Hormonal regulation of puberty onset in female rats:

Is leptin a missing link?

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Numerous factors in the hypothalamus-pituitary-gonadal axis (HPG) are involved in the timing of puberty onset. Leptin signals the nutritional status and the presence of an adequate amount of loaded adipose tissue, as a long-term resource of energy, to the brain which in response switches on the reproduction process (i.e. puberty onset). The growth hormone-insulin-like growth factor I (GH/IGF-I) axis is also thought to be involved in the timing of puberty onset. The aim of this thesis was to test the hypothesis that leptin may directly or indirectly via the GH/IGFI-I axis trigger the onset of puberty onset in female rats.

First, we carried out a series of descriptive and basic studies. Time of puberty onset was monitored by scoring the moment of vaginal opening (VO). The data showed a strong positive correlation between body fat and leptin levels, and showed leptin is increasing over the prepubertal period. Then, we aimed to find out the effects of food-restriction (FR) on puberty onset, body fat, plasma leptin levels and body temperature. FR postponed time of VO. Body fat, plasma leptin levels and body temperature in the FR rats were significantly lower than the controls throughout the experiment.

To find out if leptin is the signal initiating the onset of puberty, we used FR rats as a model for delayed puberty onset, and centrally (icv) or peripherally (sc) administered leptin, or we centrally (icv) immunoneutralized leptin. Central leptin infusion not only restored the delay in puberty onset caused by food restriction but also advanced sexual maturation in normally fed animals. Like central infusion, also peripheral leptin infusion restored puberty onset in FR animals. So, we showed an advancing effect of both centrally and peripherally infused leptin on puberty onset in prepubertal female rats. The central immunoneutralization of leptin on the other hand, postponed puberty onset. We therefore conclude that leptin is one of the crucial factors triggering puberty onset in female rats.

In the FR model system we then centrally (icv) infused GH. The infusion of GH postponed puberty onset in normally fed rats, but advanced puberty onset in FR animals. Also, the plasma

leptin levels in the GH-infused animals were significantly higher than their controls and increased as GH infusion proceeded.
Central infusion of GH antiserum (AS) advanced puberty onset in the pair-fed animals but not in the ad lib-fed animals. Also the central infusion of somatostatin worked likewise in both groups. These findings match with the results of the central infusion of GH which also advanced puberty onset in FR animals only.

Finally, we centrally (icv) infused IGF-I in FR rats. Central infusion of IGF-I significantly postponed puberty onset in ad libitum fed animals.
Immunoneutralization of endogenous IGF-I enhanced prevailing plasma leptin levels, but there was no effect on the timing of puberty onset. Centrally-present endogenous IGF-I does not appear to be involved in puberty onset although it seems to inhibit leptin secretion.

In conclusion, our findings provide further evidence that leptin, a hormone produced mainly by adipose tissue, is an important and crucial signal between the bodily nutritional status and the brain (i.e. the hypothalamus) to trigger puberty onset. Furthermore, we suggest that there is a functional interaction between growth hormone (GH) and leptin to initiate puberty in female rats.

Contents

Chapter 1

General Introduction

1) The female Hypothalamic-Pituitary-Gonadal (HPG) axis

The hypothalamus-pituitary-gonadal (HPG) axis plays a major integrative role in the control of maternal and reproductive behaviour, including sexual development and differentiation as well as sexual maturation (i.e. puberty). Important stimuli for the various aspects of reproductive functions come from a variety of extero- and interoceptive sources including gonadal steroids. The hypothalamic neuropeptide that governs the function of the reproductive processes is gonadotropin-releasing hormone (GnRH) [139]:

1-1) Hypothalamic GnRH pulse generator

Hypothalamic neurons originating in the preoptic, arcuate, ventromedial, periventricular, and paraventricular nuclei transport and secrete a variety of releasing and inhibiting hormones to the portal vessels of the median eminence [139]. These releasing and inhibiting hormones are then transported to the capillary bed of the anterior pituitary where they control the secretion of the pituitary trophic hormones.

The GnRH neurons which constitute the fundamental component of the hypothalamic GnRH pulse generator originate in the olfactory placode, and during early fetal life they migrate through the forebrain to occupy positions diffusely distributed throughout the hypothalamus [131, 139]. The cell bodies of these neurons, which are approximately 15 μm in diameter and typically bipolar, send their axonal projections to the primary capillary plexus of the portal vasculature in the median eminence. The GnRH pulse generator is an oscillating neural circuit resulting in the pulsatile secretion of GnRH from terminals in the median eminence into the pituitary portal system

The GnRH cell bodies synthesize a prohormone, which is then processed to form the mature GnRH peptide for release by exocytosis at the axonal terminals in the median eminence. The neurobiological mechanisms that synchronise the activity of GnRH neurons, and thereby generate the pulsatile discharge of the decapeptide GnRH, are not completely determined. However, the synchronous discharge of GnRH neurons resulting in GnRH release in the median eminence is robustly associated with an increased electrophysiological activity in this region of the hypothalamus [180].

1-2) Ontogeny of the GnRH pulse generator activity

The hypothalamic GnRH pulse generator becomes operational long before the onset of puberty [139, 180]. Although, the phase of fetal development has received only scant attention, it is clear that by midgestation the GnRH pulse generator is driving the fetal pituitary. GnRH pulse generator activity is robustly expressed during the first few months following birth, and the pituitary gonadotrophs of the infant respond to this hypothalamic drive with the secretion of both LH and FSH. Within the first year of life, however, the GnRH pulse generator is brought to a halt, leading to the hypogonadotropic state that imposes gonadal quiescence until the pubertal phase of development is started by a reboot of the of GnRH pulse generator activity. Indeed, if the GnRH pulse generator was not stalled during infancy, gonadal activation and development of secondary sexual characteristics would be initiated prematurely, as occurs occasionally in the condition of central precocious puberty [180]. GnRH release is affected by the negative feedback of steroid hormones at the level of the hypothalamus. Female steroid hormones (estrogens and progesterone) negative feedback decreases the frequency of pulsatile GnRH stimulation of the pituitary resulting in a decreased frequency of pulsatile LH and FSH release. In fact, steroid hormones modulate, the firing of neurons that project to GnRH neurons since the GnRH neurons themselves have no estrogen receptors [139].

1-3) The pituitary

The role of the anterior pituitary in reproduction is the secretion of gonadotropins as governed by the hypothalamic secretion of GnRH. Functional competence of the pituitary occurs early in immature rats, heifers and ewe lambs [131]. Maturational changes occurring at the level of the pituitary in prepubertal female animals might include a reduction in the number of cytosolic receptors for estradiol (E_2) in the pituitary in prepubertal heifers or changes in the sensitivity to GnRH as shown in humans and rats [131]. GnRH binds to a membrane receptor on pituitary gonadotrophs and stimulates both LH and FSH synthesis and secretion. Under physiological conditions, the GnRH receptor varies and is directly correlated with the gonadotropin secretory capacity of pituitary gonadotrophs. For example, during the rat estrous cycle, a rise in GnRH receptors is seen just before the surge of gonadotropins that occurs on the afternoon of proestrus. When there is a decline in GnRH stimulation to the pituitary, as occurs in a variety of physiological conditions including states of lactation,

undernutrition, or seasonal periods of reproductive quiescence (anestrus), the number of GnRH receptors on pituitary gonadotrophs dramatically decreases [139].

The pulsatile nature of GnRH stimulation of the pituitary leads to the release of distinct pulses of LH into the peripheral blood. A critical factor governing LH and FSH secretion from the pituitary is the rate of pulsatile GnRH stimulation of the gonadotrophs. Experimental studies in which the hypothalamus was lesioned and GnRH was replaced by pulsatile infusion of exogenous GnRH showed that different frequencies of GnRH can lead to differential ratios of LH to FSH secretion from the pituitary [139]. The female gonadal steroid hormones (i.e. estrogens and progesterone) can negatively feedback at the levels of the hypothalamus and pituitary to decrease the frequency of pulsatile gonadotropin secretion and decrease the amplitude of LH pulses. Negative feedback of steroid hormones at the level of the pituitary decreases the sensitivity of pituitary gonadotrophs to GnRH resulting in a decreased amplitude of the LH pulses but not its frequency. In contrast, E_2 elicits positive feedback by acting at the level of the hypothalamus to increase GnRH release and at the level of the pituitary to increase gonadotrophic sensitivity to GnRH.

