladie métabolique par leurs effets sur le développement de l'hypothalamus. Il est cependant clair quel est le rôle de la leptine dérégulée et la signalisation de la ghréline dans la programmation métabolique de la maladie causée par la suralimentation postnatale.

Nous montrons ici pour la première fois que la suralimentation provoque des altérations grande échelle du système de la ghréline postnatal, y compris la réduction de la sécrétion de la ghréline acyle, et altération de la sensibilité à la ghréline central lié au transport défectueux de ghréline à partir de la périphérie dans le cerveau.

On montre en outre que hyperleptinémie néonatale est cruciale impliquée dans la programmation métabolique indésirable de souris après la naissance suralimentés, puisque le blocage partiel de la signalisation de la leptine au cours du développement néonatal restaure la masse de graisse et la sensibilité à l'insuline au niveau des souris normalement nourries, éventuellement en améliorant la signalisation central du nouveau-né à la leptine. Nous démontrons ainsi l'importance des altérations temporaires nutritionnel induits dans la signalisation de la leptine au développement à long terme de la maladie métabolique.