The effects of a postnatal high-fat diet on puberty onset and estrous cyclicity in female rats – a kisspeptin study

Master's thesis in Medicinal and Molecular Biology by
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Preface

The present thesis describes a selected part of the work performed and results generated, during a one-year period aiming to attain the Master of Science Degree in Medicinal- and Molecular Biology at the Department of Science, Systems and Models, Roskilde University.

The work was carried out from May 2011 to April 2012 at the Neurobiology Research Unit, Department of Neurology, Rigshospitalet under the supervision of Jens D. Mikkelsen, MD, DMSc and Kim F. Rewitz, PhD.

A part of the work in present thesis was presented at the Third INF International Conference on Integrative Neuroendocrinology, Dourado, São Paulo, by means of a poster with the title:

The effects of postnatal high-fat diet on puberty onset and Kiss1 mRNA levels in female rats.

Lie MEK., Bentsen AH, Mikkelsen JD.

The present work is planned for publication in an article with the working title:

The effects of a postnatal high-fat diet on pubertal timing and estrous cyclicity in female rats – a kisspeptinergic study.

Lie MEK, Bentsen AH, Mikkelsen JD *(in preparation)*

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Abstract

The age of puberty onset in girls has advanced within the last few decades. This is considered to be due, at least in part, to the increasing prevalence of childhood obesity, as epidemiological studies have shown an association between early puberty onset in girls associated with obesity markers early in life or in peri-puberty. The increasing obesity epidemic has also been associated with an increased incidence of reproductive dysfunction in women and the demand for fertility treatment has increased. These associations do not prove causality and other factors can contribute to the secular trends in pubertal timing and impaired reproductive function. However, due to the increasing obesity problem it is becoming increasingly important to investigate this connection and the underlying mechanism of a nutritional regulation of puberty onset and reproductive impairment. In rodents, an association between a positive energy status during development and an advancement of puberty onset and an irregular estrous cycle, have also been reported, but like in humans little is known about the underlying mechanism.

During the last decade, it has been revealed that the hypothalamic neuropeptide kisspeptin, encoded by the *Kiss1* gene, is pivotal in the regulation of the hypothalamic-pituitary-gonadal (HPG) axis that controls maturation and reproduction, and thereby drives neuroendocrine pubertal maturation. Furthermore, kisspeptin neurons have recently emerged as a putative conduit for a metabolic regulation of the HPG axis, with leptin being a positive modulator of *Kiss1* expression.

Little is known about the impact of exposure to a high-fat diet alone in rodents, and the mechanisms have only been studied scarcely. Therefore, the question is whether an increase in fat energy percentage in the diet alone can advance puberty onset and interfere with estrous cyclicity in female rats, and if the kisspeptinergic system is involved and/or affected in this.

The first aim of this thesis was to evaluate the effects of a postnatal high-fat-diet, with 60% energy from fat, during three different periods (lactation, post weaning, or both periods) on puberty onset, revealed by vaginal opening (V.O.). To gain mechanistic insight concomitant changes in the kisspeptinergic system at peri-puberty were assessed by *Kiss1* expression determined using qRT-PCR, and kisspeptin-immunoreactivity (kisspeptin-ir).

No effects of a postnatal high-fat diet exposure, in any of the periods, on puberty onset or body weights were observed. In accordance, no changes in leptin, insulin, triglycerides, and estradiol were identified. As reported previously, a postnatal maturation of the kisspeptinergic system at peri-puberty and from peri-puberty to early adulthood was observed. However, no effects of the high-fat diet on *Kiss1* expression and kisspeptin-ir in the two hypothalamic nuclei, the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV), were detected.

The second aim was to evaluate the effect of a high-fat diet exposure from weaning and for 40 days on estrous cyclicity in adult rats in relation to changes in the kisspeptinergic system.

The high-fat diet induced an irregular estrous cyclicity by prolonging the estrous cycle in a subgroup of the rats, characterized by an unaffected body weight and increased leptin levels. Surprisingly, the high-

fat fed rats, characterized by an increased body weight with a less pronounced increase in leptin as the irregular rats, had a regular estrous cycle. *Kiss1* expression and kisspeptin-ir in ARC and AVPV were unchanged by the high-fat diet in both groups of rats.

In conclusion, these data shows that a high-fat diet induces an irregular cycle in rats by extending the diestrous phase, maybe through leptin, without modulating hypothalamic *Kiss1* expression or kisspeptin levels. The data also indicate that a postnatal high-fat diet has no effect on puberty onset or hypothalamic *Kiss1* expression and kisspeptin levels, in female rats. These findings suggest that an increase in fat energy percentage in the diet alone do not regulate puberty onset or affect the kisspeptinergic system. This is in line with several epidemiological studies unable to establish a correlation between obesity markers at puberty and an earlier puberty onset.

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Abbreviations

ARC	Arcuate nucleus
AVPV	Anteroventral periventricular nucleus
BMI	Body mass index
BSA	Bovine serum albumin
cDNA	Complementary single stranded deoxyribonucleic acid
Ct	Threshold cycles
DAB	3,3'-Diaminobenzidine
ERα	Estrogen receptor α
ERE	Estrogen response element
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GnRH	Gonadotropin-releasing hormone
H_2O_2	Hydrogen peroxide
H&E	Hematoxylin and eosin Y
HFD	High-fat diet
HPG	Hypothalamic-pituitary-gonadal
IHC	Immunohistochemistry
JLV-1	Name of antiserum against murine kisspeptin-52
Kiss1	Gene encoding kisspeptin
Kiss1R	Gene encoding the kisspeptin receptor
Kisspeptin-ir	Kisspeptin-immunoreactive
Lepr	Gene encoding the leptin receptor
LH	Luteinizing hormone
MBH	Mediobasal hypothalamus
NPY	Neuropeptide Y
ob/ob	Leptin deficiency
db/db	Leptin receptor deficiency
OD	Optical density
PBS	Phosphate buffered saline
PLCβ	Phospholipase Cβ
PeN	Periventricular nucleus
PND	Postnatal day
POA	Preoptic area
PCOS	Polycystic ovary syndrome
qRT-PCR	Quantitative real-time polymerase chain reaction
RP3V	Rostral periventricular area of the third ventricle
Rrpm	Rounds per minute
SEM	Standard error mean
TMB	3,3',5,5'-tetramethylbenzidine
V.O.	Vaginal opening

Introduction

It has recently been reported, that the age of puberty onset in girls has advanced within the last few decades both in Europe and in the US (Euling et al., 2008, Aksglaede et al., 2009b, Biro et al., 2010). This is also reflected by an increased incidence of precocious puberty amongst girls (Mouritsen et al., 2010). Pubertal development is dependent on a critical amount of body energy stores (Donato et al., 2011a), and the increased prevalence of overweight and obese children (Cole, 2006) has drawn attention to the possible relation to the secular trend in pubertal timing in girls (Kaplowitz, 2008, Ahmed et al., 2009). Epidemiological data have shown that obesity markers in early life or at peri-puberty are associated with an advanced pubertal timing in girls (He and Karlberg, 2001, Davison et al., 2003, Lee et al., 2007, Kaplowitz, 2008, Aksglaede et al., 2009a, Rosenfield et al., 2009, Maisonet et al., 2010). The importance of this is stressed by the pathological relevance given earlier pubertal maturation is associated with an increased risk of adult obesity, adult-onset diabetes, breast cancer, cardiovascular diseases (Dunger et al., 2006, Ahmed et al., 2009), and adult reproductive defects (Castellano et al., 2011).

Like pubertal maturation, reproductive function is dependent on body energy status (Donato et al., 2011a), and it is well known that reproductive function in women is impaired by both a negative (Frisch, 1994, Cunningham et al., 1999, Parent et al., 2003) and a positive nutritional status in adults (Pasquali et al., 2007). The increasing obesity epidemic is associated with an increased prevalence of reproductive dysfunction in women and the demand for fertility treatment has increased equivalently (Barber et al., 2006, Nelson and Fleming, 2007, Pasquali et al., 2007). Also, the prevalence of polycystic ovary syndrome (PCOS), which is strongly associated with an increased BMI and a reduced reproductive function, has increased and is believed to increase further in the future (Barber et al., 2006, Pasquali et al., 2007). The impaired reproductive function caused by an increased BMI is characterized by an irregular estrous cyclicity, oligo-anovulation, and infertility (Linne, 2004, Pasquali et al., 2007), which can be improved by weight loss in women with an increased BMI (Linne, 2004, Barber et al., 2006, Nelson and Fleming, 2007).

The apparent association between an increased nutritional status and earlier puberty onset in girls and an impaired reproductive function in women based on epidemiological data do not prove causality, and other factors like endocrine disrupting chemicals can be contributing (Mouritsen et al., 2010). However, due to the increasing obesity epidemic worldwide (Cole, 2006, Kaplowitz, 2008, Ahmed et al., 2009) it is becoming increasingly important to investigate this association and the underlying mechanism of a nutritional regulation of puberty onset and reproductive function.

In 2003, two research groups independently reported that humans with loss of function mutations in Kiss1R, the receptor for the neuropeptide kisspeptin encoded by the *Kiss1* gene, suffered from isolated hypogonadotropic hypogonadism, characterized by impairment of pubertal maturation and reproduction (de Roux et al., 2003, Seminara et al., 2003). The same phenotype has been observed in

Kiss1R deficient and kisspeptin deficient mice (Seminara et al., 2003, d'Anglemont de Tassigny et al., 2007, Lapatto et al., 2007). In accordance, a mutation causing enhanced Kiss1R signaling has been found in a child with precocious puberty (Dhillo, 2008). These data had significant impact on the understanding of the mechanism behind puberty onset regulation, as kisspeptin-Kiss1R signaling seems to be a key molecular mechanism involved. This is consistent with kisspeptin being the most potent positively regulator of the hypothalamic-pituitary-gonadal (HPG) axis known today (Navarro et al., 2005a, Navarro et al., 2005b), and by its ability to advance puberty onset when administered chronically in rats (Navarro et al., 2004b).

Many peripheral and central factors are implicated in the metabolic control of GnRH neurons that constitute the highest level in the HPG axis, including the adipose-hormone leptin, but intermediate networks seems to be involved since the GnRH neurons do not express leptin receptors (Cunningham et al., 1999, Castellano et al., 2010). In recent years, kisspeptin neurons have emerged as a plausible conduit for the metabolic regulation of GnRH neurons and thereby the HPG axis (Tena-Sempere, 2006a, Castellano et al., 2010, Roa et al., 2011). Several studies have documented a clear effect of a changed energy status on *Kiss1* and kisspeptin expression in the hypothalamus in rodents and higher primates (Castellano et al., 2005, Luque et al., 2007, Backholer et al., 2010, Castellano et al., 2010, Castellano et al., 2011, Wahab et al., 2011), which render it possible that kisspeptin could be implicated in the nutritional induced advancement of puberty onset in girls and impaired reproductive function in women.

In rodents, a positive energy status during development has also been associated with earlier puberty onset and an irregular estrous cycle (Boukouvalas et al., 2008, Chang et al., 2008, Brill and Moenter, 2009, Lo et al., 2009, Akamine et al., 2010, Boukouvalas et al., 2010, Castellano et al., 2011, Moral et al., 2011, Smith and Spencer, 2012). However, little is known about the effect of exposure to a high-fat diet alone, and the underlying mechanisms have only been studied scarcely in rodents (Castellano et al., 2011). Therefore, the aim of this thesis is to evaluate whether an increase in fat energy percentage in the diet alone in different postnatal periods, have an effect on puberty onset and estrous cyclicity, and whether the kisspeptinergic system is affected in relation to this.

Pubertal maturation and reproductive attainment in humans

Puberty is a developmental transition where secondary sexual characteristics and reproductive function are attained (Terasawa and Fernandez, 2001, Plant and Barker-Gibb, 2004). Pubertal maturation is comprised of two independent physiological developmental processes; gonadarche and adrenarche. Gonadarche refers to the activation of the gonads driving thelarche and menarche, whereas adrenarche refers to the activation of the adrenal cortex which drives pubarche (Plant and Barker-Gibb, 2004). The absence of adrenarche do not prevent fertility and gonadarche, and adrenarche can thus be viewed as a corollary, rather than an integral component, of the pubertal transition (Plant and Barker-Gibb, 2004). However, disorders of adrenal androgen secretion are not

clinically unimportant since hyperandrogenism is the most consistent feature in PCOS (Barber et al., 2006). In contrast to adrenarche, gonadarche occurs as a result of an activation of the hypothalamic gonadotropin-releasing hormone (GnRH) pulse generator (Plant and Witchel, 2006, Roa et al., 2008). With the previous in mind and since puberty onset is defined as thelarche, the mechanism that underline gonadarche will be the focus in the present study.

GnRH neurons have the ability to release GnRH in a pulsatile manner at infancy, but are restrained in early life so the gonads become quiescent during childhood. The restrain of the GnRH pulses during childhood is not caused by gonadal steroid suppression or the inability of GnRH neurons to secrete GnRH, but rather by central inhibition in the brain caused by gamma-aminobutyric acid (GABA) and neuropeptide Y (NPY) (Terasawa and Fernandez, 2001, Plant and Witchel, 2006). The GnRH pulse generator is reactivated at puberty leading to pulsatile releases of GnRH from the hypothalamus that stimulates the secretion of the gonadotropins, namely luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary (Plant and Witchel, 2006, Roa et al., 2008). LH and FSH act on somatic cells in the gonads to increase biosynthesis and release of gonadal sex steroids that are driving the pubertal development and attainment of reproductive capacity (Figure 1) (Plant and Barker-Gibb, 2004, Plant and Witchel, 2006, Roa et al., 2008, Terasawa et al., 2010).

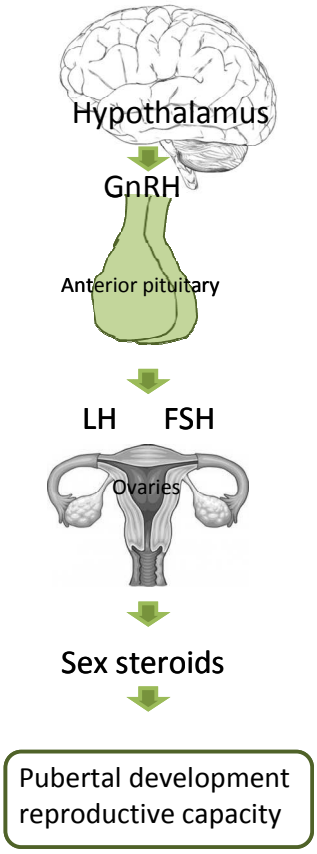


Figure 1. The hypothalamic-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) is secreted from GnRH neurons residing in the hypothalamus, and elicits a secretion of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary. LH and FSH act on the gonads and stimulate the secretion of sex steroids which drives the pubertal maturation and attainment of reproductive function. See text for details.

The upstream triggering factor(s) responsible for reactivation of the GnRH pulse generator driving the pubertal maturation has not yet been defined (Plant and Barker-Gibb, 2004, Plant and Witchel, 2006, Terasawa et al., 2010). Based on the impaired pubertal maturation and reproductive dysfunction in humans and mice suffering from a mutation in Kiss1R (de Roux et al., 2003, Seminara et al., 2003, Lapatto et al., 2007), it is highly likely that the kisspeptinergic system plays a significant role in the reactivation of the GnRH pulse generator driving pubertal maturation and attainment of reproductive capacity (Keen et al., 2008).

The Kiss1 gene and kisspeptin

Kisspeptin is derived from the *Kiss1* gene, which was first described in 1996 as a metastasis-suppressor gene in relation to human melanomas (Lee et al., 1996). The human *Kiss1* gene is translated to a 145 amino acid prepropeptide (West et al., 1998), which is cleaved giving rise to a 54 amino acid product (amino acid 68-121), termed kisspeptin-54 (Kotani et al., 2001, Muir et al., 2001, Ohtaki et al., 2001, Aparicio, 2005, Roa et al., 2008). Also, shorter forms of kisspeptin sharing the same C-terminal as kisspeptin-54, have been isolated. These are 13 or 14 amino acids in length and are termed kisspeptin-13 and -14, respectively (Figure 2) (Kotani et al., 2001). Since no obvious cleavage sites are present for these shorter peptides, it is not clear to what degree they are produced endogenously or just a result of degradation of kisspeptin-54 (Kotani et al., 2001, Kirby et al., 2010). All the different kisspeptin peptides are collectively termed kisspeptin (Tena-Sempere, 2006b).
The receptor binding part of kisspeptin, consist of the 10 amino acids that are located in the aminated C-terminal necessary for the activity of kisspeptin (Ohtaki et al., 2001), and the shorter forms of kisspeptin are just as potent activators of Kiss1R as the longer forms (Kotani et al., 2001, Mikkelsen et al., 2009).

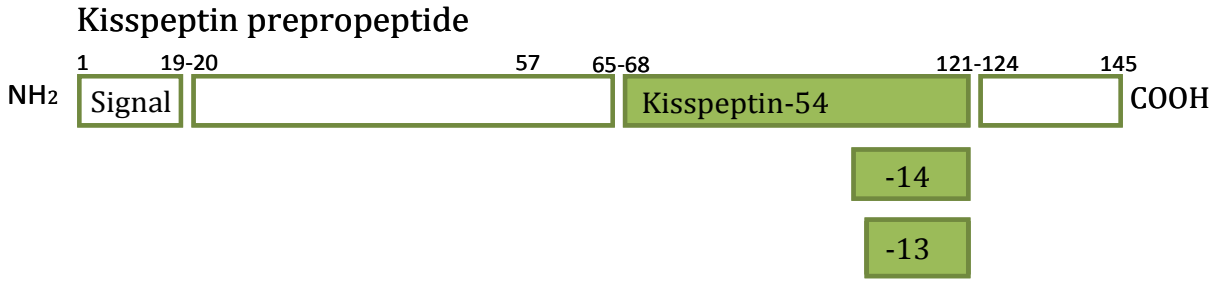


Figure 2. The human kisspeptin prepropeptide with the indication of kisspeptin-54, -14, and -13. The *Kiss1* gene encodes a 145 amino acid prepropeptide with a secretory signal sequence in the N-terminus, two potential dibasic cleavage sites at amino acid 57 and 67, and a cleavage and amidation site at amino acid 121-124. The prepropeptide is cleaved into a shorter fragment of 54 amino acids termed kisspeptin-54 with a consensus C-terminal arginine-phenylalanine-amide motif. Also, shorter forms of kisspeptin have been isolated in humans termed kisspeptin-13 and -14. These kisspeptins share the same C-terminal as kisspeptin-54. Numbers indicate amino acid residue in the primary sequence. *Modified from (Roa et al., 2008).*

In rats and mice, cleavage sites occur at the same sites in the kisspeptin prepropetide, but they give rise to a peptide that is only 52 amino acids long. The rodent kisspeptin-52 differs from the human kisspeptin-54 in several other ways than the length including a tentative disulfide bridge, and one

amino acid difference in the C-terminal (Ohtaki et al., 2001). However, the human forms of kisspeptin elicits a response in rodents that are similar to the endogenous forms of kisspeptin, indicating that the sequence dissimilarities between humans and rodent have little or no effect on the receptor binding (Mikkelsen and Simonneaux, 2008, Mikkelsen et al., 2009).

Kiss1R – the kisspeptin receptor

Kiss1R was discovered in 1999 as an G protein-coupled receptor (Lee et al., 1999), and was found to be the putative receptor for kisspeptin by three independent groups in 2001 (Kotani et al., 2001, Muir et al., 2001, Ohtaki et al., 2001). Newer studies have revealed that Kiss1R is the only receptor mediating the effect of kisspeptin (Messager et al., 2005, d'Anglemont de Tassigny et al., 2007). Kisspeptin bind and activates Kiss1R in many different tissues and have pleotropic functions including inhibition of metastasis, regulation of proliferation, and hormone secretion (Castano et al., 2009).
Kiss1R is expressed on GnRH neurons (Irwig et al., 2004, Messager et al., 2005, Herbison et al., 2010), and activation of Kiss1R, which is coupled to the $G_{q/11}$ family of the G-proteins, elicits a secretion of GnRH by activating the phospholipase Cβ (PLCβ) pathway (Kotani et al., 2001, Muir et al., 2001, Becker et al., 2005, Castano et al., 2009). Activation of PLCβ leads to an increase in inositol 1,4,5-triphosphate and diacylglycerol, and a subsequent rise in intracellular Ca^{2+} concentration which stimulates hormone release (Kotani et al., 2001, Liu et al., 2008, Castano et al., 2009). In addition to this, activation of Kiss1R has been reported to activate extracellular signal-regulated kinases 1 and 2 and p38 *in vitro* (Kotani et al., 2001). *Ex vivo* studies with hypothalamic tissue from rats support that kisspeptin induce GnRH secretion by activating PLCβ signaling as well as by activating the extracellular signal-regulated kinases 1 and 2 and p38 (Castellano et al., 2006a). Continuous activation of Kiss1R has been found to lead to a desensibilisation of the receptor (Seminara et al., 2006, Ramaswamy et al., 2007).

Neuroanatomical location of kisspeptin in adulthood

A great number of studies have been conducted especially in rodents to map the neuroanatomical location of the kisspeptinergic neurons. Initial studies carried out by *in situ* hybridization in adult mice demonstrated that *Kiss1* are mainly expressed in two hypothalamic nuclei namely the anteroventral periventricular nucleus (AVPV) in the preoptic area (POA) and the arcuate nucleus (ARC) in the mediobasal hypothalamus (MBH) (Figure 3) (Gottsch et al., 2004, Smith et al., 2005a, Smith et al., 2005b). A lower expression of *Kiss1* was detected in other hypothalamic regions in mice including the periventricular nucleus (PeN) (Gottsch et al., 2004, Han et al., 2005, Smith et al., 2005a, Smith et al., 2005b). Immunohistochemistry studies conducted later confirmed the *in situ* hybridization data, however, these studies showed that the rostral kisspeptin neurons in POA are not only located in AVPV but appear in a continuum beyond the AVPV region to PeN, which is termed the rostral periventricular area of the third ventricle (RP3V) collectively (Clarkson and Herbison, 2006, Clarkson et al., 2009b). This region is termed AVPV in the present study.

Anatomical location studies of kisspeptin populations in the primate hypothalamus is limited (Plant, 2012). However, kisspeptin cell bodies have been found in women and female monkeys in the POA in areas that may correspond to the rodent AVPV (Plant, 2012). The POA kisspeptin population in women and female monkeys is in contrast to rodents, not located immediately alongside the third ventricle (Clarkson and Herbison, 2009). The infundibular nucleus corresponding to ARC in rodents is densely populated with kisspeptin neurons in humans (Plant, 2012). The infundibular nucleus in higher primates and humans is termed ARC in the present study.

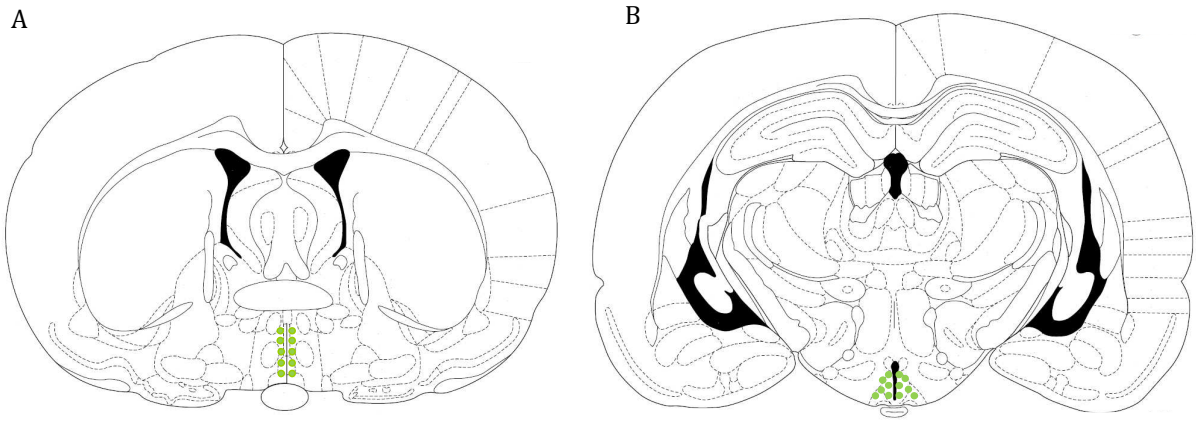


Figure 3. Localisation of kisspeptinergic neurons in the rodent hypothalamus. Kiss1 mRNA and kisspeptin are localised in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC). (A) Kisspeptin in AVPV is situated along the third ventricle from Bregma -0.26mm to -0.80. (B) Kisspeptin in ARC is situated from Bregma -2.12 to -4.52. AVPV and ARC presented here corresponds to figure 20 and 33, respectively in Paxinos and Watson (1986).

Despite the similar distribution patterns for kisspeptin in males and females and in different species, sexual dimorphism in the kisspeptin system is present. This sexual difference is very pronounced in the AVPV of rodents where male adults have fewer kisspeptin neurons than females, whereas no sexual dimorphism is present in ARC (Clarkson and Herbison, 2006, Kauffman et al., 2007, Clarkson et al., 2009b). This sexual dimorphism in AVPV seems to be organized by the effects of sex steroids during early development (Terasawa et al., 2010).

Postnatal developmental changes in kisspeptin neuronal populations

The kisspeptinergic system undergoes neuroanatomical maturation during postnatal development, and developmental profiles of Kiss1 mRNA and kisspeptin expressing neurons in ARC and AVPV have been carried out in several species including mice and rats (Han et al., 2005, Clarkson and Herbison, 2006, Bentsen et al., 2009, Clarkson et al., 2009a, Takase et al., 2009).

Developmental studies in mice and rats showed no Kiss1 mRNA before postnatal day (PND) 15 to 20 in AVPV followed by a sudden increase in *Kiss1* expression in AVPV just prior to and around puberty onset (approximately PND30-36 for female and PND45 for males) which was most pronounced in females (Han et al., 2005, Bentsen et al., 2009, Takase et al., 2009, Takumi et al., 2011). Adult levels of *Kiss1* expression in AVPV appear to be reached at puberty since a small or no change was detected at adulthood (Clarkson and Herbison, 2006, Bentsen et al., 2009, Clarkson et al., 2009a, Takase et al.,

2009, Takumi et al., 2011). Immunohistochemistry studies support the developmental expression profiles in AVPV since the number of kisspeptin cells in AVPV were found to be very low at PND10 while a slight increase occurred from PND15 to 25 and again around puberty onset where adult levels was reached in both female and male mice (Clarkson and Herbison, 2006, Clarkson et al., 2009a, Mayer et al., 2010). Whether, the number of kisspeptin cells in AVPV changes at adulthood in rats has apparently not been assessed. The above data suggests that *Kiss1* expression and number of kisspeptin cells in AVPV is low before PND15 to 25, and appear to reach adult levels around puberty onset.

In contrast, only small developmental changes occur in ARC compared to AVPV. In female rats, an increase in *Kiss1* expression in ARC has been reported from PND21 to 26, where pubertal levels were reached (Takase et al., 2009, Takumi et al., 2011). A further increase in Kiss1 mRNA occurred again at adulthood in female rats (Takumi et al., 2011). Also, the number of kisspeptin cells increased equivalently in ARC, but not before PND31 (Takase et al., 2009, Desroziers et al., 2012). A study in male rats has detected Kiss1 mRNA at PND15, where a small gradual increase in *Kiss1* expression occurred at PND30 with a peak around puberty onset (Bentsen et al., 2009, Takumi et al., 2011). The number of kisspeptin cells in ARC in male rats were similar low at PND15 after which a gradual increase occurred prior to puberty and again after puberty (Bentsen et al., 2009). These data suggests that *Kiss1* expression and the number of kisspeptin cells in ARC undergoes minor postnatal developmental changes that primarily occur just before onset of puberty and again after puberty. Though, Kiss1 mRNA in ARC has been reported to be present at embryonic day 13, and is also detectable at PND3, meaning that the ARC kisspeptin neuron population is specified well before the kisspeptinergic population in AVPV (Clarkson et al., 2010, Takumi et al., 2011). The developmental profile of kisspeptinergic neurons in AVPV and ARC in female rodents, based on the above data, is summarized in Figure 4A and B, respectively.