1-4) The ovary

The ovaries have an active role in the integration of endocrine mechanisms which is believed to have an inhibitory action on the hypothalamic-pituitary axis. Maturational changes in the developing rat ovary include an abrupt decrease in ovarian GnRH receptors together with a prolactin- and GH-induced increase in LH receptors. In addition, development of sympathetic innervations of the ovary appears to be critical for acquisition and maintenance of reproductive competence [116, 139].

During the first week of postnatal life the ovaries in female rats are relatively insensitive to gonadotropin stimulation. Nevertheless, the primary follicles can be distinguished by day 4 [131]. The ovaries become subjected to strong gonadotropin control during the second week of postnatal life as many small follicles begin to move into a more advanced developmental stage during this time. Also, ovarian production of estrogen from exogenous precursors considerably increases and FSH becomes able to induce aromatase activity. Meanwhile, early follicular growth is enhanced at this time, and since completion of follicular growth takes 15 to 19 days [131], this suggests that many of the follicles starting to grow during this period may be destined to reach a preovulatory or even an ovulatory condition at puberty.

The development of the neonatal-infantile ovary appears to be regulated by an additional modulatory mechanism of maternal origin that becomes established shortly after birth. Rat milk contains a GnRH-like substance which acts like hypothalamic GnRH, in that it can stimulate gonadotropin release from the anterior pituitary [131]. Since the rat pup suckles frequently throughout the entire day, it would be expected that milk-GnRH is almost continuously available for binding to the infant ovary which plays a physiological role in restraining neonatal-infantile development of the pup ovary [131]. This phenomenon may represent an evolutionary mechanism by which dam rat regulates gonadal development of its offspring beyond intrauterine life.

During juvenile development the ovaries grow under the influence of low serum levels of LH and FSH. In addition of those gonadotropins, two other anterior pituitary hormones seem to be involved in the regulation of prepubertal ovarian function: Both prolactin (Prl) and growth hormone (GH) have been shown to support ovarian maturation by facilitating the effects of gonadotropins. The secretion of both GH and Prl is low at the beginning of the juvenile period and increases gradually thereafter [131, 148, 149]. The most prominent Prl secretory episodes occur at midafternoon and during the early morning hours. It has been shown that Prl accelerates the onset of puberty in females, and exerts its effects by increasing the ovarian responsiveness to gonadotropins and to directly enhance aromatase gene expression. Also, it is suggested that a significant part of the stimulatory effects of Prl on the immature ovary is the facilitation of LH actions. Like Prl, GH also has facilitatory effects on the sexual maturation of female rats. Suppression of GH release can delay the onset of puberty, as determined by the age at vaginal opening (VO) and the first ovulation [131, 148, 149]. Part of this facilitatory effect of GH is likely to be exerted at the level of ovary, where GH induces LH receptors and stimulates progesterone secretion from granulosa cells in immature hypophysectomised rats [148, 149]. Although some of the actions of GH exerted in the ovary may be direct, most of them are mediated by IGF-I which is locally produced by granulosa cells, and GH facilitates in this way a number of FSH-dependent effects such as induction of aromatase activity, the progesterone secretion, and the formation of LH receptors [152].

2) Puberty

2-1) General definition

Puberty originates from the Latin word '*pubes'*, which means 'hair'. It is, therefore, possible that the onset of pubarche (development of pubic hair) had a significant influence to create the word '*puberty'*. Although, the origin of the word is simple, its definition is much more complex, as is the pubertal process itself. In female mammals puberty is defined as a single event: vaginal opening, first estrus, first ovulation, etc. All of these definitions represent good physiological markers for the onset of puberty, but they are only part of a continuum in the physiological processes comprising puberty.

Early simple definitions of the concept 'puberty' note that it is a physiological process by which the two sexes become completely differentiated, germ cells are released and reproduction first becomes switched on. Indeed, these definitions clearly distinguish puberty from sexual maturity: "The time when an animal expresses its full reproductive power" [131].

Puberty is a dynamic period of development marked by rapid changes in body size, shape and composition, all of which are sexually dimorphic. Puberty is characterized by the greatest sexual differentiation since fetal life and the most rapid rate of linear growth since infancy. Puberty is the stage of development, in which an animal first becomes capable of reproducing sexually and is characterized by maturation of the sexual organs, the development of secondary sexual characteristics, and changes in mood and behaviour. In sexually mature animals, pituitary gonadotropins regulate the processes of ovulation, spermatogenesis, and sex steroid production. The synthesis and secretion of the pituitary gonadotropins, in turn, are governed by the brain peptide gonadotropin-releasing hormone (GnRH), which is secreted by hypothalamic neurons, into a specialized portal circulation subserving the pituitary gland [101]; (see paragraph 1).

2-2) Mechanisms of action

Over the last decades a number of theories have been proposed with regards to the onset of puberty. Nevertheless, none of these theories provides all the essential information; however, it is likely that there is some truth in all of them.

The *missing link hypothesis* suggests that some component of the HPG axis is missing or non-functional. The pituitary of immature animals is competent as it secretes normal amounts of gonadotropins when transplanted under the hypothalamus of an adult animal. The ovaries (and testes) of immature animals are also potentially functional, as they too become active when transplanted into adult animals or when stimulated by gonadotropins. These observations confirm that the brain, rather than the pituitary or gonads, is the site that is functionally incompetent prior to puberty [101].

The second *critical weight* hypothesis or 'critical amount of fat' which currently is changed to 'a critical level of metabolic signals', suggests an immature female can not ovulate for the first time unless she has accumulated a critical amount of fat relative to her lean body mass. Body fat distribution, rather than body fat mass or body weight, appears to be related to early pubertal endocrine activity [88, 101].

The third hypothesis, the *gonadostat hypothesis*, believes that there is a decrease in hypothalamic sensitivity to negative feedback of gonadal steroids. Indeed, this hypothesis is supported by the observation that lesions of the hypothalamus often result in increased gonadotropin secretion. Also, castration of immature animals leads to an increase in pituitary gonadotropin secretion. Individuals lacking functional ovaries have high circulating levels of FSH and LH long before puberty, which suggests that the gonads depress gonadotropin secretion in infancy [131, 139].

In the female rat, the stimulatory effect of estrogens on LH release involves the activation of GnRH secretion from the hypothalamus. However, a precocious preovulatory surge of gonadotropins fails to occur because the ovary is not yet able to produce sufficient amounts of estrogens to stimulate LH release [131].

2-3) The onset of puberty

The timing of puberty, puberty onset, is a complex process involving many primary and secondary factors and signals. Puberty onset essentially involves the activation of the HPG axis eventually resulting in gametogenesis and the production of sex steroids by the gonads. The onset of puberty is determined by a multiplicity of interrelated physiological events, of which some root during the infantile life. The first hormonal change in rats occurs after the fourth postnatal week of life and is expressed as a diurnal change in the mode of LH release. In the

prepubertal female rat, a diurnal pattern of LH release starts around day 30 (some days before VO) and is characterised by an afternoon increase in pulse amplitude [131]. This diurnal change in the mode of LH release is physiologically relevant to the functional development of the ovary. Now, it is clear that the diurnal changes in pulsatile LH release detected at the initiation of puberty result from the activation of GnRH secretion, and that these changes in LH secretion are necessary for the puberty onset, as under the influence of these LH secretory episodes, the ovary is stimulated to produce more E2. In turn, a subtle increase in estrogens levels appears to be able to induce minisurges of LH secretion, which can further stimulate ovarian function [139, 168].