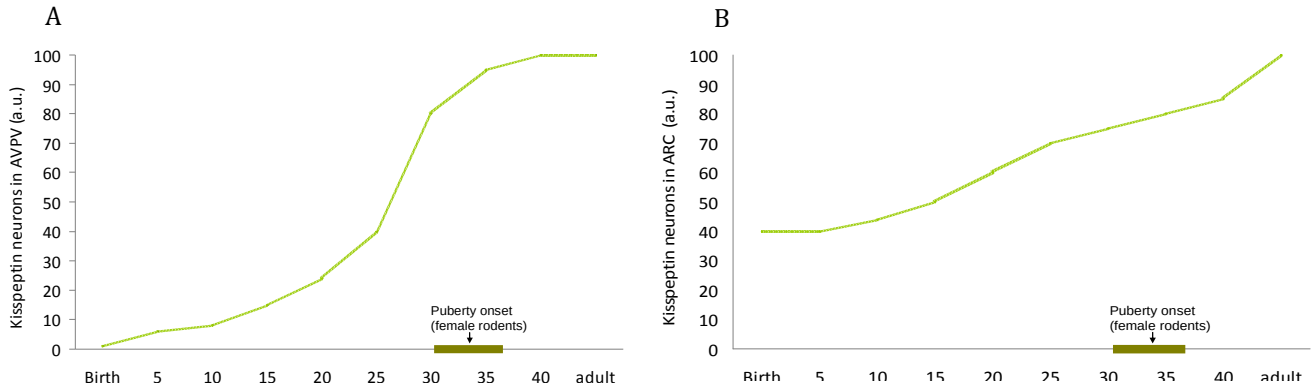


Figure 4. An illustration of the postnatal maturation of kisspeptinergic neurons in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) in female rodents based on a summary of data obtained by others (Clarkson and Herbison, 2006, Clarkson et al., 2009a, Takase et al., 2009, Takumi et al., 2011, Desroziers et al., 2012). (A) Kisspeptin neurons in AVPV, depicted by changes in Kiss1 mRNA and number of kisspeptin cells, increases dramatically just prior to and around puberty onset, where adult levels appear to be present. (B) The developmental changes in kisspeptin neurons in ARC, depicted by changes in Kiss1 mRNA and number of kisspeptin cells, are less pronounced than in AVPV, but an increase occurs primarily around puberty onset and again at adulthood. See text for more details.

Developmental changes in the kisspeptinergic neurons also occur in higher primates since an increase in *Kiss1* expression around puberty onset have been detected in MBH, containing the homolog of the rodent ARC, in both male and female monkeys (Shahab et al., 2005).

Functional activation of the kisspeptinergic system

In coincidence with the neuroanatomical maturation of the kisspeptin neuron populations in AVPV and ARC just before and around puberty onset, functional activation of the kisspeptinergic system seems to take place.

Whether *Kiss1R* expression undergoes similar developmental changes as *Kiss1* expression in ARC and AVPV is less clear. A developmental expression profile of *Kiss1R* has been conducted by the use of the Kiss1R LacZ knockin-mouse model. This has revealed a developmental change in *Kiss1R* expression since approximately 35-45% of the GnRH neurons express *Kiss1R* from birth up to PND5, while adult levels at approximately 70% are reached around PND20 prior to puberty onset in both sexes (Figure 5A) (Herbison et al., 2010). Also, an increase in the number of close appositions between kisspeptin fibres and GnRH neurons occur just prior to puberty onset since they first appeared from PND25 and PND31 onwards for female and male mice respectively (Clarkson and Herbison, 2006). A further increase in GnRH neurons with kisspeptin appositions, occurred during adulthood (Figure 5B) (Han et al., 2005). These fibre projections are believed to arise from AVPV (Clarkson and Herbison, 2006). The neuroanatomical changes in *Kiss1R* expression and AVPV kisspeptin fibre projections are associated with a change in the number of GnRH neurons responding to kisspeptin, as an increase in the number of kisspeptin-responding GnRH neurons has been reported both from puberty to prepuberty and again at adulthood in male mice (Figure 5C) (Han et al., 2005).

The above findings indicate that GnRH neurons come into contact with kisspeptin neurons just before puberty onset which is accompanied by a concomitant increase in *Kiss1R* expression at GnRH neurons which is likely to increase the number of GnRH neurons that responds to kisspeptin. This is supported by studies showing that the stimulating effect of kisspeptin on LH release is enhanced around puberty (Han et al., 2005, Castellano et al., 2006a, Bentsen et al., 2009).

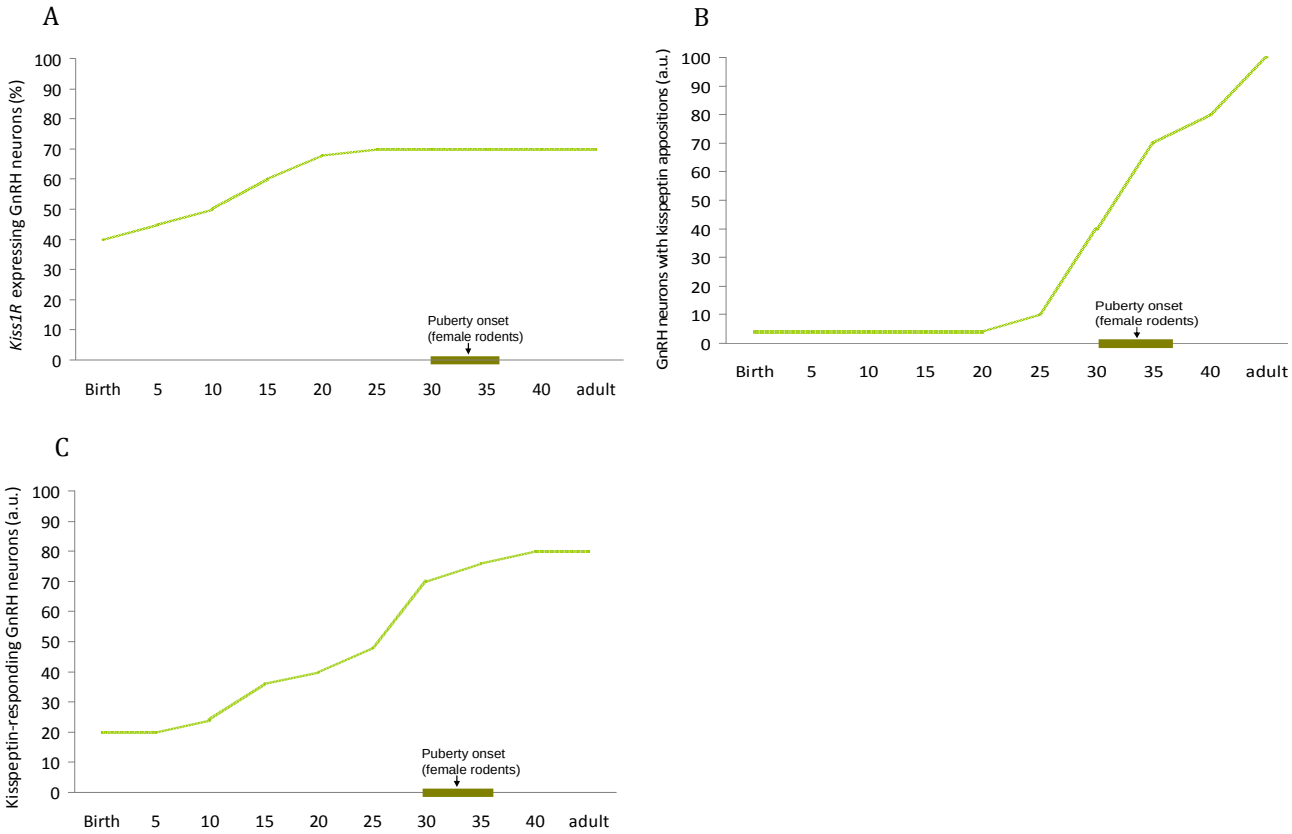


Figure 5 An illustration of the functional activation of the kisspeptinergic system just prior to puberty onset in female rodents based on a summary of data obtained by others (Han et al., 2005, Clarkson and Herbison, 2006, Herbison et al., 2010). (A) *Kiss1R* expressing GnRH neurons. (B) GnRH neurons with close kisspeptin appositions. (C) Kisspeptin-responding GnRH neurons. See text for further details.

Kisspeptin and the hypothalamic-pituitary-gonadal axis

Kisspeptin and interaction with GnRH neurons

GnRH cell bodies are scattered throughout the basal forebrain including POA in rodents (Herde et al., 2011) and POA and MBH in higher primates (Plant, 2012). The majority of the GnRH axons are projecting to the external zone of the median eminence where they terminate on portal vessels and secrete GnRH into the hypothalamohypophysial portal system to the anterior pituitary (Herde et al., 2011, Plant, 2012).

In higher primates, kisspeptin terminals are found in close proximity to GnRH cell bodies (Ramaswamy et al., 2008). In rodents, it has been known for several years that AVPV kisspeptin fibers are in direct contact with the GnRH neurons in POA (Clarkson and Herbison, 2006, Yeo and Herbison, 2011), but it is less clear whether ARC kisspeptin fibers projects directly to GnRH neurons (Roa et al., 2011). Direct projections from ARC kisspeptin neurons to POA GnRH neurons have been reported by use of anterograde and retrograde tracing in female mice (Yeo and Herbison, 2011), whereas studies in female rats have been unable to find these projections to GnRH neurons in POA (True et al., 2011). In stead they report the presence of ARC kisspeptin fibers projecting to the internal zone of the median eminence in close proximity to GnRH fibers in the female rats (True et al., 2011).

Kisspeptin – the most potent elicitor of GnRH secretion

Several *in vivo* studies have shown that central administration of kisspeptin into the cerebral ventricle elicits a secretion of GnRH and LH in several species including rodents an monkeys (Gottsch et al., 2004, Irwig et al., 2004, Thompson et al., 2004, Kinoshita et al., 2005, Navarro et al., 2005a, Navarro et al., 2005b, Shahab et al., 2005, Keen et al., 2008). Also, systemic administration of kisspeptins leads to a release of LH in rodents and humans (Matsui et al., 2004, Dhillo et al., 2005, Messager et al., 2005, Navarro et al., 2005b, Dhillo et al., 2007). Since the eliciting effect of kisspeptin is blocked by pretreatment with acyline, a potent GnRH antagonist, the regulating effect of kisspeptin on LH appears to take place in a GnRH dependent manner (Gottsch et al., 2004, Irwig et al., 2004, Mason et al., 2007). This is supported by electrophysiological recordings showing that kisspeptin depolarizes GnRH neurons (Han et al., 2005), and by immunohistochemical data showing an activation of GnRH neurons, detected by cFos expression, upon kisspeptin administration (Irwig et al., 2004, Matsui et al., 2004). According to comparative analyses, kisspeptin is the most potent elicitor of GnRH secretion (Navarro et al., 2005a, Navarro et al., 2005b), which is mediated exclusively through Kiss1R, since *Kiss1R* deficient mice fail to exhibit a gonadotropin release in response to kisspeptin (Messager et al., 2005), whilst *Kiss1* deficient mice exhibit a gonadotropin release in response to kisspeptin (d'Anglemont de Tassigny et al., 2007).

Sex steroids regulate the HPG axis through kisspeptin

GnRH neurons are regulated by feedback from sex steroids, but the GnRH neurons themselves lack steroid receptors involved in this feedback (Oakley et al., 2009, Smith, 2009). The kisspeptin neurons in ARC and AVPV express receptors for the sex steroids (Lehman et al., 2010), and sex steroids have been found to exert a negative regulation of *Kiss1* expression in ARC and a positive regulation of *Kiss1* expression in AVPV in rodents (Smith, 2009). This support that kisspeptin neurons compose an intermediate circuit mediating the feedback of sex steroids upon GnRH secretion (Figure 6) (Smith, 2009). Though, the positive feedback of estradiol upon AVPV *Kiss1* expression is not established before PND20 in rats (Clarkson et al., 2009a).

ARC kisspeptin neurons in monkeys and humans appear to forward the negative feedback of sex steroids, but POA kisspeptin neurons, homolog to rodents, are not subjected to sex steroid regulation in monkeys and humans (Dhillo, 2008).

The molecular mechanisms responsible for the differential effects of sex steroids upon *Kiss1* expression in ARC and AVPV, remains unresolved, but several hypothesis exists. One possible explanation is that estradiol exerts its different effects by differential intracellular signaling (Oakley et al., 2009). Estradiol has been found to activate two different pathways trough the estrogen receptor α (ERα), namely the classical pathway, involving genomic estrogen response, and the non-genomic pathway, which is independent of the estrogen response element (ERE) (Oakley et al., 2009). ERE independent signaling appear to be sufficient for a negative regulation of LH, while the positive

feedback is dependent on ERE signaling thus render it possible that the different effects of estrogen on *Kiss1* expression in AVPV and ARC are due to differential intracellular signaling (Oakley et al., 2009). Also, epigenetic regulation of *Kiss1* expression induced by estrogens appears to be involved in the differential effects of estrogens on *Kiss1* expression in ARC and AVPV (Tomikawa et al., 2012).

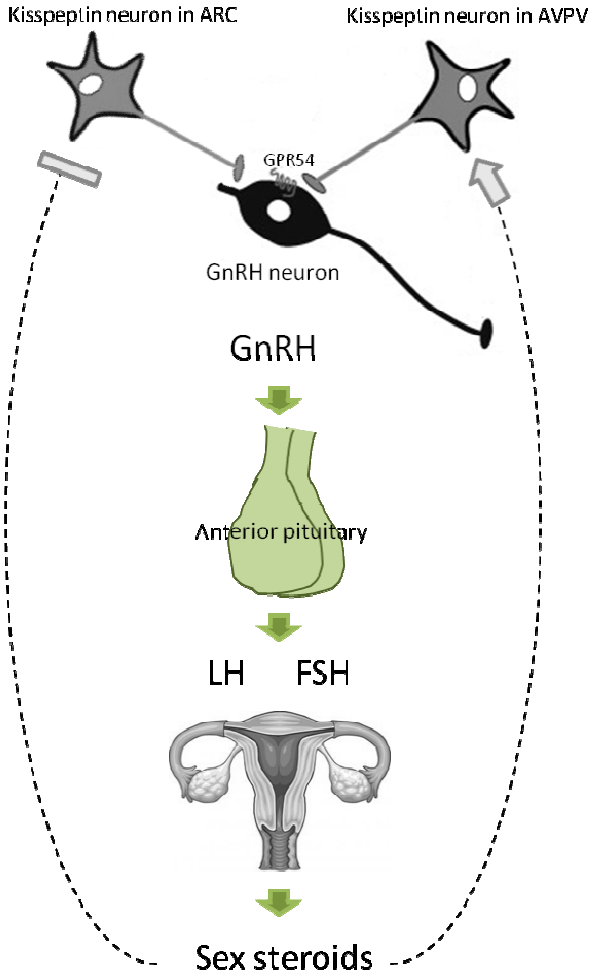


Figure 6. The kisspeptinergic system, the hypothalamic-pituitary-gonadal axis, and the regulation hereof by sex steroids in rodents. Kisspeptin fibers project from the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV) to gonadotropin-releasing hormone (GnRH) neurons in the preoptic area (POA). Kisspeptin bind to and activates Kiss1R on GnRH neurons and elicits the secretion of GnRH. GnRH induces the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary, which stimulates the secretion of sex steroids from the gonads. Sex steroids exert a negative and a positive feedback on Kiss1 mRNA in ARC and AVPV, respectively. See text for more details.

Reactivation of the GnRH pulse generator at puberty onset – the role of kisspeptin

Although the neurobiological mechanisms triggering the reactivation of the GnRH pulse generator is still not elucidated (Plant and Barker-Gibb, 2004, Terasawa et al., 2010), it is certain that different hormonal changes precede the physical signs of puberty in both boys and girls. This is illustrated in prepubertal boys and girls by an increase in pulsatile release of gonadotropins including a nocturnal increase in frequency and amplitude of LH release. These changes are associated to changes in GnRH

pulses, as the frequency and amplitude of the GnRH pulses also increases during puberty with nocturnal peaks in humans (Terasawa and Fernandez, 2001).

Studies conducted in monkeys have shown that kisspeptin secretion increases in association with GnRH during the pubertal period. The nocturnal increase in kisspeptin was present just before the nocturnal increase in GnRH secretion, and the release of both kisspeptin and GnRH were pulsatile, and approximately 76% of the kisspeptin pulses appeared 10 minutes prior to the GnRH pulses (Keen et al., 2008). The presence of an increase in kisspeptin pulse frequency around puberty was also reported in another study conducted in monkeys (Terasawa et al., 2010). In accordance, an increase in *Kiss1* expression occurs around puberty in monkeys (Shahab et al., 2005). The nocturnal increase in kisspeptin preceding the nocturnal increase in GnRH as well as the increase in *Kiss1* expression seen during the pubertal period are consistent with the hypothesis that kisspeptin plays a pivotal role in the mechanisms regulating puberty onset in higher primates.

Similar developmental studies on the secretion of kisspeptin and GnRH have not been performed on rodents, but several things support that kisspeptin is pivotal in the regulation of puberty onset in rodents as well. A very strong indicator of kisspeptin's crucial role in rodents is kisspeptin's ability to advance puberty onset, when given as chronic central infusion (Navarro et al., 2004b), or delay puberty onset when kisspeptin signaling is suppressed by a kisspeptin antagonist (Pineda et al., 2010). This is in keeping with the literature showing that kisspeptin or Kiss1R deficient mice suffer from a delayed pubertal maturation (Lapatto et al., 2007). The neuroanatomical maturation and functional activation the kisspeptinergic system undergoes just prior to and around puberty, as described above, support that kisspeptin could play a pivotal role in the reactivation of the GnRH pulse generator. This hypothesis is challenged by the presence of a normal pubertal maturation in female mice devoid of kisspeptin or Kiss1R signaling, caused by a genetic ablation induced by diphtheria toxin A of either kisspeptin or Kiss1R neurons (Mayer and Boehm, 2011). The basis for such discrepancy remains to be elucidated, however, the possibility exist that the ablation of kisspeptin and Kiss1R neurons in Mayer and Boehm (2011) were not complete, and compensatory mechanisms enabling pubertal maturation with a limited number of kisspeptin or Kiss1R neurons may have occurred. This is in line with previous studies, reporting that a severed reduction in GnRH neurons from birth did not impair female maturation and reproductive capacity (Herbison et al., 2008), whereas timed elimination of kisspeptin neurons from PND20 impaired female maturation (Mayer and Boehm, 2011).

Estrogen – the driving upstream signal?

The question is whether other upstream signals drive these pubertal changes in kisspeptin and GnRH secretion? This is not fully understood in humans (Plant and Witchel, 2006), but in nonhumans primates, one hypothesis is that a reduction in GABA leads to an increased secretion of kisspeptin which then drives the pubertal increase in GnRH pulse frequency and amplitude. Whether kisspeptin reduces the GABA tone remains to be elucidated (Terasawa et al., 2010). Furthermore, it appears like

the pubertal increase in kisspeptin and GnRH is independent from the pubertal increase in sex steroids in primates (Plant and Witchel, 2006, Guerriero et al., 2012b). Though, studies have shown that the GnRH response to kisspeptin is abolished by ovariectomy in pubertal, but not prepubertal monkeys, and partly reversed by estradiol displacement. This suggests that the GnRH response mediated by Kiss1R undergoes changes after the exposure to the pubertal increase in estrogen in primates (Guerriero et al., 2012a).

In rodents, estrogens seem to be implicated in the developmental changes in kisspeptin (Clarkson et al., 2009a, Mayer et al., 2010). This is supported by a study showing that a genetic deletion of ERα in kisspeptin neurons decreases the number of kisspeptin cells in AVPV in female mice (Mayer et al., 2010). In keeping, ovariectomy at PND15 reduces the number of kisspeptin neurons in AVPV with 70-90% in both prepubertal and adult female mice which was normalized by estradiol displacement (Clarkson et al., 2009a). Same study showed that aromatase deficient female mice display no kisspeptin neurons in AVPV (Clarkson et al., 2009a). Both Clarkson et al. (2009a) and Mayer et al. (2010) had difficulties quantifying the number of kisspeptin cells in ARC in the female mice due to a dense fiber network., however no apparent change was detected by Clarkson et al. (2009a) whereas a decrease was detected upon the genetic deletion of ERα in kisspeptin neurons (Mayer et al., 2010). The importance of an ERα dependent regulation of kisspeptin and pubertal maturation is stressed by the impaired LH secretion in ERα deficient mice and by the absence of a normal estrous cycle (Mayer et al., 2010).

In rodents, the juvenile restraint of GnRH release is believed to be mediated by an estradiol dependent inhibition of GnRH secretion through kisspeptin neurons in ARC, whereas the subsequent pubertal stimulation of GnRH secretion is believed to depend on estradiol stimulation through kisspeptin neurons in AVPV (Mayer et al., 2010). In keeping, one hypothesis is that the increase in estradiol exposure increases kisspeptin in AVPV through positive feedback, which is established around PND20 in rodents. The increase in AVPV kisspeptin leads to an increase in GnRH neuron activity and a subsequent increase in estradiol, establishing a circuit driving the pubertal development. AVPV kisspeptin neurons can then be seen as estradiol dependent GnRH neuron activity amplifier where estradiol is essential for the appearance of kisspeptin expression in AVPV (Figure 7) (Clarkson et al., 2009a, Clarkson et al., 2010, Mayer et al., 2010). Whether estradiol drives the kisspeptin expression in other species remains to be defined (Garcia-Galiano et al., 2012).

It still remains unknown whether estradiol is a permissive factor, or driving the increase in kisspeptin in AVPV and thereby pubertal timing in rodents (Clarkson et al., 2009a). The latter possibility is supported by the presence of ERα in AVPV kisspeptin neurons in rodents (Smith et al., 2006b, Mayer et al., 2010), and by the ability of estrogen to exert a positive feedback on *Kiss1* expression in AVPV established at PND20 (Clarkson et al., 2009a). The importance of estradiol is further supported by the presence of ERE motifs in the *Kiss1* promotor in mice (Smith et al., 2005a) since ERE signaling is believed to be responsible for the positive feedback of estradiol (Oakley et al., 2009).

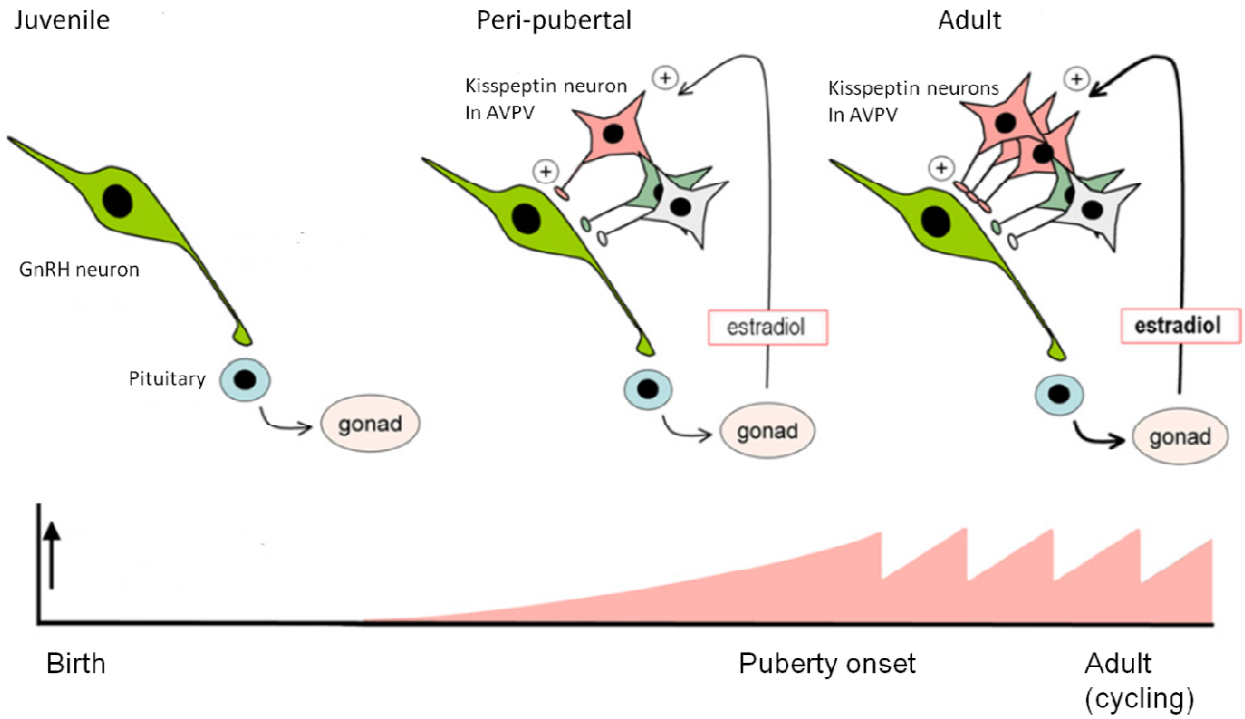


Figure 7. An illustration of the activation of AVPV kisspeptin neurons by estradiol at pre-puberty and establishment of the positive circuit composed of AVPV kisspeptin neurons, GnRH neurons, and estradiol. An initial increase in estradiol in prepuberty increases kisspeptin in AVPV through positive feedback from around PND20. At puberty, kisspeptin expression is fully developed and functioning to establish the positive circuit by amplifying GnRH neuron activity driving the pubertal development. See text for details. *Modified from (Clarkson et al., 2010).*

Reproduction – focus on kisspeptin

As described in *Kisspeptin – the most potent elicitor of GnRH secretion*, kisspeptin plays a pivotal role in the regulation of the reproductive axis, which is underlined by clinical data showing that reproductive capacity is impaired in humans and mice suffering from impaired kisspeptin-signalling (de Roux et al., 2003, Seminara et al., 2003, Lapatto et al., 2007).

Ovulation is triggered by a preovulatory LH surge, and the preovulatory LH surge, in rodents, appears as a pronounced increase in LH levels during the afternoon of proestrous followed by a drop in LH levels in the morning of estrous (Pineda et al., 2010), which is induced by a positive feedback of estrogens (Smith et al., 2006b). The mechanisms through which estrogens triggers the GnRH surge and thereby the preovulatory LH surge remains elusive (Clarkson and Herbison, 2009), but recent data suggest that kisspeptin plays a critical role in the regulation of the preovulatory LH surge (Pineda et al., 2010, Smith et al., 2011, Plant, 2012).

Kisspeptin – a central regulator of the preovulatory LH surge and estrous cyclicity

A pharmacologic approach aiming to reveal the role of kisspeptin in relation to regulation of the LH surge has primarily been conducted with kisspeptin agonists. These studies have shown that kisspeptin induces a surge-like LH secretion in mammalian species including rodents and humans

(Dhillo et al., 2005, Kinoshita et al., 2005, Dhillo et al., 2007, Clarkson and Herbison, 2009), whereas a kisspeptin antagonist blocks the preovulatory LH surge in rats and monkeys (Pineda et al., 2010) (Shahab et al., 2005). Also, a lack of LH surge is reported in rats subjected to kisspeptin neutralisation by monoclonal antibodies (Kinoshita et al., 2005). The above data is in keeping with the absence of a kisspeptin induced surge-like LH secretion in Kiss1R deficient mice (Lapatto et al., 2007). In addition, kisspeptins ability to trigger ovulation has been reported in rats primed with gonadotropins to induce ovulation, where kisspeptin administration dramatically increases the number of ovulated oocytes (Matsui et al., 2004).

The AVPV kisspeptin neurons appear to be central players in the regulation of the preovulatory LH surge in rodents (Smith et al., 2006b). Immunohistochemical data shows a huge increase in the number of AVPV kisspeptin neurons coexpressing cFos on the afternoon of proestrouns compared to the second day of diestrous, showing that AVPV kisspeptin neurons are activated during the LH surge (Smith et al., 2006b). In addition to this, a peak in *Kiss1* expression in AVPV is occurring concomitant with the LH surge in rats (Smith et al., 2006b). As reviewed in Clarkson and Herbison, several studies support that the AVPV kisspeptin neurons are critical for the regulation of the preovulatory LH surge in rodents. Unlike AVPV kisspeptin neurons, ARC kisspeptin neurons do not seem to be involved in the preovulatory LH surge in rodents, but appear to be involved in the basal LH levels which involves the negative feedback action of estradiol on ARC kisspeptin neurons (Plant, 2012).

In contrast to rodents, POA kisspeptin neurons in higher primates, homolog to AVPV in rodents, do not seem to play a role in the control of the preovulatory LH surge (Plant, 2012). However, ARC neurons seems to be involved in the positive feedback action of estradiol that regulates the preovulatory LH, since Kiss1 mRNA in ARC increases in the late folicular phase (Plant, 2012). This idea is challenged since ARC kisspeptin appears to be negatively regulated by sex steroids in higher-primates (Dhillo, 2008).

Neutralisation of kisspeptin in cyclic rats by monoclonal antibodies against kisspeptin-54, not only abolished the preovulatory LH surge, but interfered with the estrous cyclicity depicted by a persistent estrous or diestrous (Kinoshita et al., 2005). This suggests that kisspeptin is not only important for the preovulatory LH surge, but also estrous cyclicity in rats. This is in keeping with previous literature showing the absence of a normal estrous cycle in kisspeptin or Kiss1R deficient mice (Lapatto et al., 2007) and in mice with a timed elimination of kisspeptin neurons at PND20 (Mayer and Boehm, 2011). The importance of kisspeptin, in relation to regulating the estrous cyclicity, is further supported by the fact that *Kiss1* expression in AVPV and ARC are dynamic throughout the estrous cycle (Navarro et al., 2004a, Smith et al., 2006b). In female rats, a gradual increase in *Kiss1* expression in AVPV is observed from the second day of diestrous to proestrous, where it peaks on the afternoon of proestrous, and decreases again at estrous (Smith et al., 2006b). The highest *Kiss1* expression in ARC is observed on the second day of diestrous with a gradual decline at proestrous and the lowest mRNA levels in estrous (Smith et al., 2006b).