Once the diurnal pattern of LH release becomes established, new cascades of physiological events begin, which culminate in the first preovulatory surge of gonadotropins and the first ovulation. However, the occurrence and the timing of this surge clearly depends on the completion of the ovarian maturation. Only when the ovary produces sufficient E2 and for an adequate period of time, the preovulatory LH surge will occur [168]. Moreover, during the days preceding the preovulatory gonadotropin surge, the steroidal responsiveness of the ovary to gonadotropins dramatically increases. Consequently, the ability of the ovary to secrete adequate E2 is the crucial key event which determines the timing of puberty in the female rat. E2 acts on both the anterior pituitary and the hypothalamus to bring about the proestrous LH surge. In the hypothalamus it induces a discharge of GnRH release [139], and in the pituitary, it sensitises the gonadotrophs to the stimulatory effect of GnRH.

A direct stimulatory effect of progesterone (P_4) on GnRH release in prepubertal rats has also been shown, which suggests that the 2-3 fold increase in serum P_4 observed before proestrus has a role in facilitating the stimulatory effect of E2 on GnRH release [148, 149 and 201] . The attenuation of pulsatile GnRH release in late infancy and the maintenance of this reduced stimulus to the pituitary gonadotrophs during the remainder of prepubertal development are produced by either the loss of a stimulatory or the addition of an inhibitory input to or within the GnRH pulse generator [180].

The hypothalamic mechanism(s) by which E2 induces a GnRH surge at the timing of puberty is little identified. GnRH neurons have no E2 receptors [139] and hence E_2 can not directly stimulate GnRH neurons. A significant part of the stimulatory action of E_2 on GnRH

neurons and secretion is therefore exerted via a neurotransmitter system which is functionally and anatomically coupled to GnRH neurons [139]. Several neurotransmitters are involved:

- For serotonergic neurons it has been shown that blockade of serotonin synthesis depresses FSH secretion, delays VO and inhibits the gonadotropin surge in immature rats [131].
- Also neuropeptide Y (NPY) is an important hypothalamic peptide that has been implicated in the regulation of GnRH release around puberty as infusion of NPY in the adult inhibits GnRH release [139]. NPY markedly increases in the hypothalamus during the infantile-juvenile phase and is involved in the genesis of the first preovulatory surge of gonadotropins in the afternoon of the first proestrus [139]. Immunoneutralization of NPY inhibits the GnRH surge and attenuates the preovulatory increase in plasma LH [131]. The presence of E_2 receptors in NPY neurons of the arcuate nucleus suggests that E_2 directly stimulates NPY neurons to perform their secretory functions [192].
- The excitatory amino acids (EAA) are another neurotransmitter system which affects the first preovulatory surge of gonadotropins. In rats, administration of N-methyl-D-aspartic acid (NMDA) stimulated LH release and advanced the onset of puberty. The pubertal increase in GnRH release in the median eminence seems also associated with an increase in norepinephrine, and perhaps glutamate release [131, 139]. So, endogenous EAA are physiologically involved in the genesis of the first preovulatory surge of gonadotropins via activation of NMDA receptors.
- Gamma aminobutryic acid (GABA), is the major inhibitory neurotransmitter in the brain which declines with the onset of female puberty [131], and interruption of GABA synthesis or action using pharmacological and molecular probes elicits GnRH release during prepubertal development [131].

In addition to E_2, these neuronal signals might mediate the prepubertal 'brake' to the GnRH neurons. A major candidate for a glial factor serving as a developmental regulator of GnRH release is transforming growth factor alpha (TGFα). This growth factor, which is produced by glial cells of the hypothalamus, has been shown to stimulate GnRH release [180]. On the other hand, glia in the median eminence receives noticeable synaptoid-like input from GnRH nerve terminals and therefore the enhanced pubertal expression of the TGFα may be the consequence, rather than the cause, of the increased GnRH release [180].

It seems that E_2 is able to induce several neurotransmitter systems to stimulate GnRH neurons to establish the first preovulatory surge of gonadotropins. Consequently, the timing of puberty depends on a decreased sensitivity of the negative feedback of E_2 and a reduction of inhibitory neurotransmitter inputs to GnRH neurons, and it also depends on activation of excitatory inputs which functionally and anatomically are coupled to GnRH neurons. In other words, puberty onset in female rats occurs by increasing the excitatory inputs (e.g.: NPY, EAA, NE and TGFα) and decreasing the inhibitory inputs (e.g.: GABA, opioid peptides) [131].

In addition, puberty onset occurs once a critical weight is achieved irrespective of the age reached. This notion has received considerable attention over the years and has been extended from 'a critical weight', via 'a critical level of fatness', to 'a critical level of metabolic signals': Kennedy et al. [127, 128] believed that puberty onset was tightly coupled to a change in somatic metabolism. At the heart of their hypothesis was that once growth was sufficient as reflected by appropriate positive changes in energy balance, the reproductive system would become active. However, the metabolic signal(s) that could serve as the links between growth and reproduction remained unknown. This may well be one of the most important and contemporary issues in understanding puberty: to determine *how* the brain '*knows*' when the body has reached the appropriate amount of energy (or fat) to begin high frequency GnRH secretion. Although Kennedy's hypothesis still remains valuable, the critical weight per se is not the only factor affecting puberty onset [73, 88].

Later, Frisch [93] proposed that a certain degree of fatness (body fat) was the factor controlling reproduction. Adipose tissue serves as a long-term source of energy, and a critical amount of fat would be necessary for successful reproduction. Adipose tissue can influence reproductive processes in two ways: 1) the timing of puberty onset or menarche, 2) the maintenance of reproductive function (female cyclicity) during adulthood [73]. Frisch could not know that there was some factor(s) to be synthesised by adipose tissue that could be a candidate for a signal to be detected by the brain. So, the neuroendocrine mechanism by which the brain detects the amount of body fat or its likely product(s), causing it to increase GnRH secretion, was not yet identified. Frisch [93] suggested an indirect linkage by proposing that achieving greater fatness influenced reproduction via greater conversion of the androgens to estrogens by fat tissue. However, this was never considered to be a viable mechanism in terms of neuroendocrine control, because gradually increasing estrogen

concentrations would not be able to induce an adequate GnRH secretion to develop a preovulatory follicle that would then produce its own, massive rise of estrogen to trigger the preovulatory GnRH surge [88]. Despite the lack of a tenable mechanism, Frisch's work provides a highly useful point for the recent interest in fat and reproduction, and the possibility that fat may produce some signal(s) that the brain is able to distinguish to assess somatic energetics [88].

3) Nutritional and energy status link to reproduction

The ability to monitor internal and external energy availability is vital, also in order to link reproduction and energy balance, and to allow animals to prioritise their behavioural options according to fluctuations in energetic and reproductive conditions. For example, when food is sufficient and energy requirements are low, energy is available for all of the physiological processes necessary for immediate survival: protein biosynthesis, maintenance of ionic gradients, waste removal, thermogenesis, locomotion, foraging, ingestion and digestion of food. Energetic priorities include long-term investments, like growth, immune function, and later reproduction. Behaviour related to territorial defence, courtship, mating and parental care receive a high priority, and finally the surplus energy is stored as lipids in adipose tissue [196]. Conversely, when energy is not enough, the physiological mechanisms that distribute energy will tend to favour those processes that relate to survival of the animal which means some processes like foraging, hoarding and digestion receive a higher priority than reproduction because reproductive processes like gametogenesis, ovulation, pregnancy, parturition and lactation are energetically very expensive and can be postponed when the survival of the individual is in jeopardy [47-50, 196].

The metabolic signals, including hormonal mediators and neuropeptides give expression to these priorities at least in two interrelated approaches. First, they are permissive for the neuroendocrine mechanisms which control gametogenesis, ovulatory cycles and fertility. Secondly, in many species, the same metabolic signals and chemical messengers that increase the motivation to involve in reproductive behaviours also attenuate the motivation to engage in foraging and eating [197, 220].

During these energetic challenges, animals are predisposed towards behaviour, such as eating, foraging and food hoarding by a variety of metabolic sensory stimuli (e.g., decreased availability of glucose and its related metabolites), peripheral hormones (e.g. low plasma concentrations of insulin and leptin) and central feeding-stimulatory circuits [e.g. NPY, agouti-related protein(AgPR)]. The adaptive significance of the “feeding-stimulatory” circuits is related to survival (bringing metabolic fuels, other nutrients, water, salt and other minerals into the organism to maintain cell structure and function) insofar as survival is a prerequisite to reproductive processes. More important from an evolutionary perspective, the neuropeptides that stimulate eating and foraging also enhance survival during energetic challenges by

inhibiting the HPG axis. Oppositely, when food is plentiful and energy demands are low, those central circuits that inhibit eating tend to facilitate aspects of reproduction [196, 197].

Over the last decades, several metabolic signals including hormones and neuropeptides have been proposed to mediate the link between energy balance and reproduction in which most of these factors influence reproductive processes through the HPG axis. The master control of the HPG system lies within the GnRH neurons (the cell bodies of which are located in the area that spans from the preoptic area (POA) to the arcuate nucleus (Arc) in the hypothalamus. GnRH has two modes of secretion. The 'pulse mode' occurs during the follicular phase, when low concentrations of E_2 have negative feedback effects on GnRH and LH secretion; i.e. E_2 limits GnRH and LH secretion to relatively low levels. And the 'surge mode' is occurring during the preovulatory phase when high concentrations of E_2 exert positive effects on GnRH. The rising levels of E_2 have a positive feedback on GnRH and LH, and these actions of E_2 are essential for the LH surge, which triggers ovulation [196].

Metabolic challenges, such as food restriction, inhibit the HPG system at many levels. The primary locus, however, is the GnRH pulse generator, and these effects are similar in males and females [49]. Pulsatile LH secretion, follicle development and ovulation can be reinstated by pulsatile treatment with GnRH in food-restricted rats, sheep, pigs, cows, monkeys and women [16, 48, 51, 89]. Metabolic challenges inhibit the HPG system partly by increasing the sensitivity to the negative feedback effects of E_2, and partly by steroid-independent effects. Inhibition of GnRH secretion leads to a cascade of inhibitory effects, including decreased gonadotropin secretion, retarded follicle development, inhibited synthesis of gonadal steroids and in rodents decreases in steroid-induced reproductive behaviours. Thus, deficits in most of these aspects of the HPG system can be traced to metabolic inhibition of GnRH secretion [196].

4) Adipose tissue: a long-term source of energy and an endocrine tissue

Adipose tissue is a special type of connective tissue that functions as the major storage site for fat in the form of triglycerides. Adipose tissue is the body largest energy reservoir. The primary role of adipocytes is to store energy during periods of excess and to mobilise this reserve when expenditure exceeds intake [94]. Adipose tissue is found in mammals in two different forms: white and brown adipose tissue. The presence, amount and distribution of each type varies depending upon species and age.

White adipose tissue (WAT) serves three basic functions: heat insulation, mechanical cushion and most importantly, as a long-term source of energy. WAT acts as endocrine, paracrine and autocrine tissue influencing many organs, including hypothalamus, pancreas, liver, skeletal muscle, kidneys, endothelium and the immune system [94, 215]. Indeed, WAT is *the heart* of a network of autocrine, paracrine, and endocrine signals. Subcutaneous adipose tissue, is located directly below the skin, is an important heat insulator in the body, because it conducts heat only one third as readily as other tissues. Adipose tissue also surrounds internal organs and provides like a cushion, some protection for these organs [94].

As the major form of energy storage, fat provides a buffer for energy imbalance when energy intake is not equal to energy output. It is an efficient approach to store excess energy, because it is stored with very little water; consequently, more energy can be derived per gram from fat than from protein or carbohydrate.

In mature mammals, the major bulk of adipose tissue is a loose association of lipid-filled cells called adipocytes, which are held in a framework of collagen fibres. Adipocytes are capable to change their diameter 20-fold and their volumes by several thousandfold [94]. In addition to adipocytes, adipose tissue contains stromal-vascular cells including fibroblastic connective tissue cells, leukocytes, macrophages, and pre-adipocytes (not yet filled with lipid), which contribute to structural integrity [215].

Nearly 60-85% of the weight of WAT is lipid, with 90-99% being triglycerides; also small amount of free fatty acids, diglycerides, cholesterol, phospholipids and minute quantities of cholesterolester and monoglycerides are present. In WAT each adipocyte is in contact with at least one capillary; this high blood supply provides sufficient support for the active metabolism

in adipose tissue. Blood flow to adipose tissue varies depending upon body weight and nutritional status with blood flow increasing during fasting [215].

Lipogenesis is the deposition of fat which occurs in adipose tissue and in the liver at cytoplasmic and mitochondrial sites. Energy ingested as fat beyond that needed for current energy demands is stored in adipose tissue. Moreover, carbohydrate and protein consumed in the diet can be converted to fat (which is carried out primarily in the liver) and is transferred to adipose tissue for storage. Amino acids from ingested proteins are used for making new protein or can be converted to carbohydrate or fat. On the other hand, lipolysis which is the chemical decomposition and release of fat from adipose tissue, occurs when additional energy is required. The capacity of adipose tissue for energy storage is basically limitless, with potential for increasing triglyceride storage. This may occur via two mechanisms: by increasing the amount of lipid store in each cell by favouring lipogenesis over lipolysis, or via increasing the number of fat cells in the process of pre-adipocyte replication and differentiation [215].

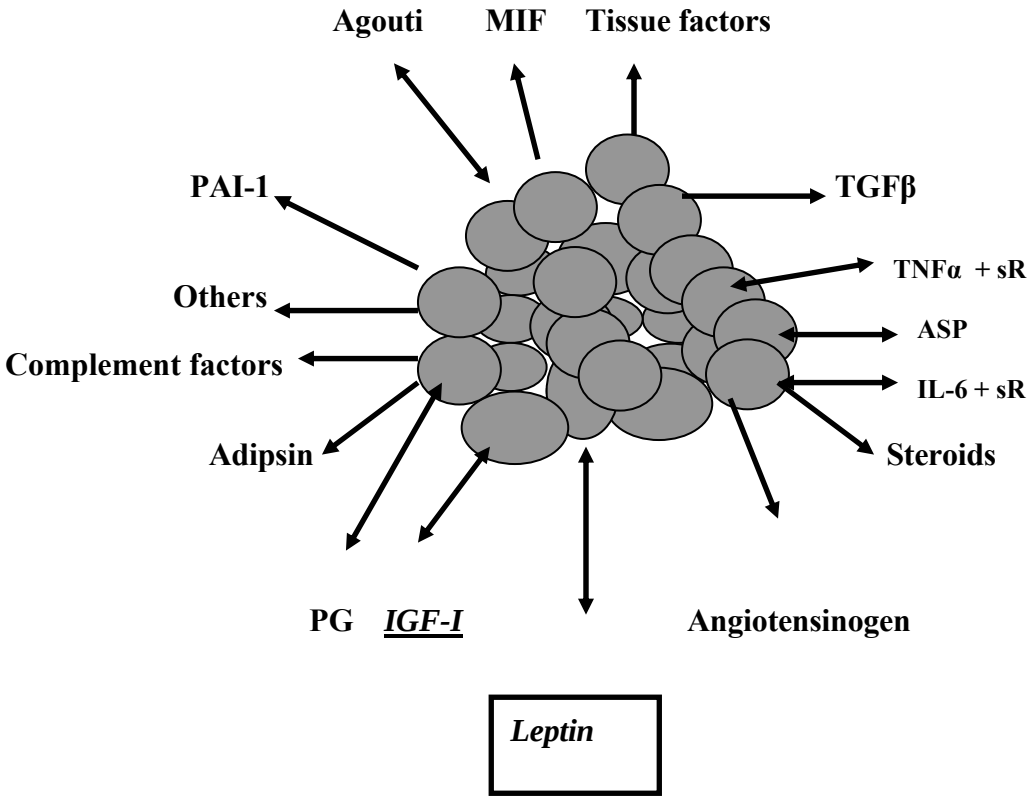

Figure 1: Dynamic view of adipose tissue, as an important endocrine tissue [modified from 94]

The sympathetic nervous system (SNS) plays an important role in the regulation of energy expenditure and in adipose tissue lipolysis [45]. The SNS and its postganglionic neurotransmitter, the catecholamine norepinephrine (NE), acting through the stimulation of WAT β–adrenoceptors is recognised as a major stimulator of lipolysis both in vitro and in vivo [45, 152, 215]. Increased sympathetic activity in WAT occurs during fasting and cold exposure, conditions also associated with decreased leptin (see paragraph 5) synthesis and secretion. Catecholamines and β–adrenoceptor agonists have been indicated to inhibit leptin production in both in vitro and in vivo studies [18].