The nutritional status and the effect on the HPG axis – focus on kisspeptin

Metabolic control of the HPG axis via kisspeptin

A critical amount of body energy stores are needed for pubertal development, normal sexual maturation, cyclicity and fertility (Parent et al., 2003, Plant and Barker-Gibb, 2004, Donato et al., 2011a). Energy status is conveyed to the brain by numerous peripherally generated signals such including leptin, insulin, and ghrelin (Havel, 2001) and also by central peptides including NPY (Wu et al., 2009) in order to maintain energy homeostasis. As described, a positive nutritional status has an effect on the HPG axis depicted by an advanced pubertal timing and impaired reproductive function in girls and women associated with obesity markers (He and Karlberg, 2001, Davison et al., 2003, Lee et al., 2007, Nelson and Fleming, 2007, Pasquali et al., 2007, Kaplowitz, 2008, Aksglaede et al., 2009a, Rosenfield et al., 2009, Maisonet et al., 2010). Also, malnutrition and extreme physical exercise are associated with delayed pubertal maturation and reproductive dysfunction in both humans and animal models (Frisch, 1994, Cunningham et al., 1999, Parent et al., 2003), implying that certain amounts of energy stores is required for a proper reactivation and subsequent function of the HPG axis (Frisch, 1994).

In recent years, much focus has been on kisspeptin's potential contribution to the metabolic control of the HPG axis. Prerequisites for this are that: (i) a change in kisspeptin-tone at the GnRH neurons as a result of a change in nutritional status, and (ii) a nutritional induced effect on pubertal timing that can be abolished by kisspeptin administration. (i) The first requirement, have been confirmed in several species including rodents and higher primates. A negative energy status has been associated with reduced hypothalamic *Kiss1* mRNA levels (Castellano et al., 2005, Luque et al., 2007, Backholer et al., 2010, Castellano et al., 2011, Wahab et al., 2011), and a decreased number of kisspeptin cells in ARC (Castellano et al., 2010, Castellano et al., 2011). In contrast, a positive energy status has been shown to increase hypothalamic *Kiss1* mRNA levels in rats (Castellano et al., 2011). (ii) Also, the second requirement has been confirmed. Female rats subjected to malnutrition experienced a delayed puberty onset, which was rescued by chronic kisspeptin administration (Castellano et al., 2005, Iwasa et al., 2010).

Many peripheral and central factors are implicated in the metabolic control of GnRH neurons (Navarro et al., 2004b, Castellano et al., 2010), but they may act though a modulation of *Kiss1* expression (Luque et al., 2007, Forbes et al., 2009, Wu et al., 2009, Castellano et al., 2010), and kisspeptin has appeared as a plausible key molecular conduit relaying the metabolic signals upon the GnRH neurons (Castellano et al., 2006b).

Leptin

The adipocyte-derived hormone leptin is a pleiotropic integrator signaling the magnitude of body energy stores to centers that regulate appetite and reproduction through modulation of GnRH neurons (Donato et al., 2011a). This is supported by hyperphagic obesity, infertility and lack of pubertal maturation in humans and mice lacking leptin (*ob/ob*) or leptin receptors (LepR) (*db/db*) (Donato et al., 2011a, Donato et al., 2011b). GnRH neurons do not express leptin receptors (Cunningham et al., 1999), which indicates involvement of intermediate circuits in the leptin-dependent modulation of GnRH neurons (Donato et al., 2011a).

Studies have shown that *ob/ob* mice have a decreased Kiss1 mRNA level in ARC, but not in AVPV, and an increased body weight (Smith et al., 2006a, Quennell et al., 2011). Kiss1 mRNA levels in ARC and body weight were partly reversed by leptin treatment (Smith et al., 2006a) suggesting that other metabolic factors regulate *Kiss1* expression. Intriguingly, total hypothalamic Kiss1 mRNA levels were unchanged in *ob/ob* mice (Luque et al., 2007). Though, the discrepancy between the *ob/ob* studies could be due to differences in the hormonal milieu and a regional dilution in Luque et al. (2007). In accordance, a negative energy status, which is associated with a lower leptin level (Ahima, 2000, Luque et al., 2007) decreases hypothalamic Kiss1 mRNA levels (Castellano et al., 2005, Luque et al., 2007, Wahab et al., 2011). One study has shown that the regional decrease in Kiss1 mRNA, induced by a negative energy status, takes place in ARC and not in AVPV (Matsuzaki et al., 2011) which is in keeping with the data obtained in *ob/ob* mice (Smith et al., 2006a, Quennell et al., 2011).

ARC kisspeptin neurons appear to be a plausible candidate as an intermediate in the circuit that relays the body energy status via leptin to GnRH neurons. In order for ARC kisspeptin neurons to relay the leptin-signaling to GnRH neurons, they have to express *Lepr*. A subpopulation of the kisspeptin neurons have been found to express *Lepr* in male mice (Smith et al., 2006a) and ewes (Backholer et al., 2010). However, several other approaches, including the use of transgenic mice, were unable to confirm this (Louis et al., 2011). The latter supporting that kisspeptin constitute an indirect relay for leptin, agreeing with the fact that genetic deletion of *Lepr* from hypothalamic kisspeptin neurons in mice have no affect on puberty or fertility (Donato et al., 2011b).

Leptin seems only to account as a permissive factor in timing the reactivation of the HPG axis, which is exemplified by several observations. First, the level of leptin increases gradually during pubertal development, but no apparent increase occurs at puberty onset in humans (Ahmed et al., 2009), which is supported by longitudinal studies conducted in male monkeys (Plant and Barker-Gibb, 2004). Secondly, leptin administration to prepubertal monkeys (Barker-Gibb et al., 2002) and rats (Cheung et al., 1997) lack the ability to advance puberty onset. Third, kisspeptin's gonadotropin-releasing ability is preserved in condition of suppressed leptin signaling caused by fasting, endogenous leptin immunoneutralisation, and leptin resistance due to leptin receptor deficiency (Navarro et al., 2004b, Castellano et al., 2005).

The above data strongly support that the kisspeptin system is downstream to or eventually independent of leptin in the control of the HPG axis, and although adequate levels of leptin are permissive for a normal pubertal development and reproductive attainment, leptin is not sufficient to trigger puberty onset. This also supports that leptin is not the only factor implicated in the metabolic regulation of kisspeptin and GnRH neurons.

Ghrelin and neuropeptide Y

Ghrelin, a gut derived hormone signaling energy insufficiency promoting food intake, has been reported to be a negative regulator of gonadotropin secretion, and a regulator of puberty onset in male rats (Fernandez-Fernandez et al., 2005, Tena-Sempere, 2008). This coulkd be mediated by kisspeptin neurons, since ghrelin has been found to be a negative modulator of *Kiss1* expression in AVPV, but not in ARC (Forbes et al., 2009). Though, since ghrelin is a functional antagonist of leptin, it is possible that the observed change in *Kiss1* expression is mediated by leptin.

Another plausible factor is NPY, an orexigenic peptide that has been found to inhibit reproduction in sheep (Backholer et al., 2010). NPY seems to play a role in regulation of the HPG axis, but both inhibitory and excitatory effects of NPY have been reported depending on the stage of maturation and sex steroid milieu (Castellano et al., 2010). Fasting for 48hour, increased NPY expression in ARC, and decreased *Kiss1* expression in AVPV in female rats (Kalamatianos et al., 2008), implying that NPY is a negative modulator of *Kiss1* expression. A positive modulation of NPY on *Kiss1* expression has been reported in NPY deficient mice and *in vitro* (Luque et al., 2007). NPY, being a putative regulator of kisspeptin, could be implicated in a nutritional regulation of pubertal timing and reproductive capacity through kisspeptin. This idea is challenged since only a small number of NPY neurons in ARC projects to AVPV where the change in *Kiss1* expression occurs during fasting (Kalamatianos et al., 2008), and by the absence of overtly infertility in NPY deficient mice (Luque et al., 2007). It should be noted that GnRH neurons express NPY receptors (Pralong, 2010) making it possible that NPY modulates GnRH release independent of kisspeptin.

Whether nutritional induced changes in puberty onset and reproductive function are associated with changes in ghrelin, and/or NPY, and subsequently *Kiss1* expression, remains unexplored.

Nutritional effects on sex steroid levels and bioavailability

Obesity is associated with increased sex steroid levels (de Ridder et al., 1992, Barber et al., 2006, Dunger et al., 2006, Ahmed et al., 2009, Brill and Moenter, 2009), and increased bioavailability hereof due to a decreased level of sex-steroid binding protein (Ahmed et al., 2009). Obesity is also associated with an increased aromatase activity thereby facilitating the conversion of androgens to estrogens (de Ridder et al., 1992). Whether an increase in androgens or estrogens is implicated in the metabolic regulation of pubertal timing and reproductive capacity is not clear. One study has shown that the high-fat diet induced advancement of puberty onset, was reversed by an androgen receptor

antagonist, suggesting that the effect of a high-fat diet on puberty onset is partly due to an increased androgen action (Brill and Moenter, 2009). Hyperandrogenamia, which is the most consistent feature in PCOS, is strongly associated with reproductive dysfunction and increased BMI (Barber et al., 2006), supporting that androgens play a role in the metabolic control of the HPG axis. However, less is known about the mechanisms that relay the sex steroid modulation on the GnRH neurons, but since *Kiss1* expression in ARC and AVPV is under the control of sex steroids (Smith, 2009) it is possible that kisspeptin is a central conduit for the nutritional regulating effect of sex steroids on the HPG axis.

Neuronal programming of neuroendocrine networks involved in puberty and reproduction

An early nutritional challenge has been associated with changes in pubertal timing, indicating that developmental changes persist over the long term (Castellano et al., 2011). Recent evidence suggests that specification of the neuroendocrine circuits implicated in regulation of puberty onset and reproduction are sensitive to nutritional influences during the gestational and postnatal period (Hilakivi-Clarke et al., 1997, Parent et al., 2003, Castellano et al., 2005, Boukouvalas et al., 2008, Chang et al., 2008, Brill and Moenter, 2009, Lo et al., 2009, Sloboda et al., 2009, Boukouvalas et al., 2010, Moral et al., 2011). Most of the rodent studies addressing the role of early nutritional programming have focused on malnutrition during pregnancy and/or lactation, and only few have focused on a positive nutritional status. The studies have mainly been descriptive, and no underlying mechanism have been identified (Castellano et al., 2011).

Leptin-dependent projections from arcuate nucleus

AVPV and ARC kisspeptin neurons undergo neuroanatomical maturation during postnatal development as depicted in Figure 4. Also a functional activation of the kisspeptin-Kiss1 system occurs prior to puberty which includes a change in the number of close appositions between GnRH neurons and kisspeptin fibres which appear from PND25 onwards in mice (Clarkson and Herbison, 2006). This indicates that either new kisspeptin projections are formed or that kisspeptin synthesis in an existing fibre is initiated around PND25.

Developmental profiling of ARC projections have been studied in neonatal mice from PND6 to PND21. Adult like fibre distribution is attainted at PND18 in mice, and ARC projections to each of the periventricular nuclei in the hypothalamus is present at PND18 (Bouret et al., 2004a), in the same regions where ARC kisspeptin fibres were identified by Yeo and Herbison (2011). The postnatal profile revealed a neuroanatomical maturation of ARC projections. For instance, the ARC projections to POA appeared to be some of the last to reach their hypothalamic targets, and were first established between PND12 and 18 (Bouret et al., 2004a). The projections from ARC to AVPV developed rapidly and the adult like distribution was achieved by PND18 (Bouret et al., 2004a).

A surge of leptin occurs during the first week of life, but is not accompanied by a reduction in food intake. This has led to the hypothesis, that leptin is involved in developmental signaling (Bouret et al.,

2004b). A developmental profile of ARC projections have also been studied in *ob/ob* mice, which has revealed how important this perinatal leptin surge is for the formation of ARC projections (Bouret et al., 2004b). This is based on a dramatic decrease in ARC fiber density to all the terminal points in leptin deficient mice, which could only be reversed by exogenous leptin treatment from PND4 to 12, and not by adult leptin treatment (Bouret et al., 2004b). The same defect in ARC projections is seen in diet induced obese bred rats, which are known to be leptin resistant before they become obese (Bouret et al., 2008). *In vitro* studies with ARC explants from mice, show how leptin exposure induce the formation of neurites from ARC explants, suggesting that leptin acts on ARC to induce axon elongation and proliferation (Bouret et al., 2004b).

The overlapping distribution of ARC projections found by Bouret et al. (2004a) (Bouret et al., 2004a) (Bouret et al., 2004a) (Bouret et al., 2004a) (Bouret et al., 2004a) (Bouret et al., 2004a) (Bouret et al., 2004a) (Bouret et al., 2004a) and ARC kisspeptin projections identified by Yeo and Herbison (2011), strongly support that the ARC kisspeptin fibre projections undergo neuroanatomical maturation in a leptin-dependent manner in the postnatal period.

Aims

So far there has been limited data on the effects of a postnatal exposure to a high-fat diet on pubertal timing and estrous cyclicity in animal models, and the possible changes in the kisspeptinergic system have only been studied scarcely. This has become a matter of translational interest given the increasing incidence of obesity during childhood and adulthood believed to be associated with earlier puberty onset in girls (Kaplowitz, 2008, Ahmed et al., 2009) and reproductive dysfunction in women (Linne, 2004, Pasquali et al., 2007).

Therefore, the first aim of this thesis was to evaluate the effects of a high-fat-diet exposure in different postnatal periods, being lactation, post-weaning, or both periods, on pubertal timing, revealed by V.O., and to assess whether the kisspeptinergic system was involved in this. Changes in the kisspeptinergic system were evaluated by assessing changes in Kiss1 mRNA and kisspeptin-immunoreativity (kisspeptin-ir). This was done in order to define the critical window of exposure on pubertal timing and kisspeptin.

The second aim was to evaluate the effects of a high-fat-diet exposure from weaning and into early adulthood on estrous cyclicity in rats, and to evaluate if the kisspeptinergic system was affected.

Several peripheral signals implicated in the signalling of body energy status and hormones, some being possible modulators of *Kiss1* expression, were also evaluated both in the pubertal and adult rats. These include leptin, insulin, triglycerides, and estradiol.

The third aim was to evaluate Kiss1 mRNA and number of kisspeptin-ir cells at peri-puberty (PND 34) and in adulthood (PND61) in order to address the profile of the kisspeptinergic system in relation to the stage of pubertal maturation based on V.O. and from peri-puberty to early adulthood.

Materials and Methods

Animals

All animal experiments were carried out in accordance with the regulations provided by the Danish Animal Experimentation Inspectorate (J. No. 2007/561-1320), and treated in concordance with the European Communities Council Directive of 24th November 1986 (86/609ECC). Female Sprague-Dawley rats were purchased from Charles River (Charles River Laboratories International, Germany) at PND14 for experiment 1, 1B, and 4, and at gestation day 14 for experiment 2 and 3. Newborn litters in experiment 2 and 3 were corrected at PND1 so that litter size did not exceed 12pups per litter. The rats purchased at PND14 were divided upon arrival to obtain experimental groups balanced for body weight. All pups were weaned at PND21. Experiment 1, 1B, and 4 were carried out simultaneously, while experiment 2 and 3 were carried out simultaneously. All rats were housed in the animal facilities at Rigshospitalet, Copenhagen University Hospital (Rigshospitalet, Denmark), and acclimatized at least 7 days prior to the experiments. All rats were maintained under conditions of 12 hour light/dark (light on at 7:00am) with free access to water and test diet *ad libitum*.

Test diets

I this study, the effects of a postnatal high-fat diet exposure in different developmental periods on pubertal timing and estrous cycle regularity were evaluated in relation to changes in Kiss1 mRNA and kisspeptin levels. In house data shows that body weight gain and changes in kisspeptin, in adult male Wistar rats, were most pronounced with a diet containing 60% energy from fat in contrast to a diet with 45% energy from fat (unpublished data). Based on this, a diet with 60% energy from fat was chosen as the high-fat diet for the present experiments. The control diet and high-fat diet were purchased as pellets from TestDiet (TestDiet, USA), and stored at -20° degrees until used. The control diet (TestDiet® Formula 58Y2, USA) contained 4.3% fat by weight corresponding to 10% energy from fat (see Appendix I for details). The high-fat diet (TestDiet® Formula 58Y1, USA) contained 34.9% fat by weight corresponding to 60.9% energy from fat (see Appendix II for details). The control and high-fat diet were balanced so an equal amount of calories leads to the same amount of ingested protein, fiber, and micronutrients.

Table 1. The nutritional profile of the control and high-fat diet from Test-Diet (see Appendix I and II).

Test diet			Control diet	High-fat diet
Fat	% fat by weight		4.3	34.9
	% energy[1]		10.2	60.9
	Fat (% energy from the different types of fat)	Soybean oil	56.56	9.25
		Lard	44.44	90.75
Protein	% energy		18.3	18.3
Carbohydrate	% energy		71.5	20.1
Kilocalories/g			3.78	5.16

[1]% of total energy based on amount of kcal/g.

Experimental designs

The effect of a postnatal high-fat diet, in different postnatal periods, on puberty onset and the kisspeptinergic system

Female Sprague-Dawley rats were fed a high-fat diet or a control diet in three different postnatal periods (Figure 8). A previous study, using small litter size as a model of overfeeding, has shown that at PND34 all overfed female Wistar rats, had entered puberty in contrast to approximately 45% of the normal fed rats (Castellano et al., 2011). Based on this study, it was chosen to sacrifice all animals in experiment 1, 2, and 3 at PND34 in order to obtain maximal differences in pubertal timing and Kiss1 mRNA and in the number of kisspeptin cells between the groups. In experiment 1B, all animals were weighted and inspected for puberty onset daily. In contrast to experiment 1, 2, and 3, they continued with the test diet and were included in experiment 4, where the effect of a high-fat diet on estrous cyclicity was evaluated.

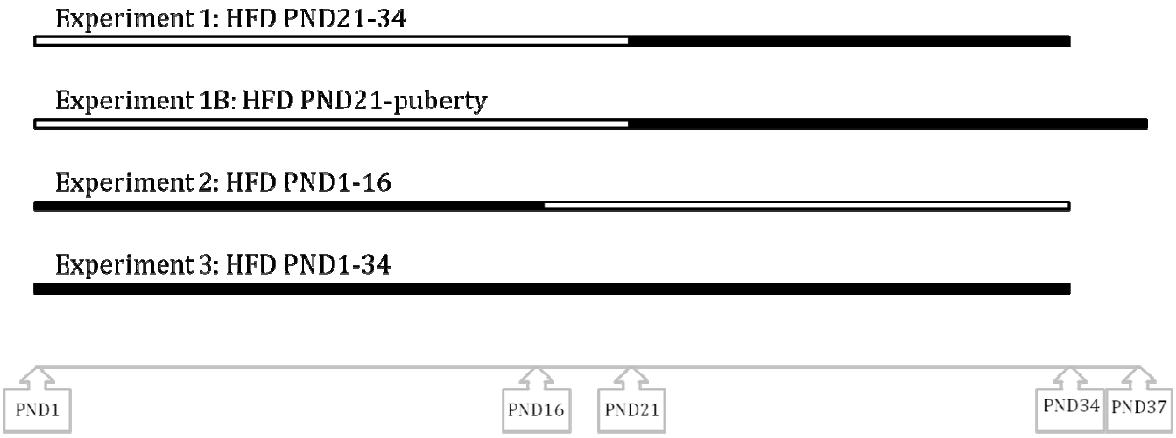


Figure 8. Illustration of the postnatal high-fat diet exposure periods conducted in female rats in respect to pubertal timing. The black filling represents the period where the rats were exposed to the high-fat diet, whereas the white filling represents the control diet period.

Experiment 1 – post-weaning high-fat diet exposure

In experiment 1, the effects of a post-weaning high-fat diet exposure on pubertal timing and the kisspeptinergic system were evaluated in rats fed either the control diet or the high-fat diet from weaning until PND34 (n=20). This group is denoted high-fat diet (HFD) PND21-34. Before weaning, all rats were fed the standard chow with 10% of the energy from fat. The rats were given identity mark at PND21, and body weight was assessed from PND21 and twice a week. V.O. defined as complete canalization of the vagina, which is an external physical sign of puberty (Navarro et al., 2004b), was inspected every morning from PND30 to 34 in which the body weights were noted as well. At PND34 after inspecting V.O., the pups were either decapitated (n=12) or transcardially perfused (n=8) in the morning, and trunk and heart blood, respectively, were collected in order to measure the levels of leptin, insulin, triglycerides, and estradiol in the plasma (see *Hormone and metabolic factor measurements* for further details). Brains removed from the decapitated rats, were used to measure

Kiss1 mRNA levels in ARC and AVPV by quantitative real-time PCR (qRT-PCR) (see *RNA analysis by qRT-PCR* for further details). Brains from transcardially perfused rats were used to determine the total number of kisspeptin-ir cells in ARC and AVPV by immunohistochemistry (IHC) which is described in more details in *Immunohistochemistry*.

Experiment 1B – post-weaning high-fat diet exposure

In experiment 1B, exposure to a high-fat diet from PND21 to puberty was carried out to evaluate the effect on body weight and V.O. The rats were fed the control or high-fat diet from weaning until puberty (n=24). This group is denoted HFD PND21-puberty. Before weaning, all rats were fed the standard chow. Body weight was noted from PND21 and twice a week, and both body weight and V.O. were inspected every morning from PND30 until all animals had entered puberty which occurred no later than PND37. The rats were not sacrificed at PND34, but continued with the respective test diet until adulthood, and were used in experiment 4.

Experiment 2 - postnatal high-fat diet exposure during lactation

In experiment 2, the effects of a high-fat diet exposure through lactation on pubertal timing and *Kiss1* expression were evaluated. This was done by feeding the dams the control diet (litter n=6, and pups n=27), or the high-fat diet (litter n=3, and pups n=12) during lactation from PND1 to 16, based on the fact, that pups begin to eat pellets around PND17. At PND17 the diet was switched to the control diet. This group is denoted HFD PND1-16. The rats were given identity mark at PND21, and body weight was assessed from PND7 and weekly until PND28. Both body weight and V.O. were inspected every morning from PND30 to 34 were the pups were decapitated in the morning after inspecting signs of V.O.. Trunk blood was collected to conduct the same measurements in the plasma as for experiment 1, and brains were collected in order to measure Kiss1 mRNA levels in ARC and AVPV.

Experiment 3 – postnatal high-fat diet from birth to puberty

In experiment 3, the effects of high-fat diet exposure from PND1 to 34 on pubertal timing and *Kiss1* expression were evaluated. This was done by feeding dams the control diet (litter n=6, and 27 pups), or the high-fat diet (litter n=3, and 16 pups) during lactation from PND1 until weaning, and by continuing with the same diet after weaning until PND34. This experimental group is denoted HFD PND1-34. Note, that the control group in experiment 3 is the same as in experiment 2 since the two experiments were carried out simultaneously. Bodyweight was assessed from PND7 and once weekly until PND28 and marked with an ID on PND21. Body weight and V.O. were assessed every morning from PND30 to 34 were the pups were decapitated in the morning, after inspecting signs of V.O.. Trunk blood and brains were collected in order to measure leptin, insulin, triglyceride, and estradiol levels in the plasma, and Kiss1 mRNA levels in ARC and AVPV.

Experiment 4 – high-fat diet from weaning and to early adulthood

In experiment 4, the effects of a high-fat diet exposure from weaning and for 40 days on estrous cycle regularity and the kisspeptinergic system were evaluated. This was carried out by feeding the rats either a control diet or the high-fat diet from PND21 and for 40 days. Body weight was noted from PND21 twice a week and weekly from PND40 to 61. Vaginal smears were taken every morning between 8am and 9am, as described by Goldman et al. (2007). The smears were collected from PND50 and for 10 consecutive days, and continued until two days of diestrous were occurring in a row for the first time. This occurred between PND61 and 68. The hematoxylin and eosin y (H&E) staining method was applied to identify the different cell types in the smear, in order to determine the estrous cycle stages (see *Estrous cycle* for further details). The rats were sacrificed on the second day of diestrous the first time it occurred after PND60. Half of the rats were decapitated in order to conduct qRT-PCR to determine the Kiss1 mRNA levels in ARC and AVPV. The other half of the rats were transcardially perfused in order to conduct IHC to determine kisspeptin-ir cells in ARC and AVPV. Trunk blood and heart blood, respectively, were collected in order to measure the levels of leptin, insulin, triglycerides, and estradiol in the plasma.

Developmental changes in the kisspeptin system at peri-puberty and to early adulthood

Experiment 1 and 4 were conducted simultaneously and rats from experiment 1 and 4 were used to evaluate the profile of the kisspeptinergic system at PND34 in relation to the stage of pubertal maturation in regard to V.O., and from peri-puberty to early adulthood. The rats, were sacrificed at PND34 (experiment 1, both control and high-fat fed rats) and between PND61 and 67 (experiment 4, only control fed rats) in the diestrous phase. The rats were either decapitated (n=12) or transcardially perfused (n=8-12), and Kiss1 mRNA and kisspeptin-ir cells in ARC and AVPV were determined with qRT-PCR and IHC, respectively.

Hormone and metabolic factor measurements

Trunk blood from decapitated rats and heart blood from transcardially perfused rats was collected, and centrifuged 10 minutes at 2,000rpm (experiment 1 and 4) or 4,000rpm (experiment 2 and 3) at 4˚C. A white layer upon the plasma was present in several of the plasma samples from the high-fat fed rats after centrifugation, regardless whether the blood was centrifuged at 2,000 or 4,000rpm. Only the plasma, not the white layer, was collected, and stored at -20˚C until use. All samples from each experiment were assayed simultaneously, and intra- and inter assay variations were calculated.

Leptin

The level of leptin was determined with a sandwich ELISA assay (90040, Crystal Chem USA) according to the manufacturer's instruction. In brief, all plasma samples and guinea pig anti-leptin antibody were applied to the 96-well plate coated with anti-leptin antibody. Sample leptin binds simultaneously to the coated plate and to guinea pig anti-leptin antibody. After 16-20 hour incubation at 4˚C, all unbound antibodies were removed by washing. Next, samples were incubated with horse radish peroxidase conjugated to anti-guinea pig IgG for 3 hours at 4° C, and excess of enzyme was removed by washing. 3,3',5,5'-tetramethylbenzidine (TMB), which is converted to a coloured product by horse radish periosidase, was applied for 30 minutes, and the product was measured spectrophotometrically on a microplate reader model 680 (BioRad, USA) at 450nm using 655nm as reference (Figure 9). The leptin levels were determined from a standard curve. The sensitivity range for the assay is 0.2 - 12.8ng/mL, and no samples were ranging outside.

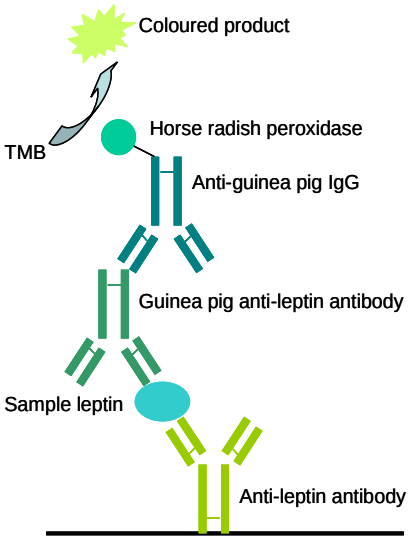


Figure 9. Plasma leptin determination by sandwich ELISA assay. Sample leptin binds to the anti-leptin antibody coated plate and is coupled to horse radish peroxidise through guinea pig anti-leptin antibody and anti-guinea pig IgG. Horse radish peroxidise converts 3,3',5,5'-tetramethylbenzidine (TMB) to a coloured product which is measured spectrophotometrically and an indirect measurement of the level of leptin in the sample. See text for details.

Insulin

Insulin levels were determined with a sandwich ELISA assay (10-1250-01, Mercodia, Sweden) using a similar method as for the leptin ELISA assay described above. However, the horse radish peroxidise conjugated to mouse anti-insulin is bound directly to the sample insulin in one step. The procedure provided by the manufacturer was followed, and the level of insulin was determined from a standard curve. Sensitivity range is 0.15 - 450µg/L, and no samples were ranging outside and excluded.

Triglyceride

Triglyceride levels were determined by an enzymatic cleavage kit (TR0100, Sigma-Aldrich, USA) according to the instructions provided by the manufacturer. Triglycerides do not circulate freely in the

blood, but are transported in lipoproteins. The method detects the triglyceride levels by an enzymatic cleavage of triglycerides in lipoproteins to glycerol and free fatty acids. Glycerol is further degraded in several enzymatic steps to a final product, a quinoneimine dye, which can be measured spectrophotometrically at 540nm. The quinoneimine dye corresponds to the initial triglycerides levels in the sample, and triglyceride levels were determined by the use of a standard curve. The sensitivity range for the kit is up to 10mg/mL, and no samples were excluded based on this. Though, it should be noted that the white layer on the plasma present after centrifugation of the blood was not included in the plasma. It is possible that lipoproteins were present in the white layer thus leading to an inaccurate triglyceride determination in the plasma samples.

Estradiol

Estradiol-17β levels, termed estradiol here, were determined by a radioimmunoassay kit (coat-a-count TKE21, Siemens Medical Solution Diagnostics, USA) according to the instructions provided by the manufacturer. The method is based on the competitive binding of ^{125}I-labeled estradiol and sample estradiol to estradiol antibody coated tubes. In brief, all samples, including the standards, were added to the estradiol antibody coated tubes along with ^{125}I-labeled estradiol, and incubated 3 hours at room temperature. After the incubation time, all liquid was decanted, and tubes were turned upside down and dried over night. The bound ^{125}I-labeled estradiol was measured with a Cobra™ II Auto-Gamma counter (Perkin-Elmer, USA) for 1 minute. The gamma counts are inversely related to the level of free estradiol in the sample, and the level of estradiol was determined from a standard curve. The sensitivity range for the kit is from 8pg/mL, and no samples were excluded based on this.