Following food intake, an integrated endocrine response facilitates the uptake of energy into the (particularly insulin-sensitive) tissues and, therefore, its removal from the circulation. Thus, a post-prandial re-distribution of nutrients from the gut lumina to the tissues via the circulation is characteristic of normal metabolic function. In the fasting state, energy is again re-distributed via the circulation. Adipose tissue has a crucial role in this process.

Adipose tissue produces a large number of molecules which include cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and members of the complement family of proteins (see figure 1). Indeed, there is ongoing debate as to whether these molecules have an immune role or a metabolic function in adipose tissue [8]. In addition, adipose tissue secretes some other components such as adipsin which may have a role in the regulation of energy metabolism: it was demonstrated that adipsin expression in humans increases with adiposity and feeding, and it is decreased during fasting [152]. Adipose tissue synthesises all components of the renin-angiotensin system and expresses angiotensin II receptors [215].

Insulin-like growth factor I (IGF-I) is also produced in adipose tissue [144]. The observation that IGF-I mRNA levels in adipose tissue are as high as in the liver, has even led to suggest that adipose tissue could be a key producer of IGF-I [218]. In rats, it has been shown that IGF-I mRNA and some IGFBPs are localized both in adipocytes and pre-adipocyte cells. Growth hormone (GH) from the pituitary is apparently the main regulator of IGF-I mRNA expression in adipose tissue. Despite a high expression of IGF-I mRNA in adipose tissue, its particular function in adipocytes is still not fully understood [144].

5) Leptin

One of the most important endocrine functions of adipose tissue is producing and releasing *leptin* which has a clear role in the regulation of energy stores and fertility. For several decades, the metabolic signal responsible for transmitting information from adipose tissue to the hypothalamus was unknown. Leptin, which is mainly produced and secreted in to the blood stream, seems to be such a link between adipose tissue and the hypothalamus.

5-1) General features

In 1994 the structure of the mouse obese gene (*ob*) and its human homologue was published [236]. The protein named 'leptin' (from the Greek word 'leptos' means 'thin') is the product of the *ob (Lep)* gene. Leptin is a 16 kD cytokine-like protein, and is mainly secreted by adipose tissue [5, 10, 61, 73, 86].

Leptin is translated as a 167 amino acid protein with an amino-terminal secretory signal sequence of 21 amino acids. This signal sequence is functional resulting in the translocation of leptin into microsomes with the subsequent removal of the signal peptide. Leptin, therefore, circulates in the blood stream as a protein of 146 amino acid residues [152]. Leptin consists of four antiparallel α-helices, connected by two long crossover links and one short loop, arranged in a left-hand twisted helical bundle which is similar to helical cytokine crystal structures like a human GH [94].

In man and rodents *ob* mRNA and leptin levels are positively correlated with the amount of adipose tissue. Adipose tissue (both white and brown) is the main site of leptin synthesis, but it is now evident that leptin is also produced in other tissues [152]. Also the placenta, GI-tract organs, and skeletal muscle produce leptin [17]. Studies have indicated physiological functions of leptin in these tissues: leptin mRNA and protein levels are increased in skeletal muscle following glucosamine treatment, consistent with an involvement in energy metabolism [7, 8]. Leptin expression in the stomach is stimulated by feeding, cholecystokinin and gastrin, suggesting a role in the regulation of energy balance [18]. Placental leptin is stimulated by hypoxia and may influence fetal outcome [155]. The brain also produces leptin, suggesting a paracrine and autocrine action; however, the physiological role in the brain is not yet determined as its concentration in CSF is about 100-fold lower than in the plasma [3, 7, 8, 137, 229].

Leptin is involved in many physiological processes: lipid and glucose metabolism, energy partitioning, angiogenesis, adipocyte apoptosis, haematopoiesis, wound healing, and bone and brain development [4-8]. Leptin circulates as a 16-kD protein partially bound to plasma proteins. The protein-bound leptin exists in equilibrium with free leptin, and the latter represents the bioactive hormone. The relationship between free and bound leptin is not static but dynamic, and the bioavailability of leptin is regulated by changes in the balance of free-to-bound hormone as it is for other hormones [137, 152]. Leptin binding activity (and circulating leptin receptor levels) reduces with age in children [182]. Studies have shown that the ratio of bound-to-free leptin is increased in obesity, pregnancy and LepRs mutations [3]. An additional pool of leptin may exist in various tissues, and may contribute to the maintenance of plasma leptin levels [4-8].

Leptin displays a diurnal rhythm with a nocturnal rise reaching its peak levels between midnight and early morning and then declines to a nadir by midafternoon [212]. The genesis of the diurnal pattern of leptin levels has not been elucidated. It has recently been shown that the suprachiasmatic nucleus (SCN) has a direct control of the diurnal rhythm in plasma leptin levels in the rat [124].

Leptin is secreted in a pulsatile manner with a pulse duration of approximately 30 min [212]. This pulsatile pattern is synchronized with that of luteinizing hormone (LH) [91].

The amount of leptin production varies according to age. In rodents, leptin is expressed widely during the prenatal periods. Some studies have indicated that leptin mRNA and protein levels decrease rapidly after birth, followed by a transient increase in the neonatal period and a steady increase in adults [3].

To date, six splice variants of the leptin receptor (LepRs), ‘LepRa’ to ‘LepRf ‘, have been identified [3, 8, 36]. LepRs belong to a family of class I cytokine receptors, which typically contain a cytokine receptor homologous domain in the extracelluar region. As with other class I cytokine receptors, e.g., IL-6, growth hormone and prolactin, the leptin signal is thought to be transmitted mainly by the JAK-STAT pathway [3, 34]. LepRa, LepRb, LepRc, LepRd and LepRf have transmembrane domains; however, only the “long receptor”, LepRb, has intracellular motifs necessary for activation of the JAK-STAT signal transduction pathway. Hence, only the LepRb isoform (the full length form of the receptor) is apparently involved in signal transduction [36, 151]. LepRe lacks both transmembrane and intracellular domains,

circulates as a ‘soluble receptor’, and is secreted into the blood in sufficient amounts so that it likely functions as a leptin buffer. LepRe isoform is the major circulating binding protein for leptin in man [151]. It is suggested that LepRc and LepRd may play a role in the clearance of leptin from the circulation [152]. Numerous reports imply that leptin receptors are expressed in peripheral tissues, including liver, heart, kidneys and adrenals, lungs, small intestine, pituitary cells, testes, ovaries, follicles, spleen, pancreas, adrenal gland, muscles, bone marrow and adipose tissue [36, 152]. This wide range of leptin receptors in various tissues suggests that leptin may play more diverse roles than initially thought and may perform various functions in different tissue.

Plasma leptin might exert its effects directly in the brain, so leptin would have to cross the blood-brain-barrier (BBB). How a protein as large as leptin, about 16 kD, can cross the BBB and enter the brain still remains unknown [23]. Leptin enters the brain via a saturable process, but the exact structures responsible for leptin transport are not yet identified [22, 23], although it has been suggested that leptin was transported by receptor-mediated transcytosis [152]. Because short LepRs are widely present in the brain microvessels, kidney, liver, lung, and are capable of binding, internalizing and translocating leptin, it is suggested that these receptors mediate leptin transport [36, 152]. Recent studies have indeed shown that leptin crosses the BBB from the circulation to enter the brain with its short receptor, LepRa. Also, LepRa mRNA is expressed at very high levels in cerebral microvessels, which constitute the BBB, again supporting the possibility that LepRa may play a crucial role in the transport of leptin from the blood into the brain [8].