RNA analysis by qRT-PCR

The principle of qRT-PCR

qRT-PCR is a sensitive technique using a fluorescent reporter molecule to detect the level of complementary single stranded DNA (cDNA), and thereby gene expression levels. Before conducting qRT-PCR, total RNA is extracted from isolated tissue, and mRNA is reverse transcribed into cDNA. The fluorescence technique applied was 2x Brilliant II SYBR® Green qPCR Master Mix (Agilent Technologies, USA) where SYBR® Green is the fluorescent reporter molecule.

RNA extraction

Rats were decapitated and brains were removed instantly. ARC was isolated from the fresh brain with a small forceps by tweezing the forceps around the hypothalamic outpouching. ARC and brains were flash frozen on dry ice. AVPV, corresponding to RV3P, was isolated by cutting the frozen brains around the AVPV from Bregma 0.48 to -0.80 on a HM500 OM cryostat (Microm, Germany) like illustrated in Figure 10. The tissues were kept at –80° C until RNA extraction.

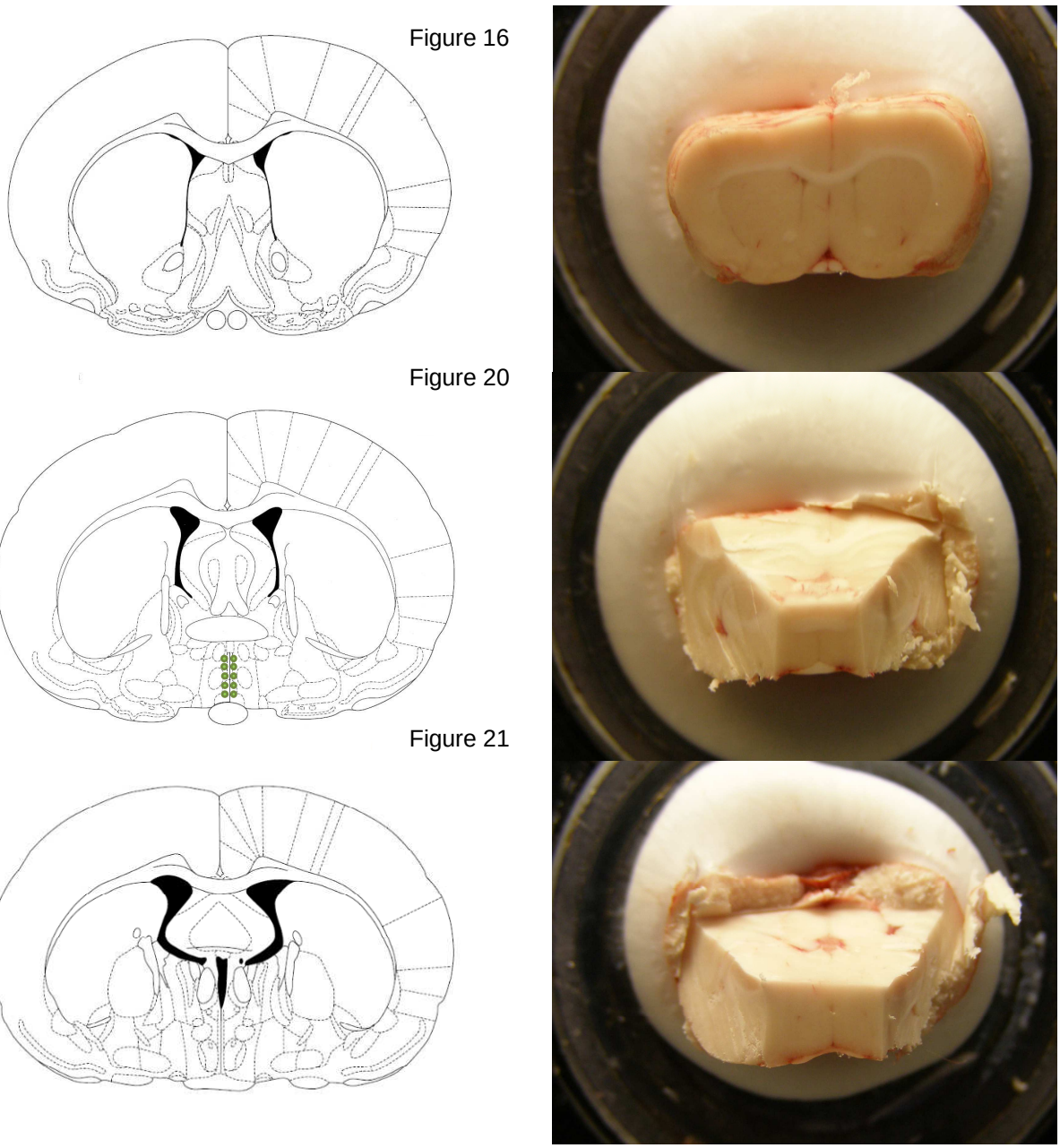


Figure 10. Isolation of the anteroventral periventricular nucleus (AVPV) for total RNA extraction. AVPV was isolated by cutting around AVPV from Bregma 0.48 to -0.80, corresponding to figure 16 to 21 in Paxinos and Watson (1986) on a cryostat.

The tissue for RNA extraction was kept on wet ice to avoid RNase degradation until the addition of TRIzol® reagent, which maintains the integrity of the RNA. The tissue was transferred to homogenization tubes, and 1mL of TRIzol (Tri-Reagent®, Sigma-Aldrich,USA) and a steel bead were added. The tissue was beaten 2 minutes on a mini-beadbeater (Biospec, USA) in order to homogenize the tissue. The homogenized tissue was kept at room temperature for 15 minutes, in which the tubes were inverted twice. The homogenized tissue was then transferred to SafeLock tubes, and 200µL chloroform (Sigma-Aldrich, USA) was applied. Chloroform denatures proteins so they become soluble

in the organic phase, while nucleic acids remain in the aqueous phase. Samples were mixed gently by inversion for 15 seconds, and left at room temperature, approximately 2-3 minutes, until the two phases were separated. Then, the tubes were centrifuged at 12,000rpm for 10 minutes at 4°C to enhance the separation. The clear water phase, containing the RNA, was transferred to new tubes, and an equal amount isopropanol was added to precipitate the RNA. The tubes were turned upside down 50 times, and incubated 15 minutes at room temperature, after which they were centrifuged like previously. Supernatant was discharged, and the pellet, containing the RNA, was dissolved in 1mL 70% EtOH, shaken, and centrifuged at 12,000rpm for 30 minutes at 4° C. The supernatant was discharged, and the pellet containing the RNA, was air dried. Next, 100µL ½x Tris-EDTA buffer (Sigma-Aldrich, USA) was added, and the samples were mixed and spun down before they were incubated for 10 minutes on wet ice in order to dissolve the RNA. The concentration of RNA was measured on a nanodrop spectophotomter ND-1000 (Saveen Werner, Sweden), and the extracted RNA was kept at –80°C until the reverse transcriptase was conducted.

Reverse transcriptase of mRNA to cDNA

The extracted RNA was transcribed to single stranded cDNA by the use of the ImProm-II™ Reverse Transcription System (Promega, WI, USA) according to the instructions. Extracted total RNA, approximately 0.5µg, was mixed with Oligo(dT)$_{15}$ (0.5µg/reaction) and incubated at 70°C for 5 minutes, and placed on ice. A mix containing Nuclease-free water, ImProm-II™ 5X Reaction buffer, 6mM $MgCl_2$, dNTP mix (final concentration of 0.5mM of each dNTP), Recombinant RNasin® Ribonuclease Inhibitor, and ImProm-II™ Reverse Transcriptase were combined according to the instructions, and added to the tubes. Subsequently, the mix was spun down, incubated 5 minutes at 25°C to enable the binding of Oligo(dT)$_{15}$ to the poly-A tail of the mRNA, and then incubated one hour at 42°C to reverse transcribe cDNA. Finally, samples were heated to 70°C for 15 minutes to inactivate the reverse transcriptase. The cDNA was diluted in 80µL ½x Tris-EDTA-buffer, and stored at –80°C.

Oligonucleotide primers for qRT-PCR

In this study, the expression levels of *Kiss1* were investigated. The expression level of the housekeeping gene *GAPDH*, encoding glyceraldehyde 3-phosphate dehydrogenase, was used as a reference gene, in order to normalize for differences in RNA concentrations e.g. due to variations in starting amount and RNA integrity. See

Table 2 for details on the primers used.

Table 2. Oligonucleotide primers used for qRT-PCR amplification of *Kiss1 and GAPDH*.

Gene	Primer*	Oligonucleotide-primer sequence 5'-3'	Length (base pairs)	Reference
Kiss1[1,3]	F R	5'-AGC TGC TGC TTC TCC TCT GT-3' 5'-GCA TAC CGC GGG CCC CCC TTT T-3'	139	Modified from Luque et al. (2007)

Kiss1[2,3]	F R	5'-TGG CAC CTG TGG TGA ACC CTG AAC-3' 5'-ATC AGG CGA CTG CGG GTG GCA CAC-3'	202	Navarro et al. (2004a)
GAPDH	F R	5'-CAT CAA GAA GGT GGT GAA GCA-3' 5'-CTG TTG AAG TCA CAG GAG ACA-3'	93	El-Sayed et al. (2011)

*F: forward primer, R: reverse primer, [1]used in experiment 1, 3, and 4, [2]used in experiment 2. [3]primer pairs span an intron.

The procedure for qRT-PCR

The qRT-PCR reaction was conducted following the protocol from the manufacturer with small modifications. In short, 5 µL of cDNA sample, 10µL mastermix containing SYBR® Green (Agilent Technologies, USA), and 15pmol of the reverse and forward primer for the cDNA template of interest (Kiss1 and GAPDH) were combined on ice. Distilled water was added to a final volume of 20µL. The 96-well plate was sealed, mixed, and centrifuged to collect all the reagents in the bottom. The qRT-PCR was performed on a LightCycler® 480 Real-Time PCR System (Roche, USA). The qRT-PCR was initiated by heating samples to 94°C for 10 minutes to activate hot-start *Taq* DNA polymerase followed by 40 PCR cycles of 94°C for 30 seconds to denature dsDNA, 60°C annealing for 45 seconds, and 72°C for elongation for 90 seconds. Melting curve for the amplicon was determined by continuously increasing the temperature from 50°C to 94°C to ensure homogeneity of the PCR product.

Dataanalysis

Data obtained from qRT-PCR reflects the number of cycles run before the exponential amplification phase occurs. The number of cycles is denoted the threshold cycles (Ct), and Ct is a measure for when the fluorescence intensity is significantly higher than the background. The Ct value reflects the initial level of the cDNA sequence of interest, and Ct is inversely related to the amount of amplicon. This means that higher initial levels of cDNA will need fewer cycles than samples with less cDNA to reach Ct. The default Ct settings for the LightCycler® 480 Real-Time PCR System was used in the present study. The fold change in expression levels were calculated by applying the comparative Ct method, described previously (Schmittgen and Livak, 2008), and normalized using the level of *GAPDH* mRNA as internal control gene as described before (El-Sayed et al., 2011). Fold change in expression within the gene of interest, *Kiss1*, and within the internal control gene, *GAPDH*, were calculated by subtracting all the samples Ct from the sample with lowest Ct by using the following equation:

$$Fold\ change\ (within\ gene\ of\ interest\ or\ internal\ control\ gene) = E^{(Ct\ lowest - Ct\ sample)}$$

Where E = efficiency = 100% = 2, Ct lowest = lowest Ct value within the group of samples to compare for the given gene, Ct sample = Ct value for each sample.

Fold change for the gene of interest was related to the internal control gene by using the following equation:

$$Fold\ change = \frac{E^{(Ct\ lowest - Ct\ sample)}}{E^{(Ct\ lowest\ GAPDH - Ct\ sample\ GAPDH)}}$$

Finally, the mean fold change in gene expression for the samples was related to the vehicle mean fold change in gene expression in order to see any change in the basal level of gene expression between groups.

Immunohistochemistry

The principle of immunohistochemistry

IHC is a method that enables detection of an antigen, e.g. peptide or protein, in fixated tissue by using antibodies directed against the given antigen. This allows detection of antigen-expressing neurons in particular brain regions. Staining of the cytoplasmic peptide kisspeptin was accomplished by using the so-called avidin-biotin complex method on free floating sections using 3,3'-Diaminobenzidine (DAB) as the peroxidase chromogen. For this method, avidin and biotin complexes to amplify the staining intensity thereby increasing the sensitivity. To stain kisspeptin, unlabeled primary antibody directed against kisspeptin is applied on the free floating sections, after which biotinylated secondary antibody directed against the Fc-part of the primary antibody is added. The avidin-biotin-peroxidase complex is applied, and binds to the biotinylated secondary antibody. Since avidin displays tetravalent affinity for biotin, it couples peroxidase to the biotinylated secondary antibody, whereby peroxidase comes into close proximity to the antigen. The chromogen DAB is converted by peroxidase to brownish precipitates in the presence of hydrogen peroxide (H_2O_2), and thereby stains the kisspeptin-containing cell compartment.

Preparation of tissue for immunohistochemistry

Anesthetized rats were transcardially perfused with isotonic saline (0.9% saline) until all blood was flushed, followed by perfusion with 4% paraformaldehyde in phosphate buffer with a pH=7.4 (Apoteket Rigshospitalet, Denmark) in order to fixate the tissue. Brains were collected and post fixated over night in 4% paraformaldehyde after which they were moved to 0.05M phosphate buffered saline (PBS) (Gibco-Invitrogen, New Zealand), and stored at 4˚C. Brains were dehydrated in 30% sucrose (Sigma-Aldrich, USA) in PBS three days prior to cutting. The brains were frozen and cut on a sliding freezing stage microtome (Bright Series 8000, firma Bright?) in 4 coronal sets in sections of 40µm thickness, and kept in cryoprotectant at –20˚C until the IHC was conducted. All groups of animals were cut in random order, and given an IHC ID number, blinding all the following steps and the quantification. All groups of animals were processed simultaneously, and one set of sections were used for each animal.

The immunohistochemistry procedure

First, the sections were rinsed 3x10 minutes in PBS, to remove the cryoprotectant, and then incubated with 1% H_2O_2 in PBS (Sigma-Aldrich, USA) for 10 minutes to block endogenous peroxidase. To block

nonspecific binding, the sections were then incubated 20 minutes in 5% normal swine serum in PBS with 1% bovine serum albumin (BSA) (Sigma-Aldrich, USA), and 0.3% Triton® X-100 (TX) (Merck, Germany), where TX is a detergent that permeabilizes the cell membrane. Subsequently, the sections were incubated in antiserum against kisspeptin (JLV-1) for 24 hours at 4˚C. The polyclonal antiserum JLV-1, directed against the long form of rat kisspeptin which is 52 amino acids, was used at 1:200 in 1% BSA and 0.3% TX in PBS. After incubation in antiserum, the sections were rinsed 3x10 minutes in PBS with 0.1% TX, in order to remove the antiserum. Sections were then incubated for 60 minutes in biotinylated donkey anti-rabbit (Jackson ImmunoResearch, USA) diluted 1:1000 with 1% BSA and 0.3% TX in PBS. After another rinse in PBS with 0.1% TX for 3x10 minutes, the sections were incubated 60 minutes in a solution containing avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector laboratories, USA) in PBS with 0.1% TX. Next, sections were rinsed for 10 minutes in PBS with 0.1% TX, 10 minutes in PBS, and subsequently 10 minutes in Tris-HCl (pH=7.6). Staining was carried out for 10 minutes using 0.1% DAB (Sigma-Aldrich, MO, USA) with 0.03% H_2O_2 in Tris-HCl resulting in a brownish precipitate in the cytoplasma. Finally, the sections were rinsed 5x6 minutes in PBS. The rinsed brain sections were mounted in 0.5% gelatine and 0.05% chromalum on gelatinised glass slides, dried, embedded in pertex (Histolab, Gothenburg, Sweden), and covered with glass slides.

Dataanalysis

The total number of kisspeptin-ir cells in AVPV and ARC were counted within the full extension of the respective regions in one series of sections under a light microscope using 10x and 40x objective lenses. The kisspeptin-ir cells were counted in the antero-posterior level in AVPV and ARC corresponding to Bregma -0.26mm to -0.80mm and Bregma -2.12mm to -4.52, respectively (Paxinos and Watson, 1986). Also, optical density (OD) was measured in AVPV using the software Image J (NIH, US), in order to determine the total level of kisspeptin-immunoreactivity in AVPV both including the soma and fibres. OD was measured on 2 brain sections corresponding to the early and late part of AVPV, and on both sides of the hemisphere, and the individual backgrounds were subtracted from each measurement. OD measurements were carried out on images taken with a light microscope (Axioskop2 plus, Zeiss, USA) using a 10x objective lens, and all images were taken simultaneously under the same conditions. OD is inversely related to kisspeptin-ir, and 1/mean OD was calculated for each animal.

Estrous cycle regularity

Evaluation of vaginal cytology and the estrous cycle

Progression through the estrous cycle can be identified by the presence of different cell types, and the distribution hereof, which corresponds to the status of the vaginal mucosa, uterus, and ovaries (Goldman et al., 2007). The H&E staining procedure is a method used to stain the nucleus and

cytoplasma, respectively, allowing the identification of different cell types. Hematoxylin stains the nuclei bluish or purplish since it is a basic dye, whereas eosin y stains the cytoplasma redish or pink since it is an acidic dye (MERCK, 2004). The stained vaginal smears were inspected under a light microscope. A predominance of round nucleated epithelial cells were considered as proestrous and denoted P, and a predominance of cornified a-nucleated epithelial cells was considered as estrous and denoted E. No distinction was made between diestrous 1 and 2 where leukocytes were present. If a proestrous smear contained a fewer number of cornified a-nucleated epithelial cells it were denoted Pe, with the dominating phase in capital letters (Figure 11) (Marcondes et al., 2002, Goldman et al., 2007).

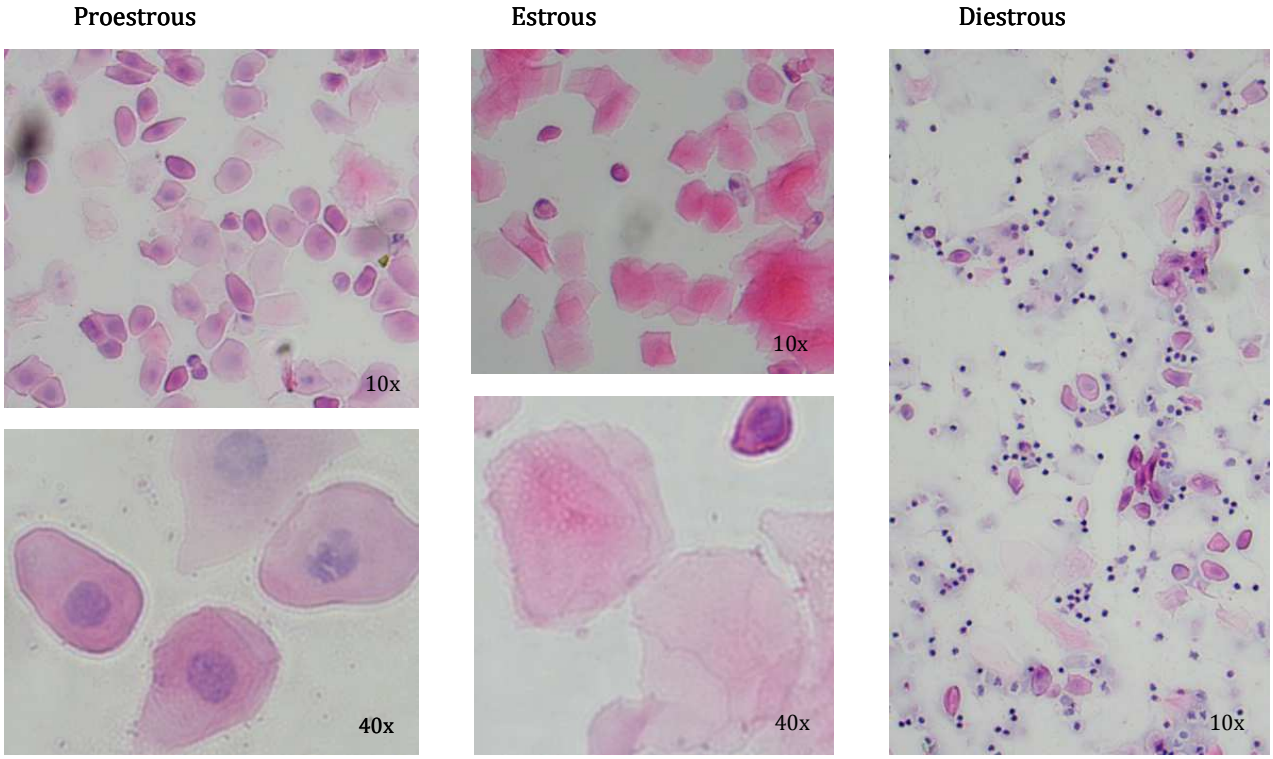


Figure 11. The phases of the estrous cycle after hematoxylin and eosin Y staining. Round nucleated epithelial cells are stained bluish or purplish in the nuclei, whereas the leukocytes will become even darker. The cornified a-nucleated epithelial cells will be stained pink. See text for details.

Preparation of vaginal smears

Vaginal smears taken early in the day or late in the afternoon will reflect the transition between the two consecutive phases. Therefore, it is important to take the samples the same time every day (Marcondes et al., 2002, Goldman et al., 2007). Vaginal smears were taken every morning between 8am and 9am, as described in Goldman et al. 2007. The smears were taken from PND50 and for 10 consecutive days, and continued until two consecutive days of diestrous were present. A clean cotton bud, moistened in isotonic saline, was inserted into the vaginal orifice, not deeper than 1 cm, and rotated. The cotton bud was then rolled on a glass slide to transfer the cells to the slide. New cotton

buds were used for each animal to avoid cross contamination of cells between animals. The glass slide with vaginal cells was dried after which the H&E staining was conducted.

H&E staining procedure

The dried glass slides were placed in hematoxylin solution Gill N°1 (Sigma-Aldrich, USA), for 3 minutes, and immersed 2 times in 0.3% HCl in 70% EtOH in order to decolorize the cytoplasma and remove excess dye from chromatin. Next, the slides were rinsed with running tap water for 1 minute, placed in eosin y solution aqueous 0.5% (Sigma-Aldrich, USA) for 3 minutes, rinsed in tap water for 30 seconds, and dehydrated by EtOH by placing them for 1 minute in 70% EtOH, 96% EtOH, and finally in 99.9% EtOH, respectively. The dehydrated glass slides were dried, embedded in pertex, coverslipped, and inspected under bright light microscope, where the phase of the estorus cycle was noted as described in *Evaluation of vaginal cytology and the estrous cycle*, without knowing the animal ID.

Data analysis of estrous cyclicity

The estrous cycle was dichotomized into a regular or irregular estrous cycle according to a modification of the classification described in Goldman et al. (2007). The estrous cycle was considered regular when it consisted of 1-2 days of E, 2-3 days of D½, and 1-2 days proestrous, whereas an irregular estrous cycle was considered to consist of 3-4 days E, 4-5 days D½, or 2-3 days P. An animal with one period or more corresponding to the characterization of an irregular cycle was noted to have an extended estrous cycle.

Presentation of data and statistical analysis

All statistical analyses were carried out using GraphPad Prism version 5.04 (GraphPad software, CA, USA). In experiment 2 and 3, the rats were siblings. Since the genetic background has an impact on pubertal timing (Maisonet et al., 2010), the genetic component was taken into consideration by using mean values per litter for statistical analysis (n=number of litters) unless otherwise states.

The impact of a high-fat diet exposure in different developmental periods on body weight was tested using Two-Way ANOVA with diet and time as independent factors. If a significant effect of main factors were found, Bonferroni multiple comparisons corrections were applied to correct for multiple comparisons, and to test for differences between subgroups. Weight gain in the test diet periods were tested by two-tailed unpaired student's t-test. It should be noted that body weights at PND16 was not available for HFD PND1-16, therefore body weights at PND18 were used instead. HFD PND1-34 was not weighed before PND 7 meaning that the weight gain is from PND7 to 34. The effect of high-fat diet exposure on pubertal timing was tested by the Log-rank. As for experiment 2 and 3, data for the pups, and not litters were included. The effects of the high-fat diet exposure on leptin, insulin, triglycerides, estradiol, and Kiss1 mRNA levels, and the total number of kisspeptin-ir cells were compared using two-tailed unpaired student's t-test in all experiments, except in experiment 4 where a One-Way ANOVA with a Dunnet's post hoc test was applied. The effect of a high-fat diet exposure on estrous

cycle regularity was tested by 2x2 contingency tables with Fischer's exact test. Correlation analyses were carried out by Pearson's correlations assuming Gaussian distribution of all the given parameters tested. Correlation analysis of data obtained in experiment 2 and 3 were based on data for the pups, and not per litter.

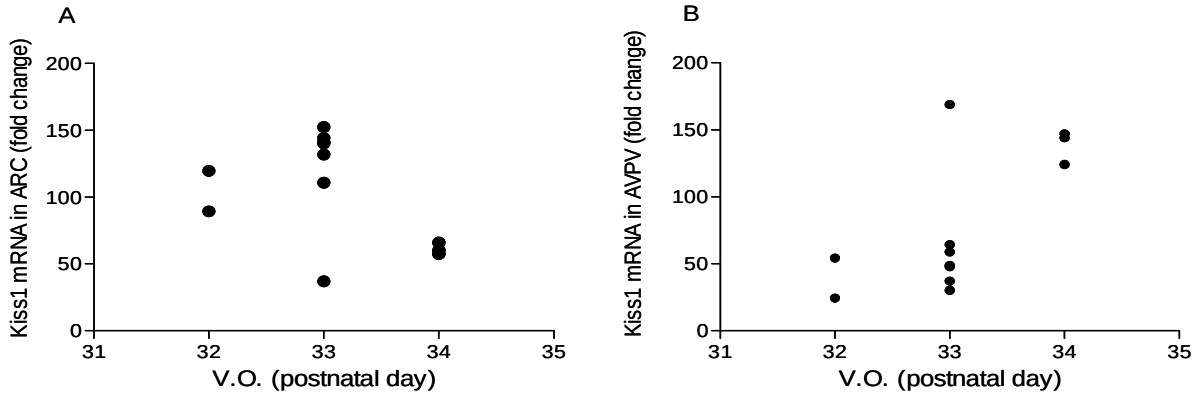
All data is presented as mean ± standard error mean (SEM) unless otherwise states, and $P<0.05$ was considered statistically significant.

Results

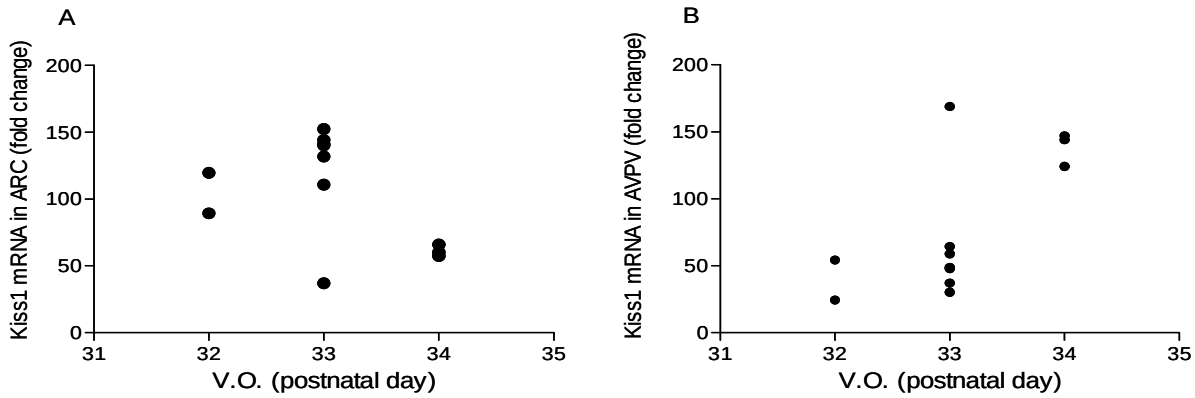
Developmental changes in the kisspeptin system

The number of kisspeptin-ir cells in ARC increases at puberty onset

Kiss1 mRNA levels as well as the number of kisspeptin-ir cells in ARC and AVPV were determined at PND34, in prepubertal rats (without V.O.) and in pubertal rats (with V.O.). This was done to evaluate changes in the kisspeptinergic system at peri-puberty in relation to the stage of pubertal maturation.
At PND34, approximately 50% of the rats had V.O. and based on these animals, a positive correlation between the day V.O. occurred and Kiss1 mRNA levels in AVPV at PND34 was present



(Figure 12B). No correlations were present between the day V.O. occurred and Kiss1 mRNA in ARC at PND34



(Figure 12A) or between the day V.O. occurred and the number of kisspeptin-ir cells in ARC or AVPV at PND34 (data not shown).

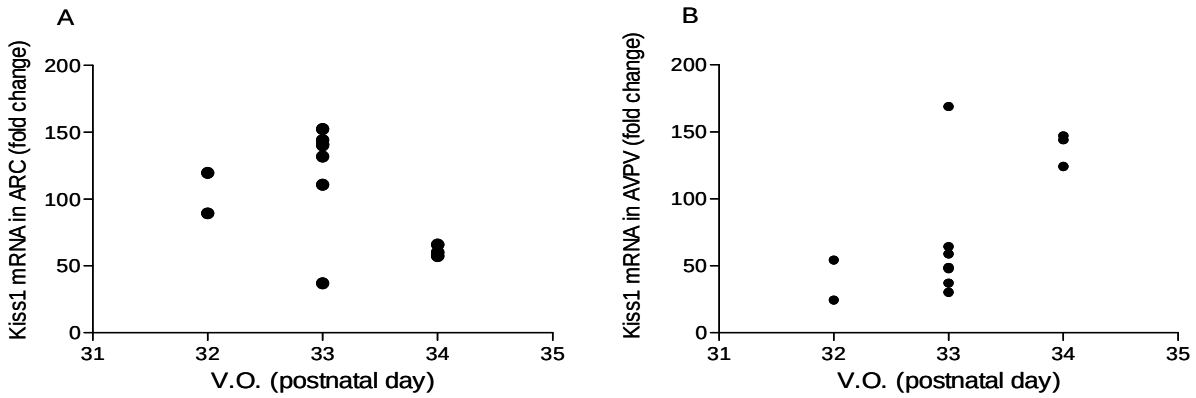


Figure 12. Correlation between the day vaginal opening (V.O.) occurred and Kiss1 mRNA levels at postnatal day 34 in (A) arcuate nucleus (ARC) and the (B) anteroventral periventricular nucleus (AVPV). There was no correlation in ARC, but a positive correlation in AVPV. *P<0.05, R^2=0,46, Pearson's correlations (n=12).

As shown in Figure 13C, the number of kisspeptin-ir cells in ARC increased at PND34 in rats with V.O., whereas no change occurred in AVPV in rats with V.O. (Figure 13D). The number of kisspeptin-ir cells in AVPV both before and after V.O. were very low, and to estimate the total level of kisspeptin-ir in the soma and fibres in AVPV, OD measurements were performed. No difference in total kisspeptin-ir, revealed by OD, was present in AVPV at PND34 in rats with V.O. (Figure 13F). Neither, were a developmental change in Kiss1 mRNA levels at PND34 in either ARC or AVPV detected in rats with V.O. (Figure 13A and B).

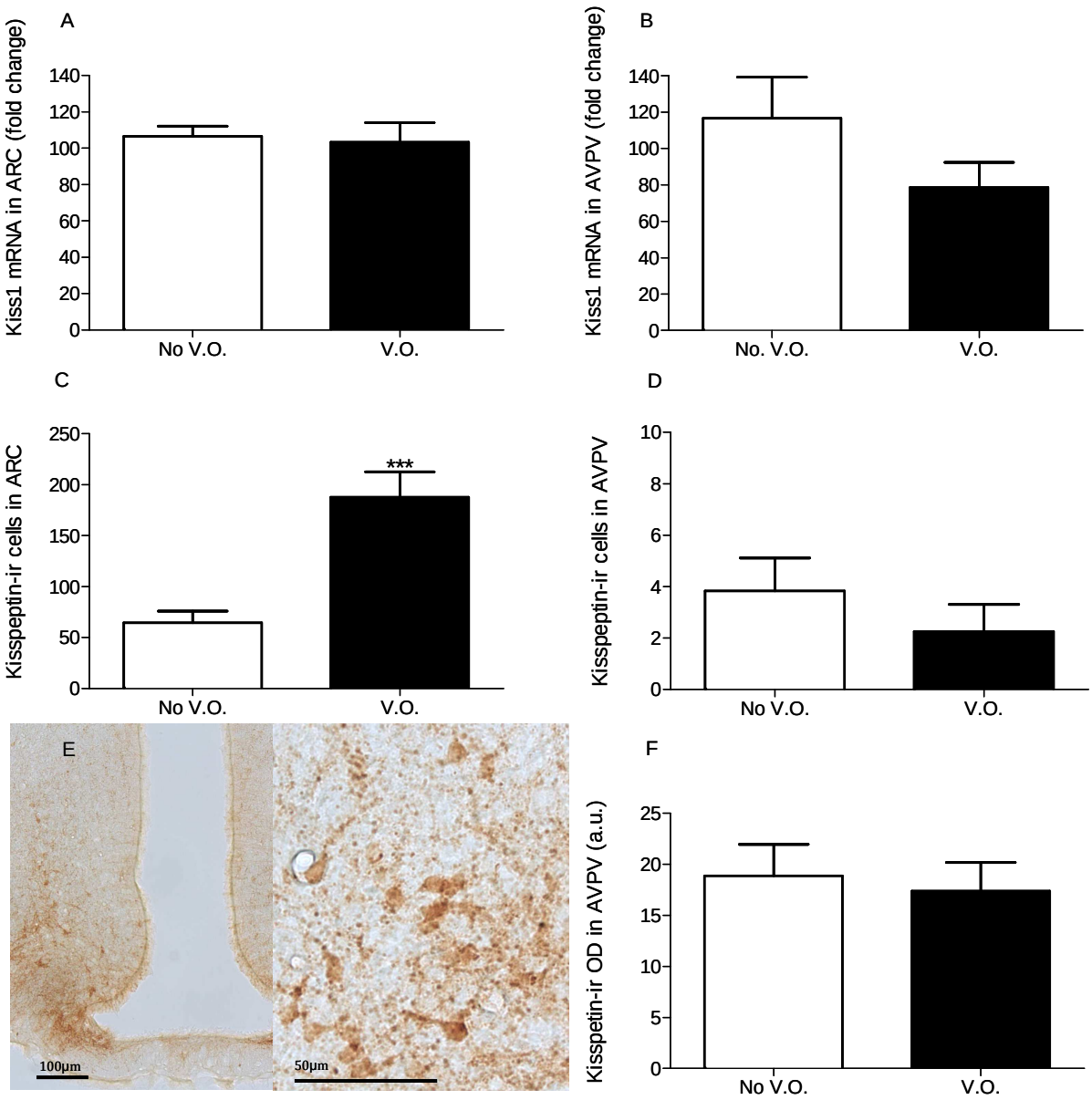


Figure 13. Developmental changes in Kiss1 mRNA and kisspeptin-immunoreactivity at postnatal day (PND) 34 in arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) in prepubertal (No V.O.) and pubertal rats (V.O) at postnatal. Kiss1 mRNA were determined by qRT-PCR at PND34, and depicted as fold change in relation to the control, in ARC (A) and AVPV (B). Kisspeptin-immunoreactivity (kisspeptin-ir) was determined at PND34 by IHC with JLV-1 1:200, as the total number of kisspeptin-ir cells in ARC (C) and AVPV (D), and also as the optical density (OD) in AVPV (F). (E) A representative illustration of kisspeptin-ir cells in ARC in a female rat with V.O.. Bars represent mean ± SEM (n=8). ***P<0.001 with unpaired student's t-test.

Kiss1 mRNA levels in ARC and AVPV as well as the number of kisspeptin-ir cells in ARC increases from PND34 to early adulthood

Developmental changes in the kisspeptin system in ARC and AVPV from peri-puberty to early adulthood, were evaluated by comparing PND34 rats with approximately PND61 rats in the diestrous phase. Kiss1 mRNA levels in ARC, and the total number of kisspeptin-ir cells in ARC, increased from PND34 to 61 (Figure 14A and B). Also, Kiss1 mRNA levels in AVPV increased from PND34 to 61 (Figure 14C). In contrast, no developmental change in the number of kisspeptin-ir cells in AVPV was

present (Figure 14D), neither when assessed by OD measurement (data not shown). It should be noted, that only around 50% of the rats had V.O. at PND34. However, only a change in the number of kisspeptin-ir cells in ARC at PND34 occurred upon V.O..

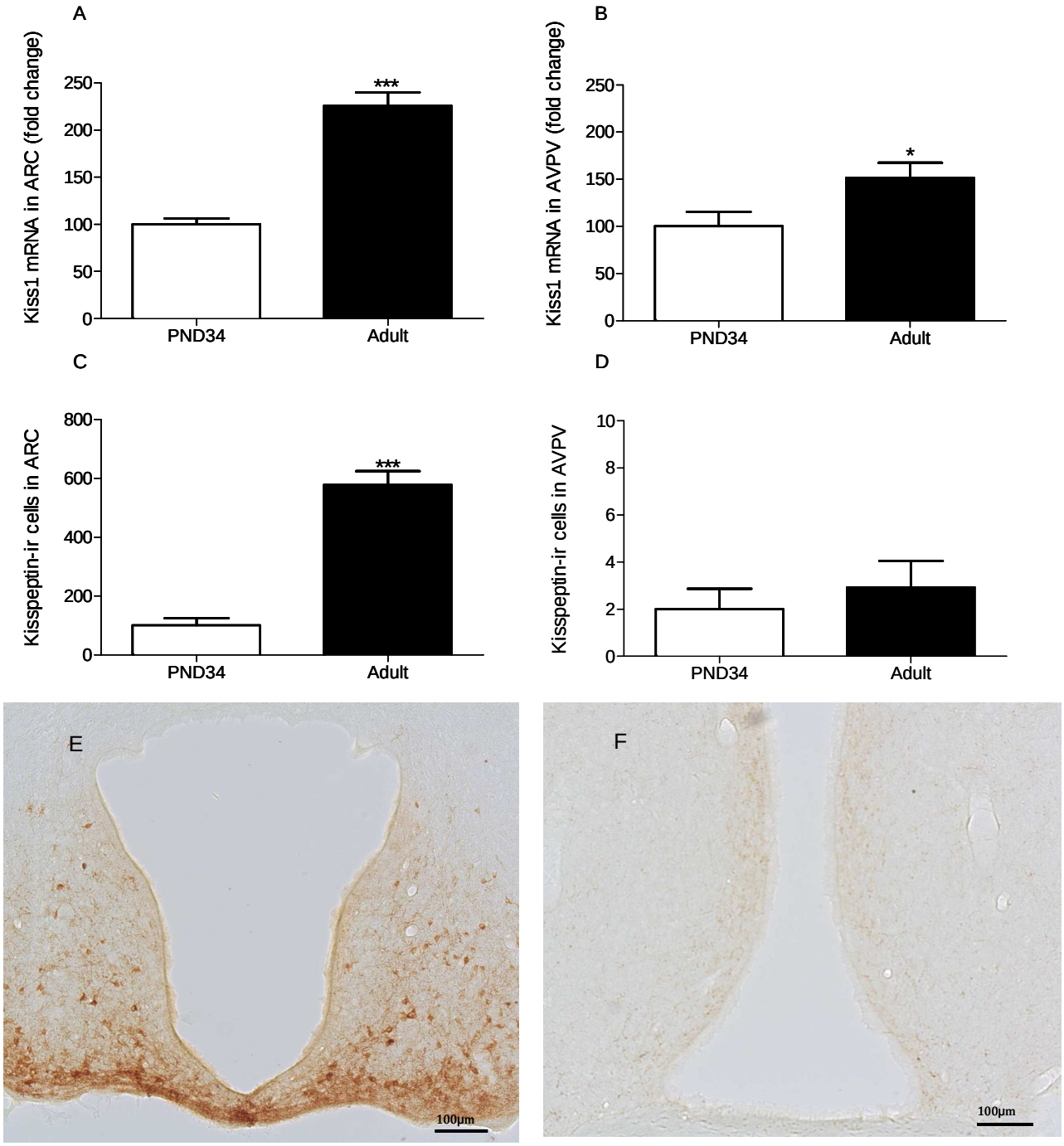


Figure 14. Developmental changes in *Kiss1* mRNA and kisspeptin-immunoreactivity in arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) from postnatal day (PND) 34 to PND61. Kiss1 mRNA were determined by qRT-PCR at PND34 and apriximately PND61, and depicted as fold change in relation to the control, in ARC (A) and AVPV (B). The number of kisspeptin-immunoreactive (kisspeptin-ir) cells was determined by IHC with JLV-1 1:200 at PND34 and approximately PND61 in ARC (C) and AVPV (D). A representative illustration of kisspeptin-ir cells in ARC (E) and AVPV (F) at PND61. Bars represent mean ± SEM (n=8). *P<0.05, ***P<0.001 with unpaired student's t-test.

The impact of a postnatal high-fat diet in different developmental periods on pubertal timing and the kisspeptinergic system

Experiment 1 – Post-weaning high-fat diet exposure

Exposure to a post-weaning high-fat diet has no effect on puberty onset

Exposure to a high-fat diet from PND21 to 34 (HFD PND21-34) was carried out to evaluate the effects on body weight and puberty onset. As shown in Figure 15A, a post-weaning high-fat diet exposure caused a minor increase in body weights at PND31, 32, and 33 in HFD PND21-34, when compared to rats fed a control diet. Furthermore, an increased weight gain from PND21 to 34 was present in the high-fat diet group (data not shown). HFD PND21-34 rats displayed a slightly earlier puberty onset since 60% had V.O. at PND34 in contrast to 45% of the control fed rats at PND34, but no statistically significant change was present (Figure 15B). There was no positive correlation between V.O. and weight gain from PND21 to 34 in the control or HFD PND21-34 group (Figure 15C). Also, no difference in body weight at V.O. was present in HFD PND21-34 when compared to the control fed rats (Figure 15D).

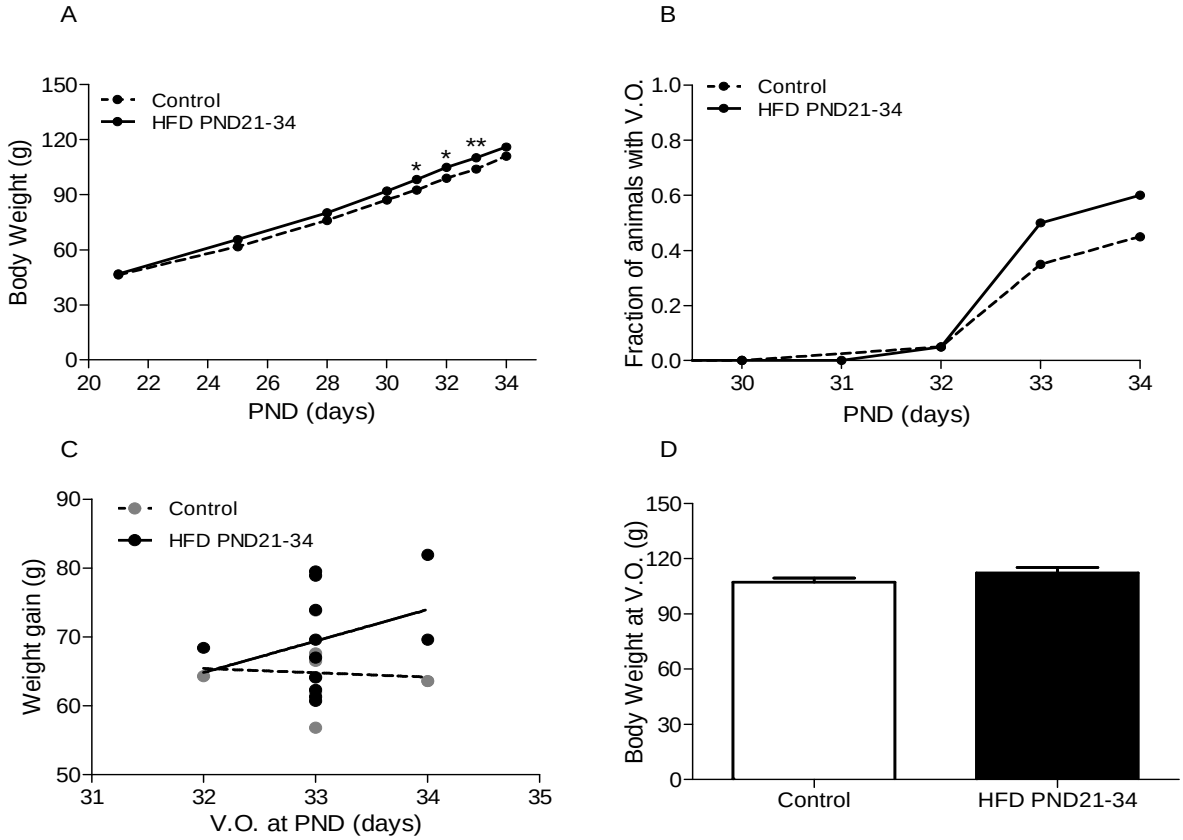


Figure 15. Body weight and vaginal opening (V.O.) in rats exposed to a high-fat diet from postnatal day (PND) 21 to 34. (A) Body weight. *P<0.05, **P=0.01, Two-Way ANOVA followed by Bonferroni multiple comparisons corrections. (B) Puberty onset assessed by V.O.. Graph represents the fraction of animals with V.O. the given day (n=20). (C) Correlation between V.O. and weight gain from PND21 to 34 (n=9-12). (D) Body weight at V.O. Points in A and bars in D represent mean ± SEM (n=20).

Exposure to post-weaning high-fat diet has no effect on leptin, triglyceride, insulin, and estradiol levels

The plasma levels of leptin, triglyceride, and insulin, which are all energy sensitive parameters, were assessed at PND34 in order to evaluate any effect of a post-weaning high-fat diet exposure. No significant changes were found in leptin, triglyceride, or insulin levels between HFD PND21-34 and control rats (Figure 16A, B and C). There were a positive correlation between body weight at PND34 and both leptin and insulin at PND34, whereas no correlation was present between body weight and triglyceride levels at PND34 (data not shown). Also, estradiol plasma levels were measured at PND34, but the fat-rich diet from weaning onwards had no impact on estradiol levels (Figure 16D). No difference in estradiol levels, between prepubertal rats (without V.O.) and pubertal rats (with V.O.) at PND34, were present (data not shown).

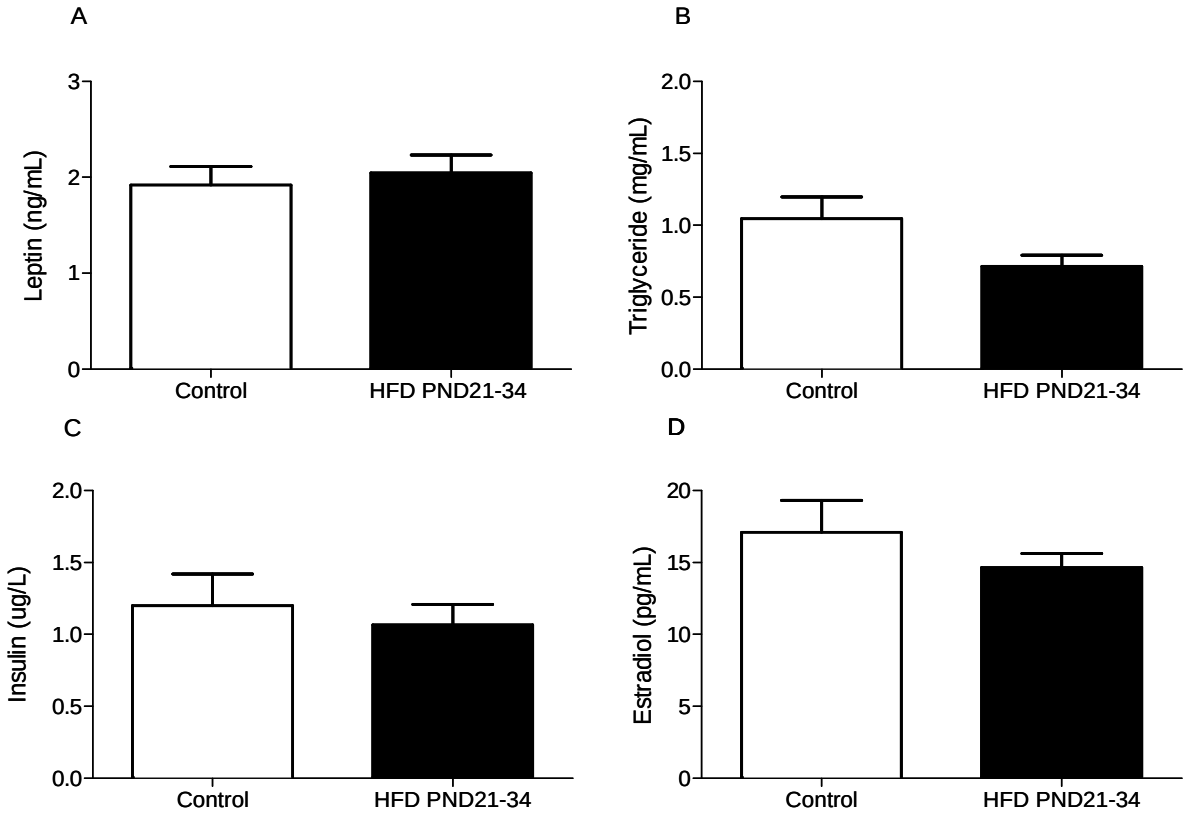


Figure 16. Leptin, triglyceride, insulin, and estradiol plasma levels at postnatal day (PND) 34 in rats exposed to a high-fat diet from PND21 to 34. (A) Leptin, (B) triglyceride, (C) insulin, and (D) estradiol plasma levels at PND34. Bars represent mean ± SEM (n=20).

Kiss1 mRNA and kisspeptin levels in the hypothalamus are not affected by a post-weaning high-fat diet

Kiss1 mRNA levels in ARC and AVPV were determined at PND34 by qRT-PCR to evaluate the effect of a post-weaning high-fat diet exposure. No changes in Kiss1 mRNA levels in ARC or AVPV were present in HFD PND21-34 (Figure 17A and B). The number of kisspeptin-ir cells in ARC and AVPV, at PND34 in HFD PND21-34 and controls, were evaluated by IHC. Exposure to a post-weaning high-fat diet had no effect on the number of kisspeptin-ir cells in ARC or AVPV at PND34 (Figure 17C and D). The number of kisspeptin-ir cells in AVPV was very low in both groups, so OD measurements were performed to

include kisspeptin-ir in both soma and fibres, but revealed no change (data no shown). No correlations were found between body weight at PND34 and Kiss1 mRNA in ARC or AVPV at PND34, or between leptin at PND34 and Kiss1 mRNA in ARC or AVPV at PND34 (data not shown). Also, no correlation was found between the number of kisspeptin-ir cells in ARC at PND34 and body weight at PND34, whereas a negative correlation between leptin at PND34 and number of kisspeptin-ir cells in ARC at PND34 was present (data not shown). No correlations for kisspeptin-ir in AVPV were conducted due to the low level of kisspeptin-ir in this nucleus. All data for experiment 1 are summarised in Appendix III, table 1.

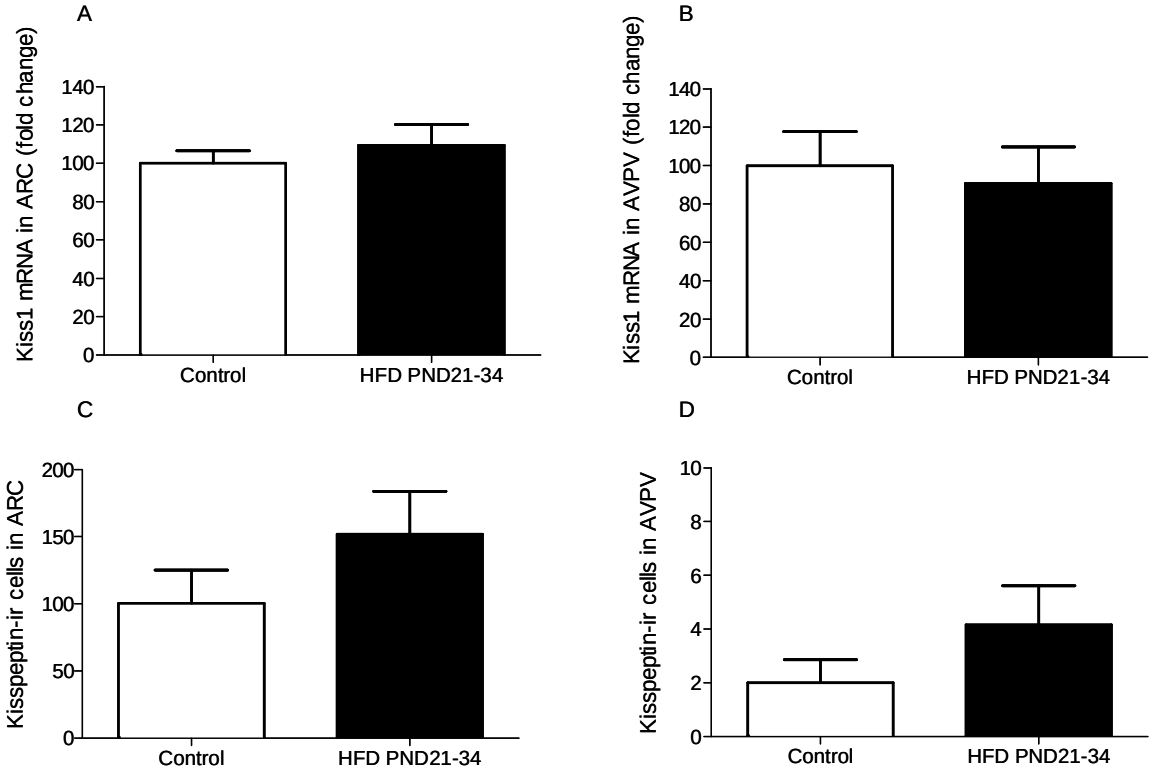


Figure 17. Kiss1 mRNA levels and kisspeptin-immunoreactivity in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) at postnatal day (PND) 34 in rats exposed to a high-fat diet from PND21 to 34. Kiss1 mRNA levels were determined by qRT-PCR at PND34, and depicted as fold change in relation to the control, in ARC (A) and AVPV (B). Kisspeptin-immunoreactivity (kisspeptin-ir) was determined by IHC at PND34 with JLV-1 1:200 in ARC (C) and AVPV (D) as the total number of kisspeptin-ir cells. Bars represent mean ± SEM (n=8-12).

Experiment 1b - Post-weaning high-fat diet exposure to puberty

Exposure to post-weaning high-fat diet has no effect on puberty onset

Exposure to a post-weaning high-fat diet from PND21 until all animals had entered puberty, at PND37 (HFD PND21-puberty), was carried out to evaluate the effect on body weight and V.O.. No changes in body weight (Figure 18A) or weight gain (data no shown) from PND21 to 37, was observed in the HFD PND21-puberty group. Neither was a change in puberty onset present in HFD PND21-puberty (Figure 18B). No correlation between V.O. and weight gain from PND21 to 37 was present, and HFD PND21-puberty did not weigh more at V.O. (Figure 18C and D). All data for experiment 1B are summarised in Appendix III, table 1.

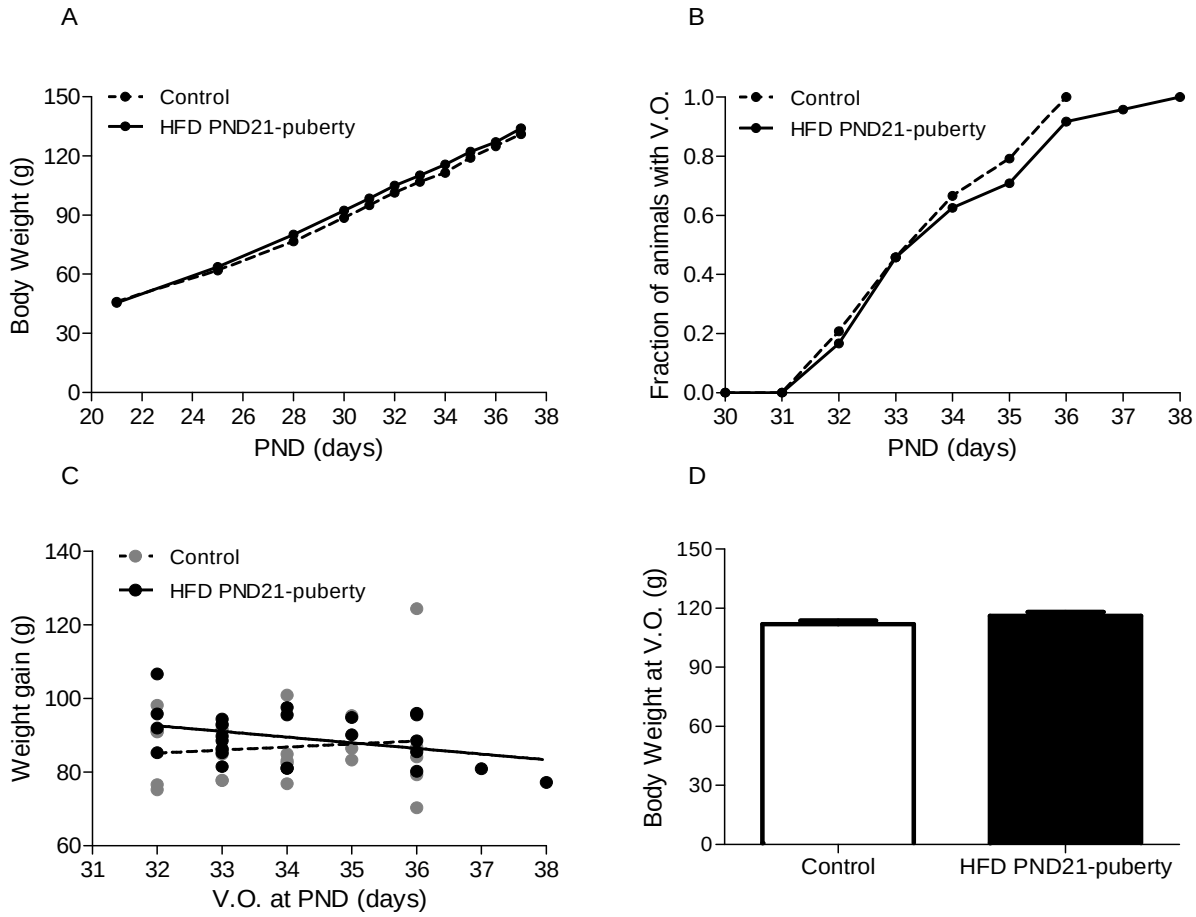


Figure 18. Body weight and vaginal opening (V.O.) in rats exposed to a high-fat diet from postnatal day (PND) 21 until puberty. (A) Body weight. (B) Pubertal timing assessed by V.O.. Graph represent the fraction of animals with V.O. the given day (n=24). (C) Correlation between V.O. and weight gain from PND21 to 37. (D) Body weight at V.O. Points in A and bars in D represent mean ± SEM (n=24).