Leptin crosses the BBB more than 20 times faster than albumin [22, 23], and enters the brain at both the endothelium and choroids plexus [8, 152, 174]. The hypothalamus transports leptin across its BBB faster than any other brain region. The hypothalamus contains the arcuate nucleus, where leptin is thought to inhibit feeding by inhibiting NPY. However, leptin has receptors throughout the brain and is transported into every region of the brain [24].

Furthermore, it is arguable that cerebrospinal fluid (CSF) is a significant source of leptin for neurons, because the leptin concentration in CSF is dramatically lower than plasma and below the dissociation constant of the LepRs [37, 229].

High prevailing leptin levels in the plasma can cause a ‘leptin resistance’, a failure of the transport mechanism across the BBB. Leptin resistance entails a direct impairment of leptin

transport across the BBB causing obesity in rats, mice and man [20, 21, 103, 125, 174]. The obesity-related defects in leptin transport across the BBB can be reversed by either fasting or central leptin treatment [21]. Also, hypertriglyceridemia has recently been shown to be an important inhibitor of leptin transport across the BBB [20]. The Koletsky rat is extremely obese and has very high leptin levels due to a mutation in the leptin receptor; but this rat has a leptin transporter which is an as yet unidentified protein [20, 152]. In spite of obesity, fasting caused a clear increase in brain uptake of leptin indicating the BBB transport of leptin are sensitive to alterations in the circulating levels of leptin [4-8, 152].

5-2) Leptin: regulation of secretion

Leptin biosynthesis and release are controlled by paracrine, endocrine, and neuroendocrine signals that impinge on the adipocyte. Plasma leptin levels and adipocyte *ob* mRNA expression are strongly correlated with total fat mass, percent body fat, and body mass index. The level of *ob* mRNA in WAT and the plasma levels of leptin are considerably increased in obesity, as has been repeatedly shown in rodents and humans. Thus greater amount of adipose tissue results in a higher level of plasma leptin concentration [3-8, 152]. Because leptin is secreted by fat cells in proportion to body fat stores, it has the potential to play a key regulatory role in fuel homeostasis.

Leptin has direct autocrine/paracrine effects on adipocytes: leptin represses acetyl-CoA carboxylase gene expression, fatty acid synthesis, and lipid synthesis; these are biochemical reactions that contribute to lipid accumulation without participation of central pathways [94]. Leptin is involved in the direct regulation of adipose tissue metabolism by both inhibiting lipogenesis and stimulating lipolysis [94]. Leptin mRNA shows higher expression in subcutaneous than in omental adipocytes [94]. In rodents, however, leptin expression may be lower in subcutaneous fat than in the internal fat depots, and the highest level of expression is generally evident in the perirenal adipose tissue [152]. In addition, a striking sexual dimorphism is shown in both *ob* mRNA expression and circulating leptin levels, with almost two-fold higher leptin levels in female than male animals [94].

It is proposed that the SNS is the main physiological regulator of leptin production providing a negative feedback loop to adipose tissue in the production of the hormone [152]. Indeed, the interaction between the SNS and leptin is two-way SNS activity in WAT and other tissue via its

hypothalamic receptors [215]. So, it is suggested that SNS actively regulates leptin expression and secretion [19].

Feeding and fasting alter both gene expression and plasma leptin levels (see below). Fasting reduces *ob* gene expression and subsequently decreases plasma leptin levels. Rapid decreases in leptin levels in response to energy restriction or fasting seem to be greater than the decrease in fat mass in both rodents and humans [3, 152]. In humans, leptin levels increase following a meal and begin to decrease several hours after a meal [4-8]. In rats, the increase in leptin mRNA and serum leptin concentration was similar during enteral and parenteral feeding [152]. These changes occur independently of circadian variations and may be partly due to secretion of leptin from gastric epithelium [18]. It is also suggested that the decreases might be mediated by SNS and β-adrenoceptors [152].

It has been shown that cold exposure reduces plasma leptin levels and leptin expression in adipose tissue, suggesting that leptin might participate in the adaptive mechanisms triggered by variations in external temperature [152]. The inhibitory effect of low temperature on circulating leptin results from an increase in the adrenergic tone induced by exposure to cold temperatures, which would in turn act through the β-adrenoceptors present in adipose tissue, inducing a reduction in the expression of leptin mRNA in rats and mice [152, 215].

Some hormones and metabolic factors have also been shown to alter leptin gene expression and secretion: insulin and glucocorticoids act directly on adipocytes to increase leptin synthesis and secretion and may function as long-term regulators of leptin expression [19].

Increased plasma insulin is associated with increased plasma leptin, and injection of insulin increases both plasma leptin and adipose tissue leptin mRNA levels [19]. In both humans and rodents, the postprandial rise in leptin follows the peak in insulin secretion [83]. In contrast, insulin deficiency results in rapid reduction of leptin mRNA and protein levels [3]. In rodents leptin peaks at night and declines during the day, and this pattern is suggested to be regulated by insulin [3].

There is also a significant gender effect on serum leptin levels, which may be the result of differences in plasma estrogen and/or testosterone levels. In man and sheep, females have higher leptin levels than males at any given level of fatness [38, 212], whereas the opposite is observed in rodents [212]. In boys, testosterone has been found to suppress the leptin synthesis by adipocytes both *in vivo* and *in vitro* [129]. Leptin levels are higher in females, even before

puberty, compared to boys, independent of differences in body composition [19]. In rats, ovariectomy causes a decrease in leptin mRNA levels in adipose tissue and a decrease in serum leptin concentration, both of which were reversed by administration of E_2 [19]. GH and thyroid hormones affect leptin synthesis and/or secretion: GH directly interacts with visceral adipose tissue to reduce leptin gene expression [120, 121].

Leptin has been shown to modulate hypothalamic-pituitary-adrenal (HPA) axis, SNS activity [45, 105]. Leptin increases POMC expression (see below) which contributes to the regulation of food intake, immune function, and some aspects of reproduction. In contrast, some reports suggest that leptin inhibits the HPA axis via release of β-endorphin (made out of POMC) and downregulates the response to stress by increasing the opioid tone [105]. Glucocorticoids are potent regulators of leptin: in vitro, dexamethasone and cortisol stimulate leptin expression in adipocytes [70]. In vivo, the daily injection of glucocorticoids, at doses that inhibit food intake and weight gain, increase leptin expression fourfold in rats, and dexamethasone doubled leptin expression and circulating leptin in humans [105].

5-3) Leptin: food intake and body weight

Over the past 20 years at least five peptides have been identified in the brain that stimulate feeding behaviour, namely, neuropeptide Y (NPY), agouti-related protein (AgRP), galanin, melanin-concentrating hormone (MCH4) and the orexins/ hypocretin; and four peptides that suppress feeding, namely, pro-opiomelanocortin (POMC), galanin-like peptide (GALP), cocaine- and amphetamine-regulated transcript (CART), and corticotrophin-releasing factor (CRF) [142, 190].

The arcuate nucleus (ARC) of the hypothalamus plays a key role in this behavioural feature.