Experiment 2 – high-fat diet during lactation

Early life exposure to a high-fat diet has no effect on puberty onset

To evaluate the effect of an early life exposure to a high-fat diet from PND1 to 16 (HFD PND1-16) on body weight and puberty onset, lactating dams were exposed to a high-fat diet the first day the pups were born until PND16. At PND16 the diet was switched to the control diet and the pups were weaned at PND21 still continuing with the control diet. A high-fat diet exposure through lactation had no impact on body weights (Figure 19A) or weight gain from PND1 to 18 (data not shown). Puberty onset was not changed in HFD PND1-16 (Figure 19B). Neither, was a correlation between V.O. and weight gain from PND1 to 18 present in the control or high-fed rats, and HFD PND1-16 did not weigh more at V.O. (Figure 19C and D).

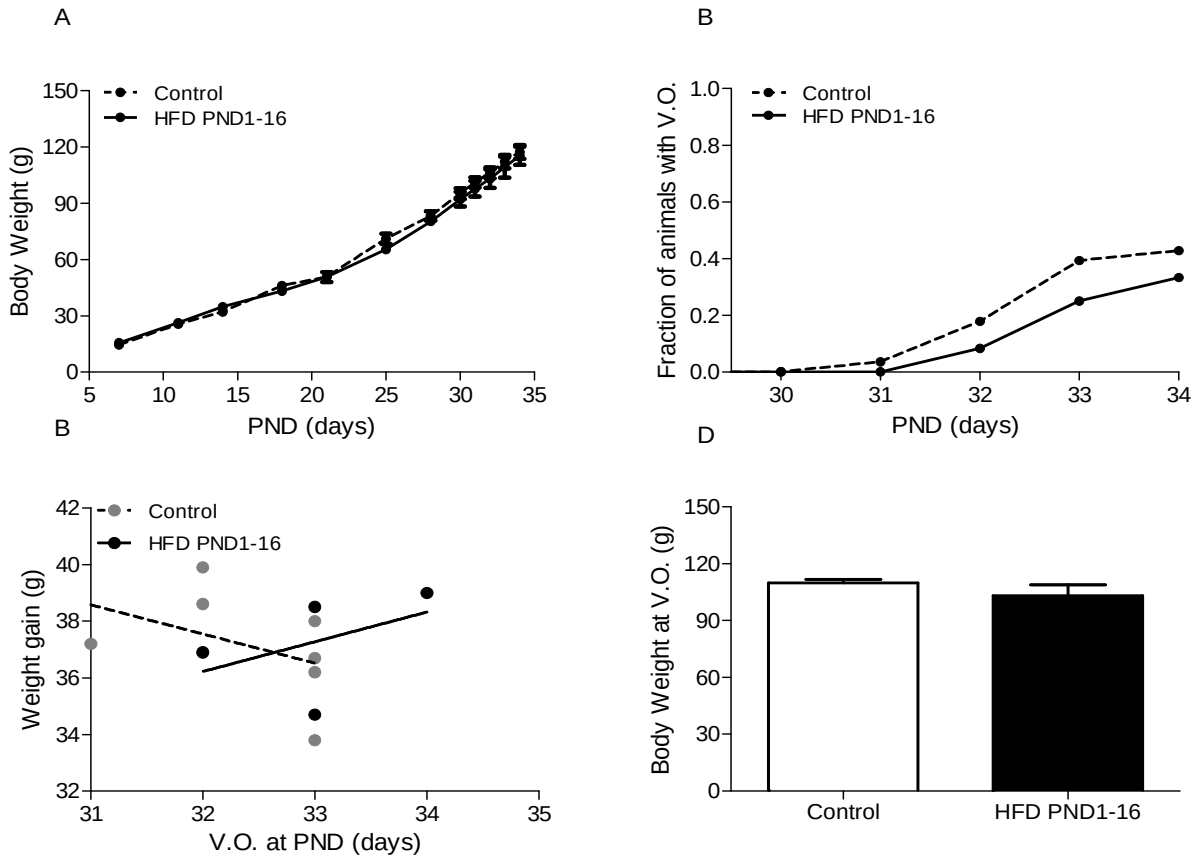


Figure 19. Body weight and vaginal opening (V.O.) in rats exposed to a high-fat diet from postnatal day (PND) 1 to 16. (A) Body weight. (B) Pubertal timing assessed by V.O.. Graph represent the fraction of animals with V.O. the given day (n=12-27). (C) Correlation between V.O. and weight gain from PND1 to 18 (n=4-8). (D) Body weight at V.O. Points in A and bars in D represent mean ± SEM (n=3-6).

Early life exposure to a high-fat diet has no effect on leptin, triglyceride, insulin, or estradiol levels in pubertal rats

High-fat diet exposure through lactation from PND1 to 16 decreased leptin, triglyceride, and insulin plasma levels insignificantly (Figure 20A, B and C). There was a positive correlation between body weight at PND34 and both leptin and insulin at PND34, whereas no correlation was present between body weight and triglyceride levels at PND34. The high-fat diet had no effect on estradiol plasma levels at PND34 (Figure 20D), and no difference in estradiol levels, between prepubertal rats and pubertal rats was present (data not shown).

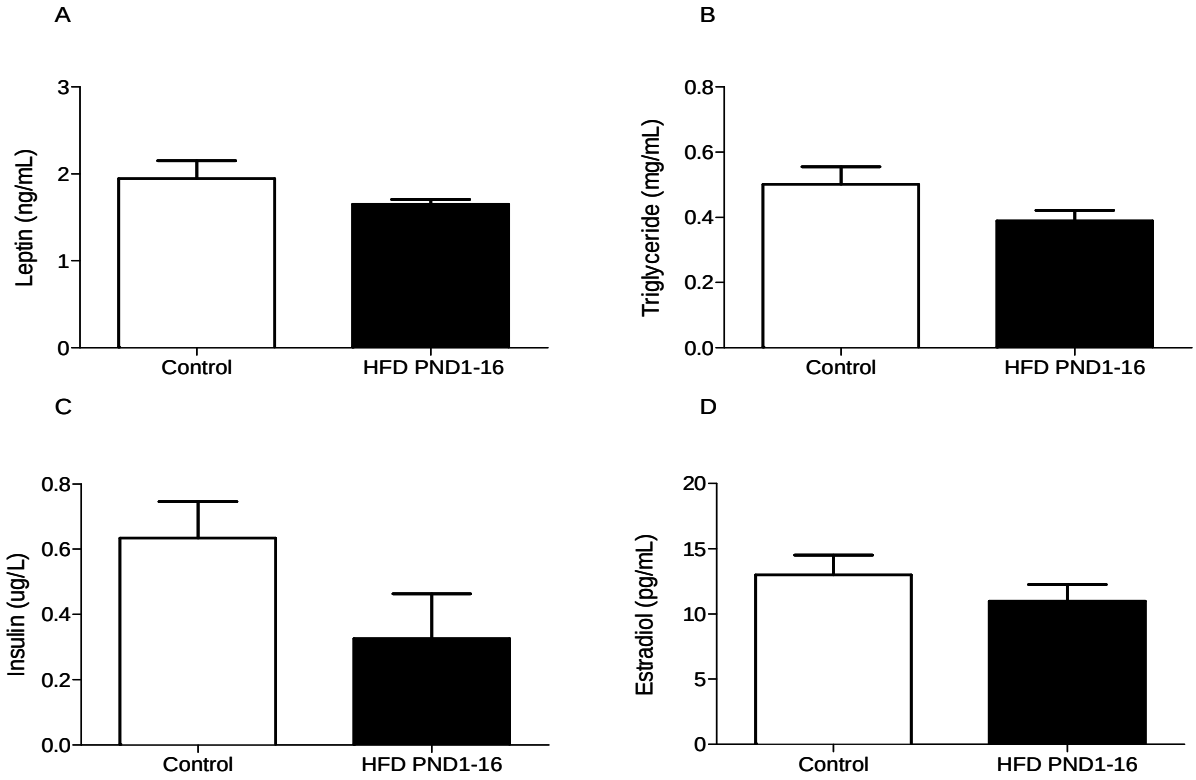


Figure 20. Leptin, triglyceride, insulin, and estradiol plasma levels at postnatal day (PND) 34 in rats exposed to a high-fat diet from PND1 to 16. (A) Leptin, (B) triglyceride, (C) insulin, and (D) estradiol plasma levels. Bars represent mean ± SEM (n=3-6).

Kiss1 mRNA levels in the hypothalamus are not affected by a high-fat diet exposure through lactation

The impact of an early life exposure to a high-fat diet, from PND1 to 16, on Kiss1 mRNA levels in ARC and AVPV were evaluated at PND34 by qRT-PCR. No changes in Kiss1 mRNA levels in ARC or AVPV were present in HFD PND1-16 (Figure 21). No correlations between body weight at PND34 and Kiss1 mRNA levels in ARC or AVPV at PND34 or leptin at PND34 and Kiss1 mRNA levels in ARC or AVPV at PND34 were present (data not shown). All data for experiment 2 are summarised in Appendix III, table 1.

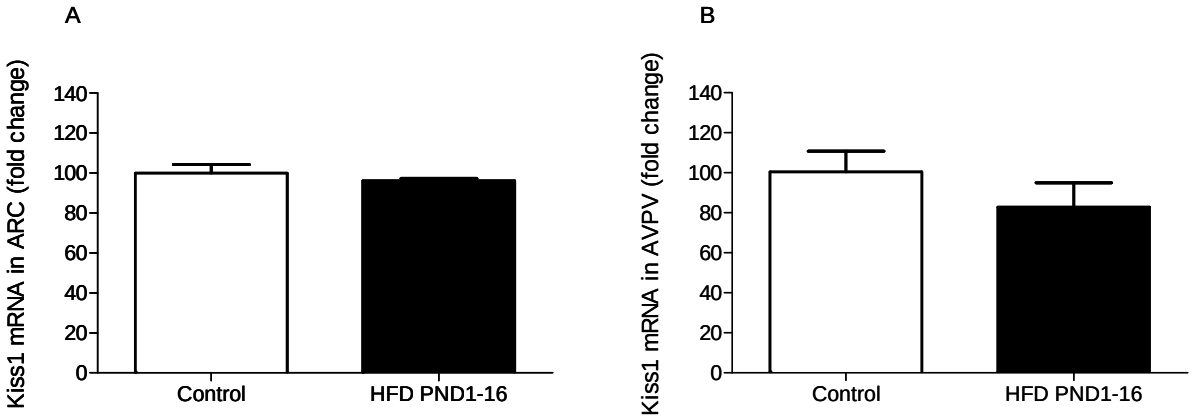


Figure 21. Kiss1 mRNA levels in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) at postnatal day (PND) 34 in rats exposed to a high-fat diet from PND1 to 16. Kiss1 mRNA levels were determined by qRT-PCR at PND34 and depicted as fold change in relation to the control, in ARC (A) and AVPV (B). Bars represent mean ± SEM (n=3-6).

Experiment 3 –high-fat diet from birth to puberty

Exposure to a high-fat diet increases triglyceride and decreases estradiol levels in lactating rats

Exposure to a postnatal high-fat diet from PND1 to 34 was carried out by feeding dams a high-fat diet during lactation. The lactating dams, exposed to a high-fat diet for three weeks during lactation, had increased plasma triglyceride levels (Figure 22B), and decreased estradiol levels (Figure 22D) the day of weaning compared to the control dams maintained on the control diet. There was no effect of the high-fat diet on leptin levels, and only a tendency to an increased insulin level was detected (Figure 22A and C).

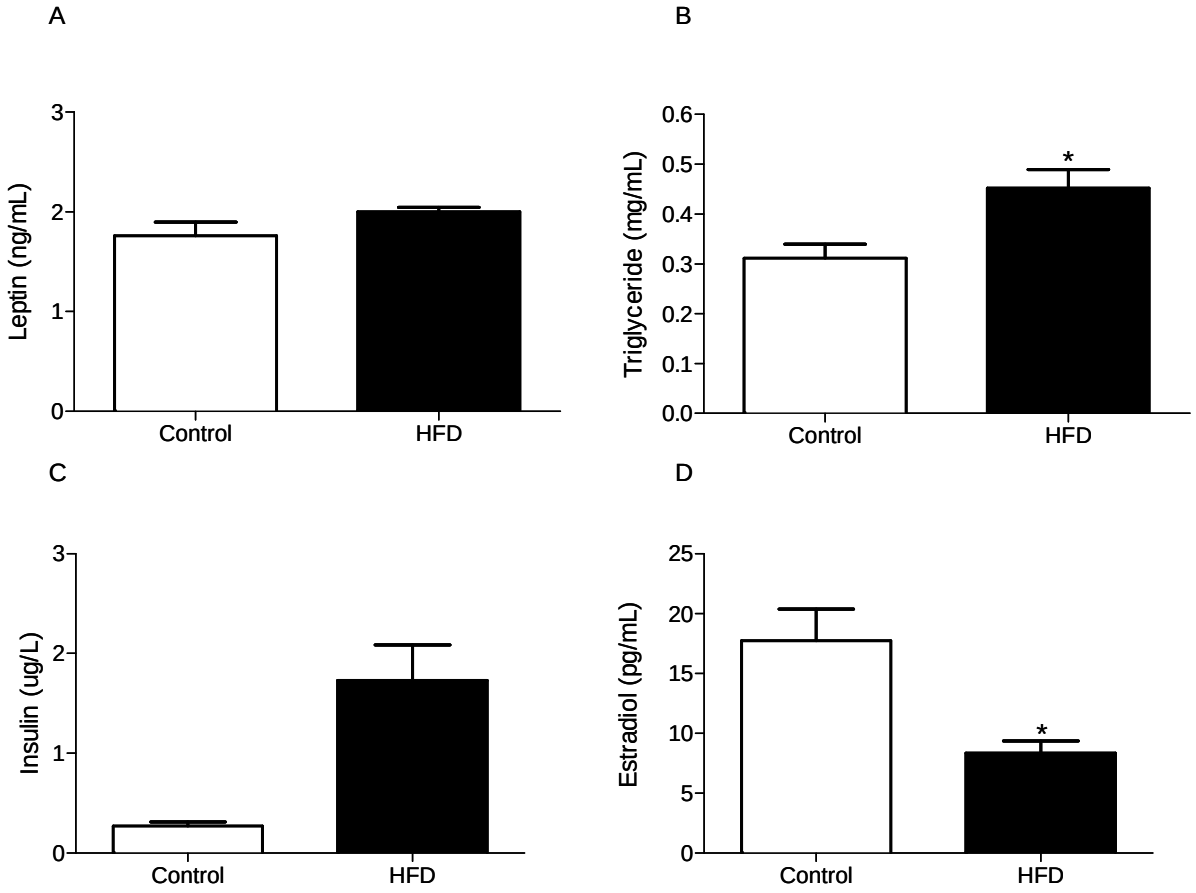


Figure 22. Leptin, triglyceride, insulin, and estradiol plasma levels in lactating rats exposed to a high-fat diet (HFD) for three weeks during lactation. (A) Leptin, (B) insulin, P=0.056, student's t-test, (C) triglyceride, and (D) estradiol plasma levels in lactating rats. Bars represent mean ± SEM (n=2-3). *P<0.05 with unpaired student's t'test.

High-fat diet exposure from birth to puberty has no effect on puberty onset

The effects of a postnatal high-fat diet exposure from PND1 to 34 on body weight and puberty onset were evaluated. Postnatal high-fat diet exposure from PND1 to 34 had no effects on body weight, weight gain (data not shown), or puberty onset (Figure 23A and B). No correlations between V.O. and body weight at V.O. in the control of HFD PND1-34 groups were present, but the HFD PND1-34 group weighted slightly more the day V.O. occurred (Figure 23C and D).

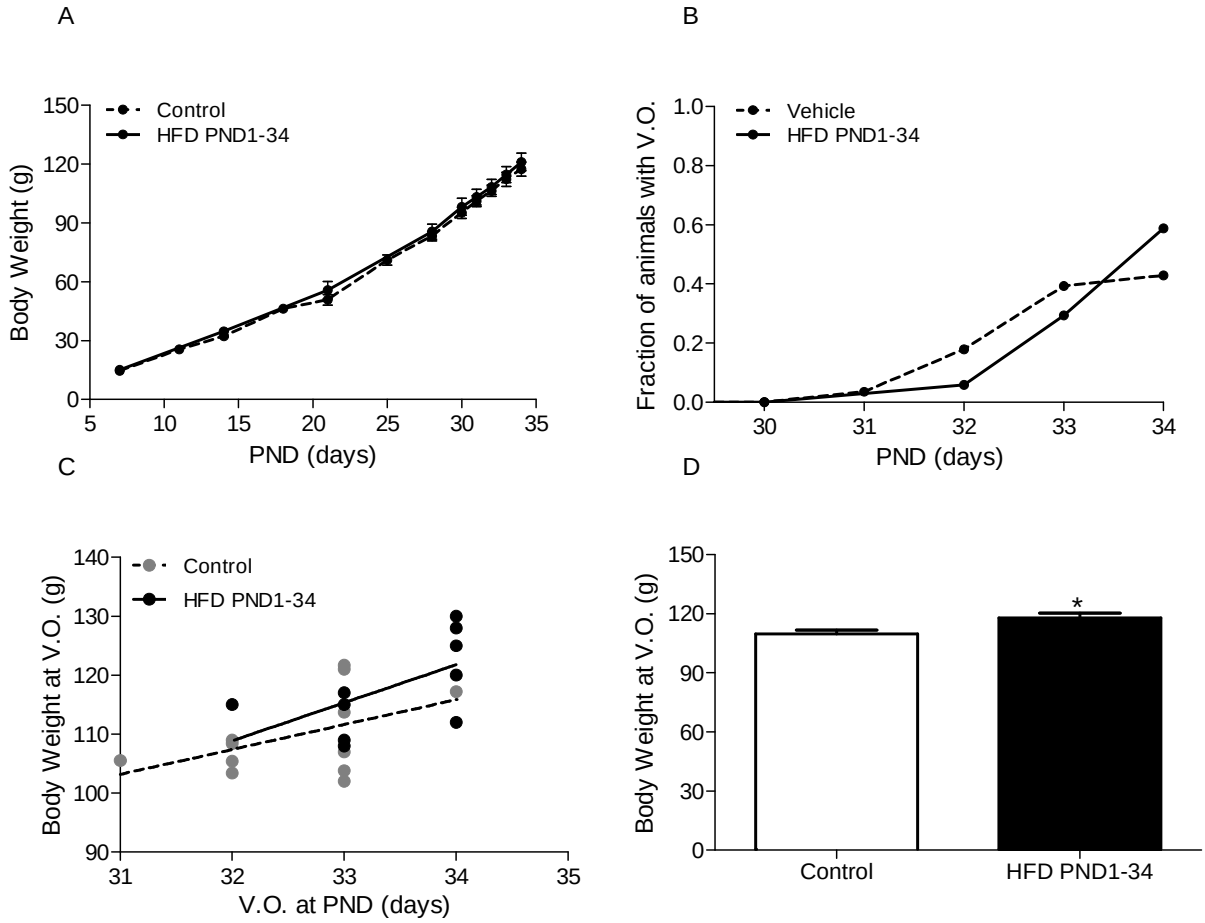


Figure 23. Body weight and vaginal opening (V.O.) in rats exposed to a high-fat diet from postnatal day (PND) 1 to 34. (A) Body weight. (B) Pubertal timing assessed by V.O.. Graph represent the fraction of animals with V.O. the given day (n=16-27). (C) Correlation between V.O. and weight gain from PND7 to 34 (n=10-12). (D) Body weight at V.O. Points in A and bars in D represent mean ± SEM (n=3-6). *P<0.05, unpaired student's t'test.

High-fat diet exposure from birth to puberty have no effects on leptin, triglyceride, insulin or estradiol levels in the pubertal rats

The plasma leptin, triglyceride, insulin, and estradiol levels at PND34 were not affected by a postnatal high-fat diet introduced from PND1 to 34 (Figure 24). There was a positive correlation between body weight at PND34 and leptin at PND34, whereas no correlation between bodyweight and insulin or triglyceride levels at PND34 were present (data not shown). No difference in estradiol levels, between prepubertal rats and pubertal rats was present (data not shown).

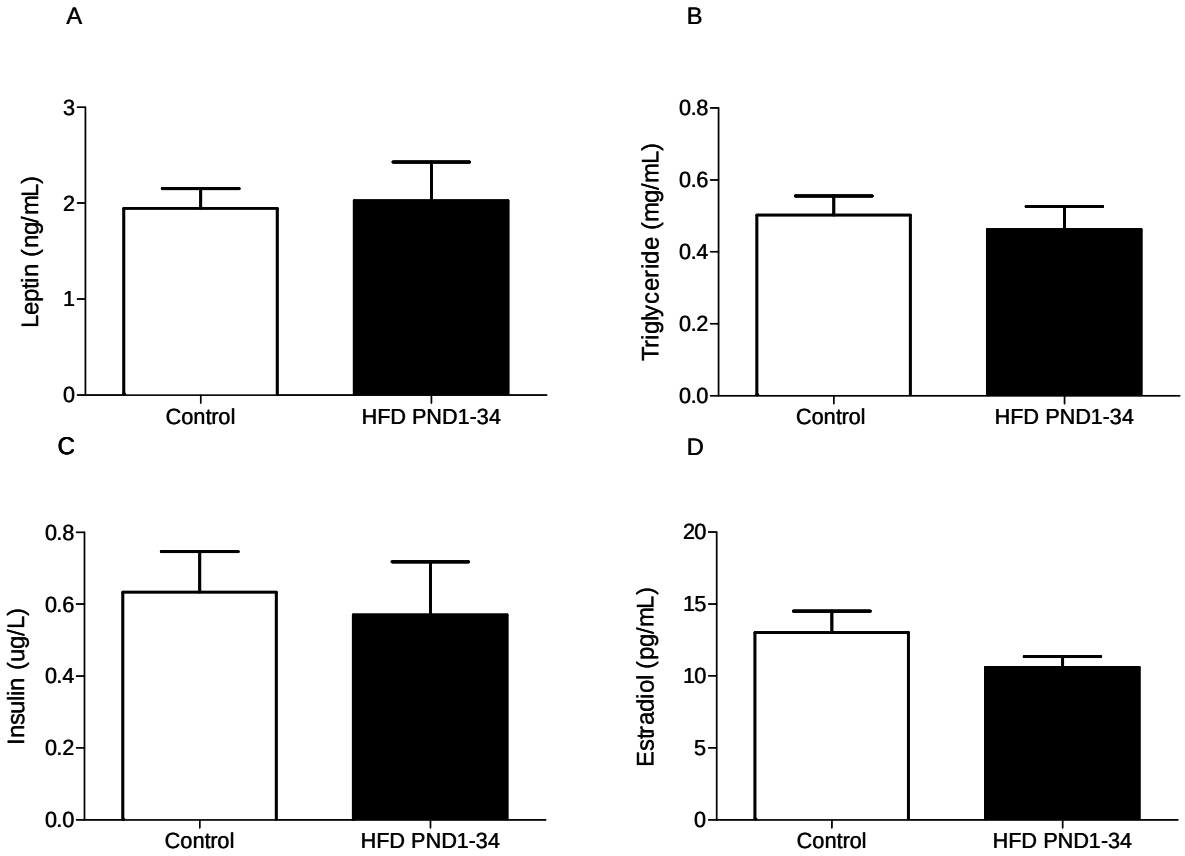


Figure 24. Leptin, triglyceride, insulin, and estradiol plasma levels at postnatal day (PND) 34 in rats exposed to a high-fat diet from PND1 to 34. (A) Leptin, (B) triglyceride, (C) insulin, and (D) estradiol plasma levels. Bars represent mean ± SEM (n=3-6).

Kiss1 mRNA levels in the hypothalamus is not affected by a postnatal high-fat diet exposure

The effect of a postnatal high-fat diet, from PND1 to 34, on Kiss1 mRNA levels in ARC and AVPV were evaluated at PND34 by qRT-PCR. HFD PND1-34 experienced no changes in Kiss1 mRNA levels in ARC or AVPV (Figure 25). Body weight at PND34 or leptin at PND34 were not correlated to either Kiss1 mRNA levels in ARC or AVPV at PND34 (data not shown). All data for experiment 3 are summarised in Appendix III, table 1.

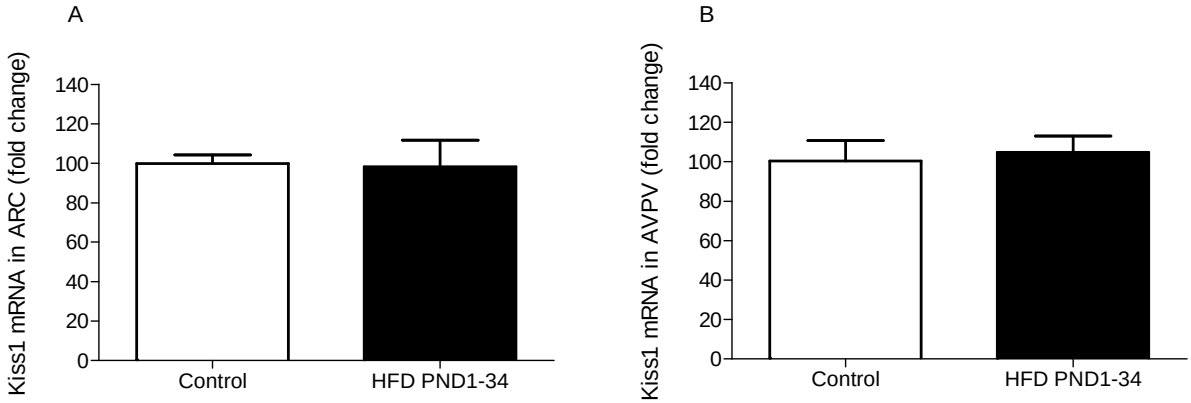


Figure 25. Kiss1 mRNA levels in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) at postnatal day (PND) 34, in rats exposed to a high-fat diet from PND1 to 34. Kiss1 mRNA levels were determined by qRT-PCR at PND34, and depicted as fold change in relation to the control, in ARC (A) and AVPV (B). Bars represent mean ± SEM (n=3-6).

Experiment 4 – high-fat diet from weaning to adulthood

High-fat diet exposure from weaning onwards extend the estrous cycle in young adult rats

To evaluate the effects of a high-fat diet on estrous cyclicity and kisspeptin, rats were exposed to a high-fat diet from PND21 and for 40 days (HFD PND21-adulthood). High-fat diet exposure increased body weight from PND54 onwards (Figure 26A), and increased the total weight gain in the test diet period (Figure 26B). To assess estrous cyclicity, vaginal smears were conducted from PND50 and for 10 consecutive days. High-fat diet exposure induced an irregular estrous cycle in a significant number of animals (33% of the animals) (Figure 26C) which were either present as an extended diestrous, estrous, or proestrous, however, an extended diestrous was most frequent. Based on this, the high-fat diet group was divided into two subgroups; one with a regular estrous cycle (HFD regular cycle), and one with an irregular estrous cycle (HFD extended cycle). Weight gain was evaluated in the subgroups, and only the HFD regular cycle group had an increased weight gain, whereas only tendencies of an increased weight gain were present in the HFD extended cycle group (Figure 26D) (see Appendix III, table 2 for a summary).

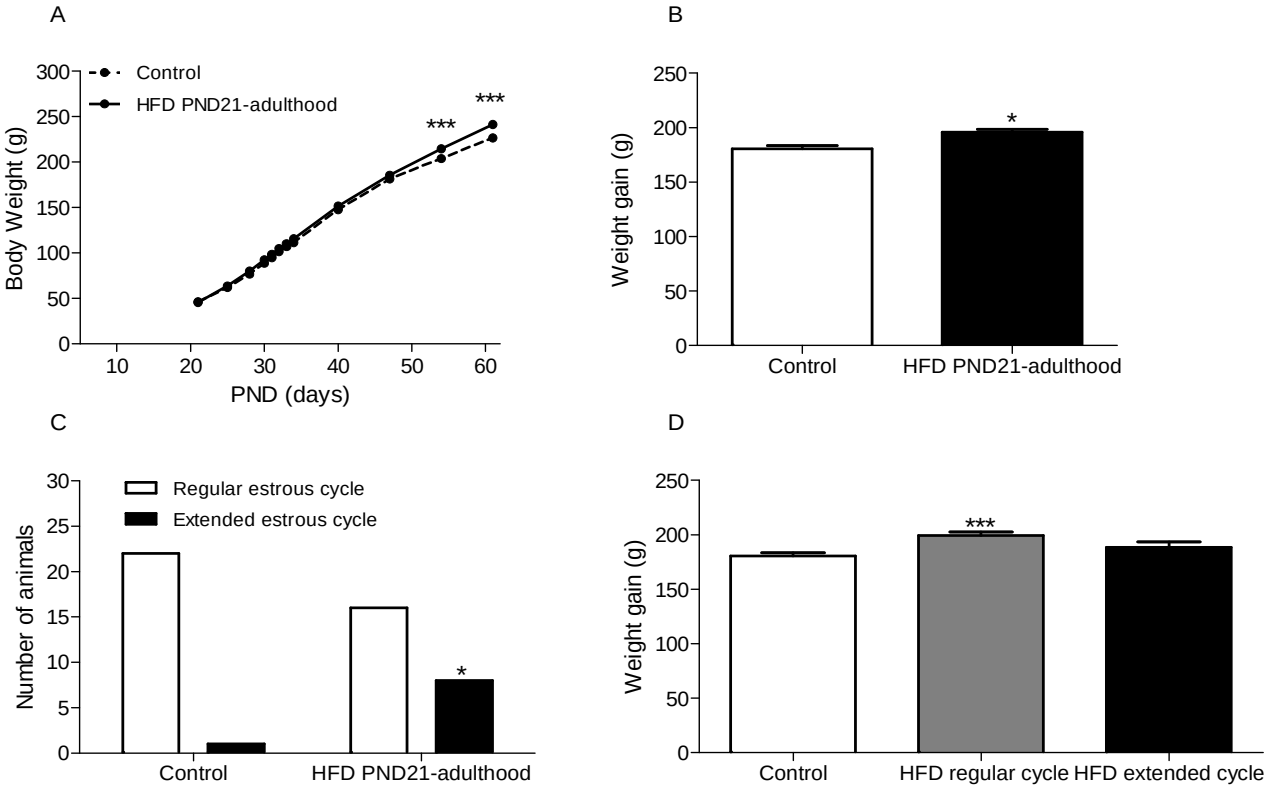


Figure 26. Body weight and estrous regularity in adult rats exposed to a high-fat diet from postnatal day (PND) 21 to approximately 61. (A) Body weight. ***P<0.001 with Two-Way ANOVA followed by Bonferroni multiple comparisons corrections (n=24). (B) Weight gain in the test diet period from PND21 to 61. *P<0.05 with unpaired student's t-test (n=24). (C) Estrous cyclicity depicted as the number of animals with a regular or irregular (extended) estrous cycle. *P<0.05 with 2x2 contingency table with Fisher's exact test (n=23-24). (D) Weight gain in the test diet period from PND21 to 61 for the HFD

subgroups. ***P<0.001 with One-Way ANOVA followed by Dunnet's post hoc test (n=8-24). Points in A and bars in B and D represent mean ± SEM.

High-fat diet exposure from weaning increases leptin levels in young adults

Leptin, triglyceride, insulin, and estradiol plasma levels were determined at diestrous around PND61. Leptin levels in the HFD regular cycle and in the HFD extended cycle group increased by the fat-rich diet (Figure 27A). A decrease in triglyceride levels were detected in the HFD regular cycle group (Figure 27B), whereas no change was detected in the HFD extended cycle group when compared to the control fed rats. No high-fat diet induced changes in insulin levels or estradiol levels were present in either high-fat diet exposed groups in comparison with the control fed rats (Figure 27C and D). A positive correlation was present between final body weight and the leptin level the same day when all animals were assessed together, but no correlations between final body weight and leptin levels were present for the control or the high-fat diet subgroups, when assessed individually. No correlations were present between final body weight and either triglycerides or insulin levels the same day.

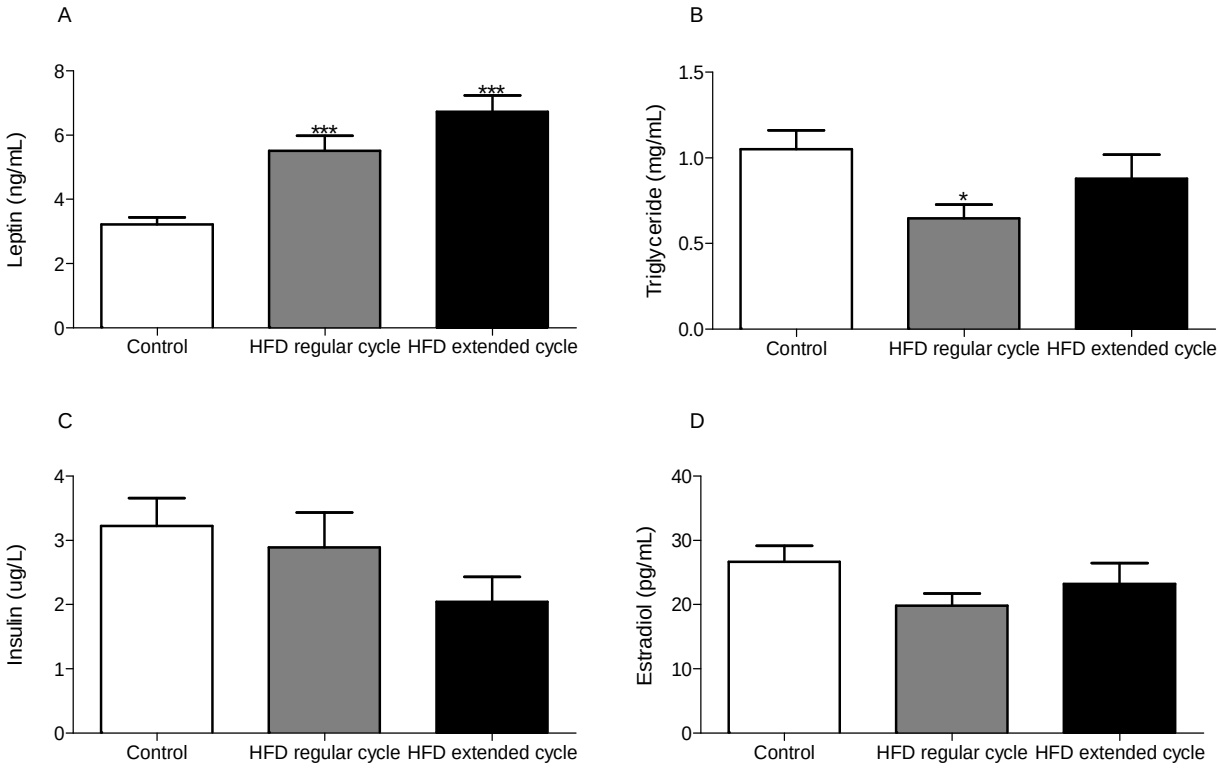


Figure 27. Leptin, triglyceride, insulin, and estradiol plasma levels at diestrous in adult rats exposed to a high-fat diet from postnatal day (PND) 21 to approximately 61. The high-fat diet group is divided into one group with a regular estrous cycle and one group with an extended estrous cycle. (A) Leptin, (B) triglyceride, (C) insulin, and (D) estradiol plasma levels. Bars represent mean ± SEM (n=8-24). *P<0.01, ***P<0.001 with One-WAy ANOVA followed by Dunnet's post hoc test.

High-fat diet exposure from weaning to adulthood has no effect on Kiss1 mRNA or kisspeptin in the hypothalamus

The impact of a high-fat diet exposure from weaning onwards on Kiss1 mRNA levels in ARC and AVPV in young adult rats was evaluated at diestrous around PND61. The high-fat diet exposure had no effect

on Kiss1 mRNA levels in ARC or AVPV at PND61 (Figure 28A and B). The number of kisspeptin-ir cells in ARC and AVPV at diestrous around PND61 was evaluated by IHC by use of our kisspeptin antibodu JLV1 diluted 1:200. Exposure to a high-fat diet from weaning onwards had no effect on the number of kisspeptin-ir cells in ARC or AVPV (Figure 28C and D). The number of kisspeptin-ir cells in AVPV was low in the adults. OD measurements were performed to include total kisspeptin-ir in cell bodies and fibres, but revealed no change in AVPV (data not shown). All data for experiment 4 are summarized in Appendix III, table 2.

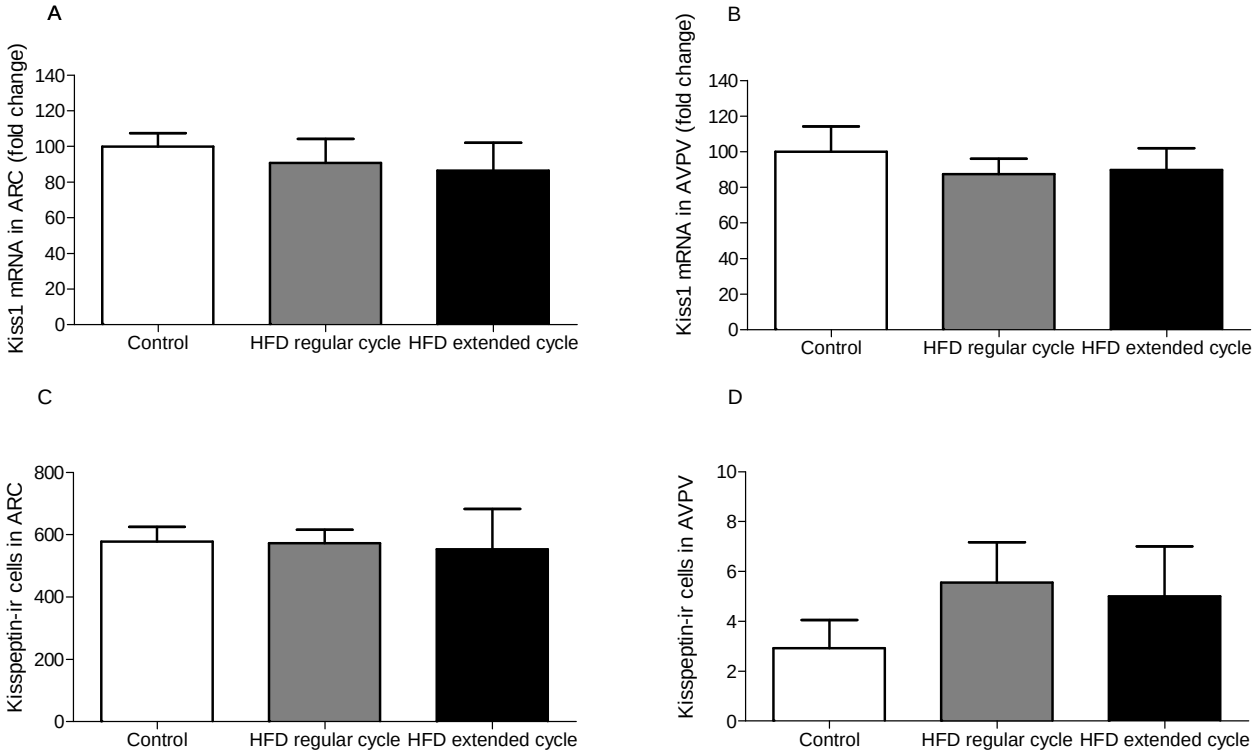


Figure 28. Kiss1 mRNA levels and kisspeptin-immunoreactivity in the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV) at diestrous in adult rats exposed to a high-fat diet from postnatal day (PND) 21 to approximately 61. The high-fat diet group is divided into one group with a regular cycle and one group with an extended cycle. Kiss1 mRNA levels were determined by qRT-PCR at approximately PND61, and depicted as fold change in relation to the control, in ARC (A) and AVPV (B) (n=5-12). Kisspeptin-immunoreactivity (kisspeptin-ir) was determined by IHC with JLV-1 1:200 as the total number of kisspeptin-ir cells in ARC (C) and AVPV (D) (n=2-12). Bars represent mean ± SEM.

Discussion

The secular trend in pubertal timing in girls and increased incidence of reproductive dysfunction in women believed to be linked to the rising incidence of obesity (Kaplowitz, 2008, Ahmed et al., 2009) (Linne, 2004, Pasquali et al., 2007) highlights the importance of understanding the mechanisms coordinating nutrient-dependent growth and timing of sexual maturation, one of the most fundamental, yet unsolved, questions in biology.

In rodents, little is known about the impact of a postnatal exposure to a high-fat diet on pubertal timing and estrous cyclicity and even less is known about the underlying mechanism. In the following, the data obtained during the present thesis focused on the effects of a postnatal high-fat diet on puberty onset and estrous cyclicity in relation to changes in the kisspeptinergic system will be discussed in relation to relevant scientific literature.

The effects of a postnatal high-fat diet exposure on puberty onset and the kisspeptinergic system

High-fat diet exposure from weaning

In the present study, no effect of a high-fat diet exposure from weaning onwards, on puberty onset revealed by V.O. was found. This is in contrast with previous studies were a post-weaning high-fat diet advance puberty onset in female rodents without changing the body weight (Boukouvalas et al., 2008, Brill and Moenter, 2009, Sloboda et al., 2009, Boukouvalas et al., 2010, Moral et al., 2011). As in the other studies, no effect of the high-fat diet on final body weight was present. Furthermore, no correlations between V.O. and weight gain in the control or high-fat diet group were found, and the high-fat fed rats did not weight more at V.O.. Caloric intake or fat pads were not assessed, but the lack of change in body weight at PND34, together with the unchanged leptin, insulin, and triglyceride levels at PND34, indicates that the female rats are not metabolic disturbed by the post-weaning high-fat diet. Only one other study reports leptin levels, but like here found no change after a post-weaning high-fat diet (Boukouvalas et al., 2010). A lowered insulin level has been reported in female rats, whereas other finds no change in female mice (Brill and Moenter, 2009). Whether the advanced puberty onset reported in the other studies (Boukouvalas et al., 2008, Brill and Moenter, 2009, Sloboda et al., 2009, Boukouvalas et al., 2010, Moral et al., 2011) was accompanied by a metabolic change is unclear, but it is certain that the metabolic change is not reflected in a change in body weight. A previous study have shown that the caloric intake increases just after switching the diet from normal chow to a high-fat diet, but that rats slowly adjust their food intake so the caloric intake per day becomes the same as before the diet was switched (Kamara et al., 1998). This, together with the short period of high-fat diet

exposure, can be the reasons why no change in body weights at peri-puberty was detected after the post-weaning high-fat diet exposure.

Multiple reasons may explain the different effects of a high-fat diet exposure from weaning on puberty onset reported here and elsewhere (Boukouvalas et al., 2008, Brill and Moenter, 2009, Sloboda et al., 2009, Boukouvalas et al., 2010, Moral et al., 2011). One possibility is that differences in food quality and content like the presence of phytosteroids in soybean oil (elSattar ElBaltran, 2001) in the high-fat diet affects pubertal timing. However, no differences in the pubertal timing were observed in the present studies arguing against this possibility. None of the other studies used a diet with a fat energy percentage over 45% (Boukouvalas et al., 2008, Brill and Moenter, 2009, Sloboda et al., 2009, Boukouvalas et al., 2010, Moral et al., 2011). Our in-house data show a more pronounced effect of a post-weaning diet with 60% energy from fat compared to a diet with 45% energy from fat, on both body weight and kisspeptin-ir cells in ARC (unpublished), but it is possible that 60% energy from fat does not have the same effect on V.O. as a diet with a lower fat energy percentage. A difference in the genetic background is another consideration that may explain contrasting results obtained by the different groups. The majority of the studies have been conducted on Wistar rats, which are very isogenetic compared to Sprague Dawley rats (Dourmashkin et al., 2006). This is underlined by the major diversity in body weight gain and timing of puberty onset from one litter to another observed here (data not shown). This is the reason why the Sprague Dawley rats used in these studies came from different litters. Theoretical, the advantage of using rats with an isogenetic profile, like Sprague Dawley rats, is that it could reveal whether the high-fat diet sensitive rats also experience earlier V.O. in contrast to the high-fat diet insensitive rats. Though, no correlations between body weight gain and timing of V.O. were present in the control or high-fat fed rats in the present study. In contrast to this study, a post-weaning high-fat diet advanced puberty onset in Sprague Dawley rats from different litters (Moral et al., 2011). However, both fat type and fat energy percentage were different, and V.O. occurred quite late in Moral et al. (2011) in comparisons with these studies, which may be implicated in the different outcomes. The relevance of differences in fat type is underlined by the lack of change in timing of V.O. in Sprague Dawley rats exposed to a post-weaning high-fat diet composed of olive oil (Moral et al., 2011).

An important issue to be made is the large variability in puberty onset reported in the different studies both for the high-fat and control fed rats ranging from PND33 to 42 (Boukouvalas et al., 2008, Sloboda et al., 2009, Boukouvalas et al., 2010, Moral et al., 2011). This difference in V.O. suggests that many other factors than a higher fat energy percentage is involved in the regulation of pubertal timing. This is in agreement with epidemiological data showing that multiple factors are involved in the regulation of pubertal maturation including race, maternal age at menarche, primiparity, endocrine disrupting chemicals, and maternal overweight or obesity (Rosenfield et al., 2009, Maisonet et al., 2010, Mouritsen et al., 2010).

At PND34, only a tendency of an earlier puberty onset and an increase in the number of kisspeptin-ir cells in ARC and AVPV, and Kiss1 mRNA in ARC, were present in the HFD PND21-34 group. It is possible that the genetic variability in Sprague Dawlay rats could mask the effects of a high-fat diet and explain the lack of effect on puberty onset and the kisspeptinergic system observed here. This idea is consistent with the lack of effect of a high-fat diet on Kiss1 mRNA levels in mice not prone to obesity-induced infertility, whereas a change in Kiss1 mRNA levels was present in mice that are susceptible to obesity induced infertility (Quennell et al., 2011). However, no correlations between timing of V.O. and Kiss1 mRNA or kisspeptin-ir in ARC or AVPV neither between body weight at V.O. and Kiss1 mRNA or kisspeptin-ir in ARC and AVPV were present. Surprisingly, a negative correlation was present between leptin and kisspeptin-ir in ARC, which is in contradiction with data reporting that leptin is a positive modulator of *Kiss1* expression (Smith et al., 2006a, Quennell et al., 2011).

Changes in the kisspeptinergic system in relation to a high-fat diet induced advancement of puberty onset, has only been assessed by one other group. Hypothalamic *Kiss1* expression was evaluated just prior to V.O. and was found to increase insignificantly in Sprague Dawlay rats fed the high-fat diet (Moral et al., 2011). The failure to detect a change in Kiss1 mRNA in the entire hypothalamus by Moral et al. (2011) may reflect dilution of restricted regional changes in *Kiss1* expression in ARC and/or AVPV. It is also conceivable that the regional changes would rather be reflected in kisspeptin-ir. Last, it is possible that the genetic difference is implicated in the lack of a clear effect on Kiss1 mRNA levels in Moral et al. (2011).

Based on the limited data available, it is not possible to conclude whether the post-weaning high-fat diet induced change in puberty onset, reported elsewhere (Boukouvalas et al., 2008, Brill and Moenter, 2009, Sloboda et al., 2009, Boukouvalas et al., 2010, Moral et al., 2011), is associated with an advanced maturation of the kisspeptinergic system. However, according to the data obtained here, a post-weaning high-fat diet has no effect on either puberty onset or the kisspeptinergic system.

High-fat diet exposure through lactation

In the present study, high-fat diet exposure through lactation from PND1 to 16 did not interfere with puberty onset or body weight, and no correlation between timing of V.O. and weight gain in the diet period was present. Also, Kiss1 mRNA levels in ARC and AVPV and plasma estradiol, leptin, triglyceride, and insulin levels were unchanged by the high-fat diet exposure. These findings are consistent with previous data where a high-fat diet from PND1 to 15 through lactation neither affected V.O. nor changed the levels of leptin, insulin, or triglycerides (Chang et al., 2008).

Since the high-fat diet exposure was through lactation, an obvious question is what the offspring was exposed to through the milk. Dams, subjected to a high-fat diet only during the lactating period for three weeks, had an increased triglyceride level and a decreased estradiol level, whereas no changes in leptin or insulin levels were detected in the present study. Although, it should be mentioned that the triglyceride levels measured may not be representative due to methodological complications

described in *Triglyceride*. It has previously been shown that dams exposed to a high-fat diet with 60% of the energy from fat, have the same caloric intake during lactation as control fed dams (Chang et al., 2008). Furthermore, a diet with 60% energy from fat induced from the second day of gestation onwards, increases fat content in the milk from PND10 onwards from 11% to 14%, without changing the level of leptin in the milk (Purcell et al., 2011). It is uncertain whether the fat content of the milk was changed at any time in the present study, given the high-fat-diet was not induced before the first day of lactation. Based on this, it is possible that the pups, subjected to the high-fat diet only through lactation, are not metabolic challenged as the dams may have functioned as a barrier, and the reason why no change in puberty onset was present.

In contrast, female rats subjected to overfeeding, by being raised in small litters, a widely used model of overfeeding, had advanced V.O. and a pronounced increase in body weight and leptin, that persisted after the overfeeding period (Castellano et al., 2011, Smith and Spencer, 2012). This model, in contrast to a high-fat diet, increases the amount of available food per pup. Manipulations of litter size can have an impact of additional factors e.g. sex steroids and the adrenal stress axis that may have an effect on puberty onset (Castellano et al., 2011). Maternal care could also have been more intense in the small litters and an important bias towards an earlier puberty onset, but whether this is the case remains unknown.

As reported her, no change in the kisspeptinergic system was present in rats subjected to a high-fat diet through lactation, but whether the change in timing of V.O., induced by overfeeding, is caused by a change in the kisspeptinergic system is ambiguous (Castellano et al., 2011, Smith and Spencer, 2012). One study report an increase in hypothalamic Kiss1 mRNA at PND36 in the overfed rats, whereas the number of kisspeptin cells in ARC or kisspeptin fiber number and density in AVPV only tends to increase at PND36 in the overfed rats (Castellano et al., 2011). It is possible that the observed differences in Kiss1 mRNA and kisspeptin-ir would be larger if they were assessed at PND34 where all overfed rats had V.O., but only a smaller fraction of the normal fed rats had V.O.. Since *Kiss1* expression was determined in the entire hypothalamus it is not clear where the regional changes in Kiss1 mRNA occurs. Another similar study assessed Kiss1 mRNA levels the day of V.O., and found no difference in Kiss1 mRNA in either ARC or AVPV in the overfed rats with earlier V.O. (Smith and Spencer, 2012). Since Kiss1 mRNA primarily increases prior to V.O. (Han et al., 2005, Clarkson and Herbison, 2006, Bentsen et al., 2009, Clarkson et al., 2009a, Takase et al., 2009) and since no change in Kiss1 mRNA occur upon V.O. in the present study, this lack of difference in Kiss1 mRNA could be due to the fact that Kiss1 mRNA is assessed at V.O. as opposed to a fixed day like in present study and by Castellano et al. (2011). Considering the limited data available, it is not possible to conclude whether the advancement in puberty onset induced by pre-weaning overfeeding (Castellano et al., 2011, Smith and Spencer, 2012) is associated with an earlier increase in Kiss1 mRNA and/or kisspeptin-ir in ARC and/or AVPV. Though, it is tempting to propose that the persistent increase in leptin and a subsequent positive modulation of *Kiss1* expression is involved in the precocious puberty, which is supported by Castellano

et al. (2011). Leptin was not measured in the perinatal period in the overfed fats (Castellano et al., 2011, Smith and Spencer, 2012), but it is possible that the pre-weaning overfeeding interfered with the prenatal leptin-surge and thereby altered the formation of kisspeptin projections from ARC. It has been shown that an impaired perinatal leptin-surge impair the formation of ARC projections (Bouret et al., 2004b, Bouret et al., 2008), but whether an increased leptin level in the period can have the opposite effect remains undefined. Since the high-fat diet was not induced before PND1 in this study, and given that no change in leptin was present at PND34, it is likely that leptin was not affected in the perinatal period in the present study.

Other factors affected by the energy status and possible modulators of *Kiss1* expression like NPY and ghrelin could play a role in the advancement of pubertal timing induced by overfeeding (Castellano et al., 2011, Smith and Spencer, 2012). Since puberty in female rats do not appear to be sensitive to ghrelin (Fernandez-Fernandez et al., 2005), ghrelin do not seem to be a relevant factor in this respect. Also, an increase in sex steroid levels or the bioavailability hereof due to obesity (de Ridder et al., 1992, Barber et al., 2006, Dunger et al., 2006, Ahmed et al., 2009, Brill and Moenter, 2009) may modulate *Kiss1* expression and thereby advance V.O. in the overfed rats.This idea is consistent with the fact that litter size manipulations increased body weight after the overfeeding period and can interfere with sex steroid levels (Castellano et al., 2011).

In contrast to the effect of pre-weaning overfeeding on puberty onset (Castellano et al., 2011, Smith and Spencer, 2012), an increase in fat energy percentage during lactation has no effect on timing of V.O. or the kisspeptinergic system in the offspring according to the data presented here.

High-fat diet exposure through lactation and post-weaning

In the present study, high-fat diet exposure in the entire postnatal period did not interfere with the timing of V.O.. This finding is in contradiction with a similar study were a high-fat diet exposure from PND1 to puberty advanced V.O. in Sprague Dawley rats (Lo et al., 2009). No correlation between V.O. and weight gain from PND7 to 34 was present in this study, but the HFD PND1-34 group weighted slightly more at V.O.. However, no effect of the high-fat diet was reflected in any of the metabolic factors. Also, no change in body weight occurred in the high-fat fed rats in this study, which is consistent with Lo et al. (2009).

Several differences between the studies can contribute to the different outcomes regarding timing of V.O.. The fat percentage was lower and the fat source in the Lo et al. (2009) study was exclusively safflower, which is believed to contain phytosteroids (Bieber, 1986). Both factors could be implicated in the advanced puberty onset reported in Lo et al. (2009)(Lo et al., 2009) since no change in puberty onset was present here. In addition, V.O. occurred quite late in the study by Lo et al. (2009) in comparisons with this study, which suggests that other factors are regulating puberty onset as described above.

At PND34, no changes in Kiss1 mRNA levels in ARC or AVPV were present in the HFD PND1-34 group. Since no concomitant characterization of the kisspeptinergic system was evaluated by Lo et al. (2009) it is not possible to deduce if the advanced puberty onset induced by high-fat feeding in Lo et al. (2009) is accompanied by an increase in Kiss1 mRNA or kisspepetin-ir in ARC or AVPV. However, according to the data presented here, an increase in fat energy percentage alone in the postnatal period has no effect on puberty onset or the kisspeptinergic system.

Nutritional induced changes in pubertal timing in rodents and humans – translational aspects

Epidemiological data have shown that an increased weight gain, BMI or fat percentage in early life or at peri-puberty are associated with advanced puberty onset in girls (He and Karlberg, 2001, Davison et al., 2003, Lee et al., 2007, Aksglaede et al., 2009a, Rosenfield et al., 2009, Maisonet et al., 2010). However, not all epidemiological surveys have found an association (de Ridder et al., 1992, Aksglaede et al., 2009b), and numerous methodological issues have to be encountered before making this association plausible.

An expert panel has recommended some methodological guidelines for assessing this link which includes longitudinal studies, pubertal maturation assessment by Tanner definitions with breast palpation, and body fat and weight covariates like BMI (Euling et al., 2008, Biro et al., 2010). Only a very limited number of the epidemiological surveys meet these recommendations. Many epidemiological surveys, assessing puberty onset as thelarche (Marshall and Tanner, 1969), do not palpate the breast tissue (Ahmed et al., 2009, Rosenfield et al., 2009), and increased adiposity can thus be mistaken as early breast development (Ahmed et al., 2009). Only few of the studies measure percent body fat, but measure only BMI, which is a surrogate marker for fat percentage (Kaplowitz, 2008). Also, different definitions of puberty onset are applied e.g. peak height velocity, onset of growth spurt (He and Karlberg, 2001, Aksglaede et al., 2009a), thelarche (Ahmed et al., 2009, Rosenfield et al., 2009, Biro et al., 2010), and menarche (Dunger et al., 2006) which are occurring at very different times. Peak height velocity and pubertal growth spurt being considered early markers, whereas menarche is a late marker (Karaolis-Danckert et al., 2009). It is likely that the different pubertal markers are not equally susceptible to a metabolic regulation as observed in both epidemiological surveys (Karaolis-Danckert et al., 2009) and animals studies (Moral et al., 2011), and thereby contribute to the different findings. These methodological guidelines are extremely important to encounter before drawing the conclusion that obesity markers at different developmental periods, are implicated in the secular trend in pubertal timing, to avoid incorrect conclusions. This can also reveal whether there is a critical window or sensitive period during which the regulation of pubertal maturation are susceptible to an obesity induced change in girls (Kaplowitz, 2008).