NPY, first isolated in 1982, is a 36-amino acid, is one of the most abundant known neuropeptides in the nervous system of mammals, and is expressed at high levels in several CNS regions, including the hypothalamus [226]. This peptide, a potent stimulant of feeding, is highly expressed in the ARC which sends dense projections to multiple areas of the hypothalamus [19, 31, 36, 90]. The ARC NPY neurons are strategically located to integrate a variety of nutritional and neural signals, and to interact with and influence other neuronal systems [226]. It is suggested that ARC is a principal monitor of leptin signaling in the brain; importantly, the distribution of ARC projections overlaps projections to other key parts of the

hypothalamus: these include the paraventricular (PVN), ventromedial (VMN), and dorsomedial (DMN) and lateral nuclei [31, 42, 142, 181, 226].
It is also demonstrated that for a normal postnatal development of ARC projections require leptin, suggesting the postnatal leptin surge is a key developmental signal affecting the architecture of hypothalamic circuits mediating feeding [36, 42, 181]. Neurons synthesizing NPY in the ARC are highly responsive to states of energy deficiency and a higher metabolic demand. Their expression is stimulated by food deprivation, increased exercise, cold and pregnancy.
Several studies suggest an important interaction between NPY leptin [8, 19, 31, 181]. The co-localisation of leptin receptors and NPY suggests that leptin is able to act on NPY release to affect food intake [161]. Indeed, leptin administration has been shown to reduce NPY gene expression, ARC NPY peptide levels and NPY-stimulated feeding, and consequently to remove the inhibitory action of NPY on pulsatile GnRH release [19,142, 181]. This effect may be mediated by leptin receptors on NPY neurons and may involve a direct, inhibitory effect of leptin on the firing of NPY neurons [72].
Although NPY is a major leptin target, deletion of NPY or its receptors had little effect or did not completely reverse the obese phenotype in ob/ob mice, indicating that other neuropeptides and neurotransmitters than NPY also play significant roles in the transmission of the leptin signal in the hypothalamus [3, 181]. The AgRP in the ARC is similar to NPY in being stimulated by conditions of energy deficiency and inhibited by leptin [142].
Also POMC is synthesized in the ARC, but in the anterior pituitary as well as POMC, a 267-amino acid precursor protein, might be one of the neurotransmitters. Again there is considerable evidence relating the effects of leptin on POMC in the ARC [8, 19, 142, 181, 190]. POMC neurons express the leptin receptors and leptin stimulates POMC expression [72]. Leptin-deficient ob/ob mice exhibit a substantial reduction in POMC throughout the ARC, which is restored by leptin replacement [213]. Also, obesity with elevated leptin is accompanied by an increase in POMC mRNA [214]. So, POMC gene expression is stimulated under conditions of positive energy balance including excess caloric intake and elevated leptin levels [142, 181].

The interesting point and importance of leptin is that it is a signal integrating various central functions but originating from the periphery, the adipose tissue, and carrying this peripheral information to the CNS, mainly to the hypothalamus [147, 190]. Ghrelin, a novel protein mainly produced in the stomach, is another peripheral originating hormone which stimulates feeding and reduces energy expenditure leading to an increased body weight [172]. Ghrelin is also produced in the hypothalamus, and its receptors are localised in the hypothalamus particularly on NPY/AgRP neurons [190]. Leptin attenuates ghrelin's actions on NPY neurons which may be one of the important mechanisms of leptin signaling in the hypothalamus [190].

In general, leptin expression and serum levels decrease in response to weight loss both in humans and rodents [73, 86,170]. It has been shown that leptin gene expression in ovine adipose tissue is reduced by underfeeding [38, 153]. Serum leptin declines during short-term fasting (24 h) in the absence of any change in adipose tissue mass in human [69]. A short food deprivation (12 to 48 h) results in a rapid and drastic fall in leptin gene expression. As little as a 10% reduction in body weight in obese subjects results in a 53% reduction in serum leptin levels, whereas a 10% increase in body weight causes a 300% increase in serum leptin [114]. Leptin treatment in animals has been shown to cause a dose-dependent decrease in food intake, a loss of body weight and fat depots, and an increase in energy metabolism [114, 165].

During starvation, leptin levels fall, thus activating a behavioural, hormonal and metabolic response that is adaptive when food is unavailable. Weight gain increases plasma leptin concentration and elicits a different response, leading to a state of negative energy balance. It is not yet known whether the same and/or different neurons respond to increasing or decreasing leptin levels. The range of leptin's effects is likely to be complex, as different thresholds exist for several of leptin actions [92]. Increasing leptin leads to fatty acid oxidation and a reduction in adipose tissue mass, whereas leptin deficiency, as seen in ob/ob mice or in mice receiving a leptin antagonist, is associated with an increase in fat deposition [92].Thus, leptin not only functions as an 'adipostat' to signal the status of bodily energy stores to the brain (and perhaps other tissues) but also acts as a 'sensor' of energy balance [14, 38, 92].

It is believed that besides leptin, insulin also acts as a peripheral hormone providing peripheral information to the CNS concerning the amount and distribution of body fat [35]:

plasma insulin levels are directly correlated with adiposity, and insulin is a key peripheral regulator of food intake and body adiposity. It has been proposed that insulin serves as an ‘adiposity signal‘ whose levels in the brain reflect the size of adipose stores integrated over time and which serve to complete a negative loop that links the behaviour of feeding with the size of adipose stores [82]. Insulin levels correlate better with visceral than with subcutaneous fat. Compared to leptin insulin is the short term signal: Insulin signals the changes of energy balance and the size of the fat mass to the brain within minutes to hours rather than days like leptin. Leptin is secreted from adipocytes in direct proportion to the amount of stored fat, especially the amount of subcutaneous fat. Leptin secretion rate and leptin mRNA expression are two to three times higher in subcutaneous than visceral fat, in part due to the larger adipocytes relative to visceral fat [35].
Obese animals and humans are insulin-resistant and more insulin is required to maintain a normal level of blood glucose. Insulin-resistance and visceral fat correlate with levels of insulin, type 2 diabetes mellitus and obesity. Like leptin, insulin receptors and receptor mRNA are widely found in CNS regions involved in the regulation of food intake and body weight, in particular in the arcuate nucleus of the hypothalamus [82]. Both in humans and rodents, the postprandial rise in leptin follows the peak insulin secretion and insulin deficiency causes a rapid reduction of leptin mRNA and protein levels in WAT [3].

So, leptin and insulin together inform the brain not only about the size of fat mass but also about its distribution and important recent changes in metabolic status [35]. Insulin also enters the brain via a saturable transport system and even with very high plasma levels, in contrast to leptin, the entry of insulin remains relatively constant; this is important for the regulation of body weight because some types of obesity are known to be associated with disruptions in the insulin transport system. From a broader perspective, potential interactions among insulin, leptin, metabolic status and brain may explain the regulation of food intake and body weight.