A link between a postnatal positive energy challenge and an earlier puberty onset has been reported in rodents where V.O. is used as a marker of puberty onset (Hilakivi-Clarke et al., 1997, Boukouvalas et

al., 2008, Chang et al., 2008, Brill and Moenter, 2009, Lo et al., 2009, Sloboda et al., 2009, Boukouvalas et al., 2010, Castellano et al., 2011, Moral et al., 2011). V.O., an external sign of puberty onset in rodents, is a functional marker indicating that a proper functional activation of all the levels of the HPG axis has occurred (Navarro et al., 2004b), and more likely correspond to menarche in girls. It is conceivable that V.O. is not a proper marker for evaluating the effect of a metabolic induced change in pubertal timing in rodents since it may not be as sensitive as an earlier pubertal marker. This is consistent with the lack of effect of a high-fat diet on V.O. reported here. In accordance, only small effects of a high-fat diet on V.O. were present in the other studies (Hilakivi-Clarke et al., 1997, Boukouvalas et al., 2008, Brill and Moenter, 2009, Lo et al., 2009, Sloboda et al., 2009, Boukouvalas et al., 2010, Moral et al., 2011). This is in line with the secular trends observed in Danish girls, where menarche has declined only by few months in contrast to thelarche, which has declined by 1 year within the last decades (Aksglaede et al., 2009b, Mouritsen et al., 2010), which suggests that the advanced puberty onset observed in girls is not accompanied by a full activation of the HPG axis, which V.O. is a marker for. Another possible reason for the lack of effect of a high-fat diet on puberty onset reported here, and the small effects observed elsewhere (Hilakivi-Clarke et al., 1997, Boukouvalas et al., 2008, Brill and Moenter, 2009, Lo et al., 2009, Sloboda et al., 2009, Boukouvalas et al., 2010, Moral et al., 2011), could be that a nutritional regulation of puberty onset are absent in rodents. This idea is in keeping with epidemiological surveys being unable to find a link between earlier thelarche and pubertal BMI (Aksglaede et al., 2009b), or thelarche and pubertal body fat mass or distribution (de Ridder et al., 1992).

Only few studies have investigated the mechanisms underlying the possible nutritional induced change in pubertal timing in rodents and humans. Animal models, like rodents, are invaluable in this context. Though, by using V.O. as a marker for puberty onset it may be difficult to see metabolic induced changes in the systems believed to be pivotal in the reactivation of the HPG axis, since the HPG axis is already fully activated at V.O. This is consistent with the fact that both Kiss1 mRNA and kisspeptin-ir in ARC and AVPV primarily increases prior to puberty onset in rodents (Han et al., 2005, Clarkson and Herbison, 2006, Bentsen et al., 2009, Clarkson et al., 2009a, Takase et al., 2009). Therefore, it would be beneficial to find and implement the use of an earlier marker for pubertal maturation in rodents which may correlate better to nutritional induced changes in puberty onset, and changes in the kisspeptinergic system or other factors believed to play a role in the pubertal maturation. However, V.O. is an easy inexpensive external pubertal marker, and an earlier marker would probably meet methodological obstacles since it would not be external, but require blood samples or the assessment of the reproductive organs.

To translate the findings obtained in rodents, the physiological differences between rodents and humans need to be considered. In rodents, estradiol seems to play a central role in the reactivation of the HPG axis and the maturation of the kisspeptinergic system (Clarkson et al., 2009a, Clarkson et al., 2010), which is not the case in humans and higher primates, where the reactivation of the HPG axis

appears to be independent of an increase in sex steroids (Guerriero et al., 2012b, Plant, 2012). This is extremely relevant in regard to the kisspeptinergic system, since *Kiss1* expression in ARC is regulated by sex steroids in both rodents and higher primates, but in different ways as *Kiss1* expression in AVPV in rodents, and not in the corresponding POA in higher primates, are subjected to a regulation by sex steroids (Dhillo, 2008, Smith, 2009). To exploit the application of animal models, like rodents, in a translational aspect many more epidemiological and rodent studies are required to elucidate the physiological differences as well as to define the critical window, if any, for a nutritional regulation of pubertal timing. This should be done in order to reveal when and how, e.g. by changing food content, amounts etc., the nutritional challenge should be applied in rodents, in order to model the human scenarios best.

Even if a nutritional induced effect on pubertal timing is present in girls and rodents, one needs to have in mind that many other factors seems to be involved in the regulation of pubertal timing, which is revealed by epidemiological surveys (Aksglaede et al., 2009b, Rosenfield et al., 2009, Maisonet et al., 2010, Mouritsen et al., 2010), and by the large viability in V.O. in rodents reported her and by others (Boukouvalas et al., 2008, Chang et al., 2008, Brill and Moenter, 2009, Lo et al., 2009, Boukouvalas et al., 2010, Castellano et al., 2011, Moral et al., 2011, Smith and Spencer, 2012).

Estrous cyclicity

According to the present study, a high-fat diet from weaning induced an irregular estrous cycle in a subgroup of the rats primarily by extending the diestrous phase. This was accompanied by a pronounced increase in leptin. This subgroup of rats had no change in body weight in contrast to the high-fat fed with a regular cycle, which weighted more, and had an increased leptin level which was not as high as in the HFD extended cycle group. This reveals that some Sprague Dawley rats are prone to diet induced obesity, while others are resistant, like reported previously (Dourmashkin et al., 2006).

A positive correlation between final body weight and leptin levels was present when based on all animals, but not when the control and high-fat fed subgroups were assessed individually. Surprisingly, the irregular estrous cycle was present in the rats resistant to diet induced obesity, but since leptin levels were almost doubled in the HFD extended cycle group it suggest that adiposity increased without a concomitant increase in body weight. This is consistent with the lack of correlation between final body weight and leptin levels in this subgroup. Why this subgroup of animals experienced a much higher leptin increase than the high-fat fed rats with a regular cycle it not certain.

A high-fat diet induced irregular estrous cycle has been reported both after 120 days (extended by one day) and 180 days (cycle duration doubled) exposure which was accompanied by an increased body weight and body fat (Akamine et al., 2010). The high-fat diet exposure for 120 or 180 days induced hyperinsulinemia, insulin resistance, and increased progesterone levels, but did not change estradiol levels (Akamine et al., 2010). In contrast to Akamine et al. (2010), the high-fat diet induced irregular

estrous cycle observed here do not appear to be mediated by changes in insulin, but whether leptin is affected by the high-fat diet in Akamine et al. (2010) was not assessed.

This study show that even 40 days of high-fat diet exposure, not reflected by a change in body weight, extend the estrous cycle. A phenomenon, that is more pronounced after longer exposure to a high-fat diet accompanied by a more distinct increase in body weight (Akamine et al., 2010), which is consistent with the clinical findings (Linne, 2004, Nelson and Fleming, 2007, Pasquali et al., 2007).

An association between an impaired kisspeptinergic system, due to a decreased kisspeptin fiber density in ARC and AVPV, and an irregular estrous cycle has been reported in adult rats subjected to neonatal exposure to phytoestogens or estrogen specific ligands (Bateman and Patisaul, 2008). In the present study, the high-fat diet had no impact on *Kiss1* expression or number of kisspeptin-ir cells in ARC or AVPV. To my knowledge, no characterization of the kisspeptinergic system in relation to a high-fat induced extension of the estrous cycle has been conducted. However, a previous study has detected a decrease in Kiss1 mRNA levels in AVPV in mice fed a high-fat diet for approximately 150 days (Quennell et al., 2011). This decrease was only reported in mice being susceptible to obesity-induced infertility, and could not be detected in mice not prone to obesity-induced infertility, even though both mice strains weighted more (Quennell et al., 2011). This mice study emphasizes how important the genetic background is for a metabolic regulation of fertility and even *Kiss1* expression. According to the present findings, the kisspeptinergic system is not likely to be involved in the high-fat diet induced irregularity of the estrous cycle. Though, it cannot be excluded that the high-fat diet period is too short to reveal an effect on Kiss1 mRNA levels or kisspeptin-ir.

It is conceivable that leptin could be mediating the metabolic regulation of estrous cyclicity in a kisspeptin independent manner given Kiss1 mRNA levels were unchanged in the present study. This hypothesis, which is highly speculative, is supported by the lack of effect of a genetic deletion of the leptin receptor from kisspeptin neurons in mice, on pubertal timing and fertility (Donato et al., 2011b).

As described above, several other factors including NPY and ghrelin are affected by a nutritional challenge (Fernandez-Fernandez et al., 2005, Luque et al., 2007), but neither of these factors appear to be involved in the high-fat diet induced irregular estrous cycle, given estrous cyclicity are unaffected by chronic ghrelin administration (Fernandez-Fernandez et al., 2005) and due to the absence of overtly infertility in NPY deficient mice (Luque et al., 2007). Nor, seems a change in the level of sex steroids or the bioavailability hereof to be involved given no changes in either estradiol or insulin, a known modulator of sex steroid levels and bioavailability (Ahmed et al., 2009), were present in the HFD extended cycle group.

These types of animal studies aiming to reveal the underlying mechanisms of a nutritional regulation of reproductive function are becoming increasingly important due to the increasing obesity epidemic and the equivalent need of fertility treatment (Nelson and Fleming, 2007, Pasquali et al., 2007). However, the physiological differences in regulating reproductive function between human and

rodents, e.g. the kisspeptinergic control of the preovulatory LH surge (Plant, 2012), needs to be considered before translating the findings in rodents to humans.

Maturation of the kisspeptinergic system

We detected big variations in Kiss1 mRNA levels in ARC and AVPV at PND34 which can be due to different stages of HPG activation in both the rats with and without V.O.. Also, it cannot be excluded that the rats, with V.O. at PND32 or PND33 are cyclic since they typically become cyclic immediately after V.O. (Goldman et al., 2007). If so, Kiss1 mRNA in AVPV and ARC at PND34 would be affected by circulating sex steroids.

No changes in Kiss1 mRNA levels at PND34 in either ARC or AVPV were present in rats with V.O. in contrast to rats without V.O.. This is in line with the fact that Kiss1 mRNA levels in ARC and AVPV primarily increases prior to puberty onset in both ARC and AVPV (Han et al., 2005, Clarkson and Herbison, 2006, Bentsen et al., 2009, Clarkson et al., 2009a, Takase et al., 2009, Takumi et al., 2011). To my knowledge, no studies have evaluated changes in Kiss1 mRNA on a fixed day in relation to V.O., and developmental studies of *Kiss1* expression with a week interval around V.O. have lead to mixed results for AVPV, whereas no change in ARC Kiss1 mRNA was detected (Takase et al., 2009, Takumi et al., 2011). In the present study, a positive correlation between V.O. and Kiss1 mRNA in AVPV at PND34 was present, meaning that Kiss1 mRNA levels in AVPV were higher if assessed the same day V.O. occurred. Whether this is due to an actual peak in Kiss1 mRNA in AVPV at V.O. remains uncertain since Kiss1 mRNA levels were not measured in one animal before and after V.O.. However, if a peak in Kiss1 mRNA in AVPV occurs at V.O. it would indicate that AVPV kisspeptin neurons may be involved in the regulation of V.O.. Another possible interpretation of the positive correlation is that rats with earlier V.O. have a lower Kiss1 mRNA level in AVPV, but the lack of correlation between V.O. and Kiss1 mRNA in AVPV at PND61 argues against this possibility.

As reported before (Takase et al., 2009), the number of kisspeptin-ir cells in ARC at PND34 was higher in rats with V.O. than without V.O.. The physiological meaning of this increase in kisspeptin-ir in ARC upon V.O. is undefined. No change in the number of kisspeptin-ir cells in AVPV was present after V.O. which is in contradiction with two studies reporting an increase in AVPV upon V.O., after use of colchicin (Sun et al., 2007, Takase et al., 2009). It appears that kisspeptin-ir cells in AVPV are easily visualized in mice in contrast to rats, where kisspeptin is virtually absent unless colchicin, which is an inhibitor of axonal transport, is applied (Castellano et al., 2011). The difference in detecting kisspeptin-ir in ARC and AVPV is probably due to physiological differences in the transport, processing, and secretion of kisspeptin in the two nuclei (Castellano et al., 2011). Colchicin was not applied in the present study since colchicin cause unphysiological conditions and may affect kisspeptin expression. Due to this, kisspeptin-ir in AVPV was also assessed by OD to include both soma and fiber kisspeptin-ir, but also this approach revealed no change in kisspeptin-ir in AVPV upon V.O.

In keeping with previous literature (Takumi et al., 2011, Desroziers et al., 2012) the present study shows that Kiss1 mRNA levels in ARC and the number of kisspeptin-ir cells in ARC increases from peri-puberty at PND34 to early adulthood at PND61 in female rats. Whether the same is true for kisspeptin in AVPV is ambiguous since only an increase in Kiss1 mRNA were detected here and not by others (Takumi et al., 2011), and due to the methodological obstacles regarding assessing kisspeptin-ir in AVPV in female rats without the use of colchicin as experienced here and reported by others (Desroziers et al., 2012). Though, it should be noted that only approximately 50% of the animals assessed at peri-puberty had V.O., however, since no changes in Kiss1 mRNA levels in either ARC or AVPV occurred after V.O., this seems not to cause artificially low Kiss1 mRNA levels at PND34. Whether the same holds for kisspeptin-ir in ARC is questionable since kisspeptin-ir in ARC increases after V.O..

Based on the limited data available it is not possible to reveal a consistent developmental profile for the kisspeptinergic system at peri-puberty and from peri-puberty to early adulthood in female rats. However, according to the data reported here, it is clear that the kisspeptinergic system undergoes a postnatal maturation both at peri-puberty and from peri-puberty to adulthood which is consistent with the literature (Han et al., 2005, Bentsen et al., 2009, Clarkson et al., 2009a, Takase et al., 2009, Takumi et al., 2011, Desroziers et al., 2012). Based on the present findings, Kiss1 mRNA levels and the number of kisspeptin-ir cells increases from peri-puberty to adulthood, which is not the case around V.O. where the number of kisspeptin-ir cells in ARC increases upon V.O. without any simultaneous increase in Kiss1 mRNA. This could be due to the fact that Kiss1 mRNA in ARC primarily increases prior to puberty onset (Han et al., 2005, Takase et al., 2009) and perhaps are unchanged around V.O.. Another possibility is that Kiss1 mRNA is translated more rapidly around V.O. where the kisspeptinergic system is central for stimulating the GnRH neurons. The physiological implication of these postnatal changes at peri-puberty and trough adulthood still remains to be elucidated.

Conclusion

A developmental maturation of the kisspeptinergic system occurs both at peri-puberty and from peri-puberty to early adulthood in female rats. The number of kisspeptin-ir cells in ARC at PND34 increased upon V.O. whereas both an increase in the number of kisspeptin-ir cells in ARC and in Kiss1 mRNA levels in ARC and AVPV occurred from peri-puberty to early adulthood.

The postnatal high-fat diet exposure through lactation, post-weaning, or both periods, had no effect on puberty onset, revealed by V.O., or body weights. In accordance, no changes in the metabolic factors and hormones including leptin, insulin, triglycerides, and estradiol, were present at PND34 in any of these high-fat fed rats. Further supporting the lack of effect, high-fat diet, in the three different postnatal feeding periods, did not affect the kisspeptinergic system, reflected by changes in *Kiss1* expression and in the number of kisspeptin-ir cells in ARC or AVPV.

The high-fat diet induced an irregular estrous cycle in a subgroup of the rats, characterized by an unaffected body weight, but a pronounced increase in leptin levels. Surprisingly, no changes in estrous cyclicity were present in the high-fat fed rats that had an increased body weight as well as an increased leptin level that were lower than in the high-fat fed rats with an irregular estrous cycle. It is highly speculative, but since *Kiss1* expression and the number of kisspeptin-ir cells were unchanged by the high-fat diet, leptin could be implicated in the high-fat diet induced extension of the estrus cycle independent of a modulation of the kisspeptinergic neurons. To elucidate whether this is plausible and whether the mechanisms are the same after a longer high-fat diet exposure requires more studies.

The present studies have added knowledge to the understanding of the effect of a postnatal high-fat diet on pubertal timing and estrous cyclicity. In conclusion, these data shows that a high-fat diet, during lactation, post-weaning or both periods, is unable to affect puberty onset and the kisspeptinergic system. This suggests that an increase in fat energy percentage in the diet alone cannot change the timing of V.O. or affect the kisspeptinergic neurons. However, the high-fat diet induces an irregular estrous cycle, perhaps through leptin, without modulating kisspeptinergic neurons.

Perspectives

It is still ambiguous whether the secular trend in pubertal timing in girls and impaired reproductive function in women are due to the rising incidence of obesity, and if so, what the link is. To shed light on this matter, more studies, conducted in agreement with the expert panels guidelines, are required, also to elucidate whether a critical developmental window for a nutritional regulation of puberty exist.

Animal models like rodents are invaluable when investigating the underlying mechanisms of a nutritional regulation of puberty onset. Surprisingly, no effects of the high-fat diet on puberty onset were observed here, making it impossible to assess whether the high-fat diet induced advancement of puberty onset observed in rodents by others are accompanied by an advanced maturation of the kisspeptinergic system. Therefore, it still remains unclear whether an increase in fat energy percentage in the diet alone can affect the pubertal timing and the kisspeptinergic system since conflicting results have been reported. To elucidate this, further studies are required, and also to define how and when the nutritional challenge should be applied in rodents to model the human scenario best. To use rodents as a model for human scenarios, the physiological differences between humans and rodents, e.g. regarding regulation of the reactivation of the HPG axis and the metabolic response to a nutritional challenge, needs to be addressed.

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DIO Rodent Purified Diet w/10% Energy From Fat - Yellow 58Y2

DESCRIPTION

Diet Induced Obesity Rodent Purified Diet with 10% Energy From Fat, Dyed Yellow is based on AIN-76A Semi-Purified Diet, Rat or Mouse 5800-B. See Van Heek et al., J. Clin. Invest. 99:385-390, 1997, for initial use of lower-fat versions of this formula. Originally manufactured as "D12450B".

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be

*Product Forms Available**	*Catalog #*
1/2" Pellet	58124
1/2" Pellet, Irradiated	56834
Meal	1810727
Meal, Irradiated	1810728

*Other Forms Available On Re

INGREDIENTS (%)

Sucrose	33.1290
Dextrin	29.8560
Casein - Vitamin Free	18.9560
Powdered Cellulose	4.7390
Maltodextrin	3.3170
Soybean Oil	2.3700
Lard	1.8960
Potassium Citrate, Tribasic Monohydrate	1.5640
Dicalcium Phosphate	1.2320
DIO Mineral Mix	0.9480
AIN-76A Vitamin Mix	0.9480
Calcium Carbonate	0.5210
L-Cystine	0.2840
Choline Bitartrate	0.1900
Yellow Dye	0.0500

NUTRITIONAL PROFILE [1]

Protein, %	***17.3***
Arginine, %	0.66
Histidine, %	0.49
Isoleucine, %	0.91
Leucine, %	1.64
Lysine, %	1.38
Methionine, %	0.49
Cystine, %	0.35
Phenylalanine, %	0.91
Tyrosine, %	0.96
Threonine, %	0.73
Tryptophan, %	0.21
Valine, %	1.08
Alanine, %	0.52
Aspartic Acid, %	1.22
Glutamic Acid, %	3.87
Glycine, %	0.37
Proline, %	2.23
Serine, %	1.05
Taurine, %	0.00
Fat, %	***4.3***
Cholesterol, ppm	18
Linoleic Acid, %	1.39
Linolenic Acid, %	0.19
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.19
Total Saturated Fatty A	1.14
Total Monounsaturated Fatty Acids, %	1.30
Polyunsaturated Fatty Acids, %	1.59
Fiber (max), %	***4.7***
Carbohydrates, %	***67.4***
Energy (kcal/g) [2]	***3.78***

From:	kcal	%
Protein	0.692	18.3
Fat (ether extract)	0.384	10.2
Carbohydrates	2.697	71.5

Minerals	
Calcium, %	0.57
Phosphorus, %	0.43
Phosphorus (available), %	0.43
Potassium, %	0.57
Magnesium, %	0.05
Sodium, %	0.12
Chloride, %	0.21
Fluorine, ppm	0.9
Iron, ppm	44
Zinc, ppm	34
Manganese, ppm	55
Copper, ppm	5.7
Cobalt, ppm	0.0
Iodine, ppm	0.20
Chromium, ppm	1.9
Molybdenum, ppm	1.55
Selenium, ppm	0.15
Vitamins	
Vitamin A, IU/g	3.8
Vitamin D-3 (added), IU/g	0.9
Vitamin E, IU/kg	49.3
Vitamin K (as menadione), ppm	0.48
Thiamin Hydrochloride, ppm	5.7
Riboflavin, ppm	5.7
Niacin, ppm	28
Pantothenic Acid, ppm	14
Folic Acid, ppm	1.9
Pyridoxine, ppm	5.5
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	9
Choline Chloride, ppm	950
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only; NOT for human consumption.

6/28/2007

ISO 9001:2000 CERTIFIED

TestDiet
www.testdiet.com

DIO Rodent Purified Diet w/60% Energy From Fat - Blue 58Y1

DESCRIPTION

Diet Induced Obesity Rodent Purified Diet with 60% Energy From Fat - Dyed Blue is based on AIN-76A Semi-Purified Diet, Rat or Mouse 5800-B. See Van Heek et al., J. Clin. Invest. 99:385-390, 1997, for initial use of this formula. Originally manufactured as "D12492".

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet	58126
1/2" Pellet, Irradiated	56833
Meal	1810473

**Other Forms Available By Request*

INGREDIENTS

Lard	31.6600
Casein - Vitamin Free	25.8450
Maltodextrin	16.1530
Sucrose	8.8470
Powdered Cellulose	6.4610
Soybean Oil	3.2310
Potassium Citrate, Tribasic Monohydrate	2.1320
Calcium Phosphate	1.6800
AIN-76A Vitamin Mix	1.2920
DIO Mineral Mix	1.2920
Calcium Carbonate	0.7110
L-Cystine	0.3880
Choline Bitartrate	0.2580
Blue Dye #1	0.0500

Part of the TestDiet® "Blue-Pink-Yellow" DIO Series ("van Heek" Series)

DIO Rodent Purified Diet w/10% Energy From Fat - Yellow
- 1/2" Pellet - Catalog # 58124 (58Y2)
- Meal - Catalog # 56834 (58Y2)

DIO Rodent Purified Diet w/45% Energy From Fat - Red
- 1/2" Pellet - Catalog # 58125 (58V8)
- 1/2" Pellet, Irradiated - Catalog # 55629 (58V8)
- Meal - Catalog # 1810729 (58V8)
- Meal, Irradiated - Catalog # 1810730 (58V8)

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only, not for human consumption.

NUTRITIONAL PROFILE [1]

Protein, %	***23.1***
Arginine, %	0.90
Histidine, %	0.67
Isoleucine, %	1.24
Leucine, %	2.24
Lysine, %	1.88
Methionine, %	0.67
Cystine, %	0.48
Phenylalanine, %	1.24
Tyrosine, %	1.31
Threonine, %	1.00
Tryptophan, %	0.29
Valine, %	1.47
Alanine, %	0.71
Aspartic Acid, %	1.66
Glutamic Acid, %	5.28
Glycine, %	0.50
Proline, %	3.04
Serine, %	1.43
Taurine, %	0.00
Fat, %	***34.9***
Cholesterol, ppm	301
Linoleic Acid, %	4.70
Linolenic Acid, %	0.39
Arachidonic Acid, %	0.06
Omega-3 Fatty Acids, %	0.39
Total Saturated Fatty Acids, %	13.68
Total Monounsaturated Fatty Acids, %	14.00
Fiber (max), %	***6.5***
Carbohydrates, %	***25.9***
Energy (kcal/g) [2]	***5.10***

From:	kcal	%
Protein	0.924	18.1
Fat (ether extract)	3.140	61.6
Carbohydrates	1.035	20.3

Minerals

Calcium, %	0.79
Phosphorus, %	0.59
Phosphorus (available), %	0.59
Potassium, %	0.77
Magnesium, %	0.07
Sodium, %	0.15
Chlorine, %	0.25
Fluorine, ppm	1.2
Iron, ppm	64
Zinc, ppm	46
Manganese, ppm	76
Copper, ppm	7.8
Cobalt, ppm	0.0
Iodine, ppm	0.27
Chromium, ppm	2.6
Molybdenum, ppm	2.11
Selenium, ppm	0.29

Vitamins

Vitamin A, IU/g	5.2
Vitamin D-3 (added), IU/g	1.3
Vitamin E, IU/kg	67.2
Vitamin K (as menadione), ppm	0.65
Thiamin Hydrochloride, ppm	7.8
Riboflavin, ppm	8.7
Niacin, ppm	39
Pantothenic Acid, ppm	21
Folic Acid, ppm	2.8
Pyridoxine, ppm	7.5
Biotin, ppm	0.3
Vitamin B-12, mcg/kg	18
Choline Chloride, ppm	1,290
Ascorbic Acid, ppm	0.0

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly. Nutrients expressed as percent of ration on an As Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

9/14/2010

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Appendix III

Table 3. A comparative summary of HFD PND21-34, HFD PND21-puberty, HFD PND1-16, and HFD PND1-34.

Experiment	HFD PND21-34		HFD PND21-puberty		HFD PND1-16		HFD PND1-34	
Diet	Control	High-fat	Control	High-fat	Control	High-fat	Control	High-fat
Final weight at PND34	111.2±1.2	115.8±1.0	-	-	117.4±3.7	115.3±4.9	117.4±3.7	121.1±4.5
Weight gain (g)[1]	64.7±1.2	69.1±1.5*	85.1±1.5[2]	88.6±1.2[2]	38.4±0.7[3]	36.9±0.5[3]	103.6±1.8[4]	105.7±1.8[4]
% with V.O. at PND34	45%	60%	67%	63%	44%	33%	44%	59%
Leptin (ng/ml)[5]	1.92±0.19	2.05±0.19	-	-	1.95±0.21	1.65±0.05	1.95±0.21	2.03±0.40
Insulin (ug/ml) [5]	1.20±0.22	1.07±0.14	-	-	0.63±0.11	0.33±0.14	0.63±0.11	0.57±0.15
Triglyceride (mg/ml) [5]	1.05±0.15	0.71±0.08	-	-	0.50±0.05	0.39±0.03	0.50±0.05	0.46±0.06
Estradiol (pg/mL) [5]	17.10±2.23	14.65±0.97	-	-	13.01±1.50	10.98±1.3	13.01±1.50	10.59±0.75
***Kiss1* mRNA in ARC[6]**	100.0± 6.4	109.4±10.9	-	-	99.8±4.4	96.0±1.4	99.8±4.4	98.2±3.6
***Kiss1* mRNA in AVPV[6]**	99.9±17.8	90.7±19.0	-	-	100.4±10.5	82.7±12.3	100.3±10.5	104.8±8.2
Kisspeptin-ir cells in ARC[7]	100.3±25.0	151.8±32.0	-	-	-	-	-	-
Kisspeptin-ir cells in AVPV[7]	2.0±0.9	4.2 ± 1.4	-	-	-	-	-	-

[1]Weight gain during the high-fat diet periods. [2]Weight gain from postnatal day (PND) 21 to 37. [3]Weight gain from PND1 to 18. [4]Weight gain from PND7 to 34. [5]Plasma levels at PND34. [6]Kiss1 mRNA levels were determined by qRT-PCR at PND34, and depicted as fold change in relation to the control. [7]Number of kisspeptin-immunoreactive (kisspeptin-ir) cells were determined by IHC with JVL-1 1:200. All data is presented as mean±SEM. *P<0.05, unpaired student's t-test.

Table 4. A comparative summary of HFD PND21-adulthood and subgroups.

Experiment	HFD PND21-adulthood		
Group	Vehicle	HFD regular cycle	HFD extended cycle
Diet	Control	High-fat diet	High-fat diet
Final weight at PND34	226.6±3.1	245.8±3.3***	232.7±5.1
Weight gain (g)[1]	180.6±2.8	199.4±3.2***	188.4±5.3
Leptin (ng/mL)[2]	3.22±0.23	5.51±0.47***	6.72±0.51***
Insulin (ug/mL)[2]	3.23±0.43	2.89±0.54	2.05±0.39
Triglyceride (mg/mL)[2]	1.05±0.11	0.65±0.08*	0.88±0.14
Estradiol (pg/mL)[2]	26.68±2.46	19.86±1.89	23.24±3.21
***Kiss1* mRNA in ARC[3]**	99.9±7.5	90.7±13.6	86.4±15.6
***Kiss1* mRNA in AVPV[3]**	100.0±14.1	87.3±8.6	89.8±12.1
Kisspeptin-ir cells in ARC[4]	577.7±47.4	573.3±42.8	553.5±129.5
Kisspeptin-ir cells in AVPV[4]	2.9±1.1	5.6±1.6	5.0±2.0

[1]Weight gain is from PND21 to 61. [2]Plasma levels at PND61 to 68 in the diestrous phase. [3]Kiss1 mRNA were determined by qRT-PCR at PND61 to 68, and depicted as fold change in relation to the control. [4]Number of kisspeptin-immunoreactive (kisspeptin-ir) cells were determined by IHC with JVL-1 1:200. All data is presented as mean±SEM. *P<0.05, One-Way ANOVA, n=8-24, ***P<0.001, One-Way ANOVA, n=8-24.