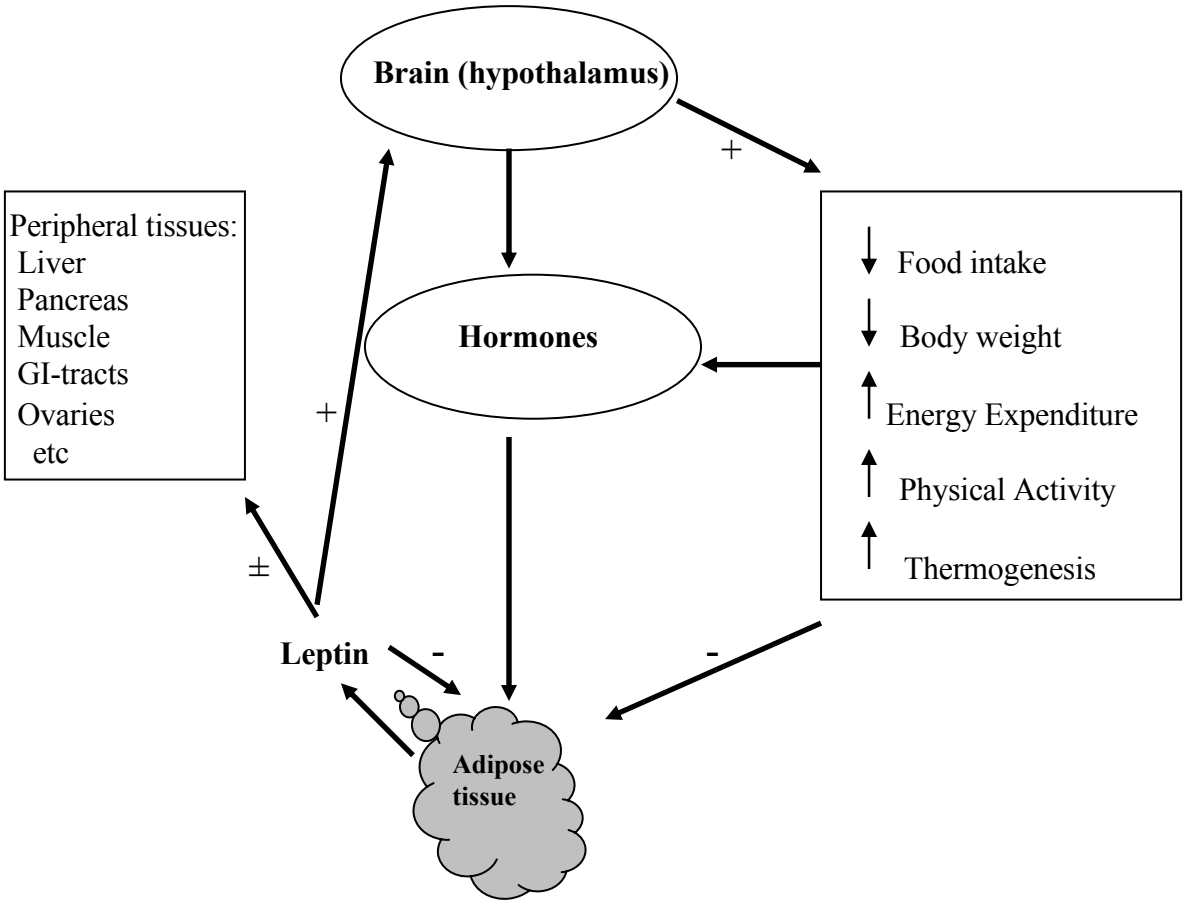

Figure 2: Leptin is secreted from adipose tissue and is transferred to the brain and peripheral tissues like ovaries, liver, GI-tracts and skeletal muscles which may directly affect their metabolism and function. In the brain (particularly in the hypothalamus) leptin stimulates or inhibits neurotransmitters release like NPY, which inhibit food intake and stimulate physical activity and thermogenesis per se, resulting in the stimulation of some other hormones that inhibit leptin synthesis in adipose tissue [adapted from 113].

5-4) Leptin: reproduction, general views

Since leptin was found, several independent groups of researchers have been trying to find out the effects of leptin in various aspects and stages of reproduction.

Reports on the direct relationship between leptin and ovarian function are inconsistent. It has been shown that granulosa, thecal, interstitial and cumulus oophorus cells have a receptor for leptin [130]. Human and murine ovary express mRNA for the leptin receptor [58, 152]. In fact, the human and porcine ovaries have one of the highest levels of leptin receptor mRNA compared to other tissue. Leptin inhibits follicular growth in immature and adult mice probably by impairing the proliferation and E_2 production of granulosa cells [130]. Leptin at physiological concentrations inhibits insulin-induced E_2 production by granulosa cells from both small and large bovine follicles. In vitro studies showed that leptin directly inhibits steroidogenesis and ovulation in rodent, bovine and primate ovaries [202]. Expression of leptin receptors in human granulosa cells suggests leptin might play a role in follicular growth and maturation.

Human and animal studies suggest that placental leptin is likely to affect maternal, placental and fetal function via both autocrine and paracrine mechanisms. It is possible that placental leptin may have physiological effects on the placenta itself including angiogenesis, growth, immunomodulation, and regulation of fetal and uterine metabolism [152]. Leptin receptors (LepRa and LepRb) were found in murine, rat, and human placenta. It is also believed that placental leptin acts as a signal of energy status between the mother and fetus. The placenta also expresses leptin receptors, suggesting that the organ is a target for the action of leptin as well as a source of the hormone [3, 8, 152].

Several independent studies have shown that correction of leptin deficiency in ob/ob mice by peripheral infusion of recombinant leptin activates the reproductive axis and restores fertility in both sexes [25, 60, 166]. Leptin-treated female ob/ob mice had significantly elevated serum levels of LH, increased ovarian and uterine weight compared to controls [152].

It has been shown, both in vivo and in vitro, that gonadal steroids regulate leptin synthesis and release from the adipose tissue [69]. Estrogens increase leptin mRNA expression in vitro but, testosterone inhibits leptin gene expression both in vitro and in vivo [145]. Plasma leptin level is always higher in women than in men for any given percentage of body fat [105]. In children and adults, leptin is negatively correlated with testosterone in males and positively associated with estrogen in females, after correcting for fat mass [105]. These finding suggest

that androgens and estrogens modulate leptin expression at the mRNA level through sex steroid receptor-dependent transcriptional mechanisms.

In females of most mammalian species, high leptin levels may signal sufficient long-term energy stores (i.e. fat) that are crucial for successful reproduction and pregnancy: in both humans [154] and mice [60], serum leptin level was high throughout pregnancy compared to the non-pregnant state. It is suggested that this high level of leptin during pregnancy may be due to increased production by maternal fat, increased expression by the placenta, or increased levels of binding proteins in the maternal circulation; but, hyperleptinaemia during pregnancy is not associated with decreased food intake or a decline in metabolic efficiency, as might be expected from leptin's roles as a satiety factor. It is probably because of increased nutritional demands during pregnancy and it is not the actions of leptin as a satiety factor, why leptin is increased [152]. Potential roles of leptin in pregnancy may also include facilitation of the endocrine response to pregnancy, maintenance of maternal fuel homeostasis during a period of increased nutrient intake and requirement and/or optimization of fetal growth and development [112].

Leptin receptor mRNA and leptin receptor protein were also identified in numerous fetal tissue including brain, lung, kidney, heart and liver. So, high levels of leptin during pregnancy raise the possibility that leptin may play a key role in nutritional signaling between mother and fetus throughout pregnancy [111].

Leptin receptor is expressed in human endometrium [97], with the peak in the early secretory phase of pregnancy [132]. These studies suggest that there is steroid hormone regulation of leptin within the human endometrium and that progesterone, acting through its receptor, is able to suppress the expression of the leptin receptor. Steroid hormone may contribute to the sensitivity of the endometrium to leptin and may be important in establishing the implantation cascade. Also, leptin may be implicated in the implantation process itself due to its ability to effect a marked change in the endometrium, thereby facilitating trophoblastic invasion. Leptin may hence play an autocrine or paracrine role in cytotrophoblast invasion during implantation [97].

Leptin also plays an important role in lactation, as was shown in ob/ob female mice which completely failed in this respect. Leptin acts as a functional link between adipocytes and the epithelial cells of the mammary gland, providing information on the adequacy of energy

stored in adipose tissue. Thus, absence of leptin may result in failure of mammary gland growth and subsequently in failure of lactation [205].

Collectively, the diverse functions of leptin indicate that its effects extend beyond the basic roles initially attributed in the lipostatic model.

5-5) Leptin: reproduction, a missing link hypothesis

Food intake/energy expenditure and reproduction are intimately related [147]: Inadequate nutrition delays or prevents the onset of puberty [29, 52, 73, 88, 212]. Acute changes in the animal's energy status result in modulation of the HPG axis, and a suppression of the pulsatile LH secretion has been documented upon fasting or food restriction in rats, mice, hamsters, sheep and primates [88]. When food availability is low, short-lived and small species (e.g. rats) with a high metabolism and a reduced growth may not even attain puberty. In longer-lived species, puberty is merely delayed for months (or even years) until more food becomes available [88]. A reduction or postponement of the GnRH secretion by the hypothalamus is assumed to be the cause of this delay. An important question therefore is, *how* the brain *'knows'* when the (stored) nutrients are adequate for starting up reproduction. The brain should be able to read, interpret and integrate a wide range of signals that describe the animal's nutritional status and its immediate environment, and to make appropriate adjustments in food intake, energy expenditure and metabolism as a result of the information received through these signals [226].

The mechanisms responsible for communicating nutritional status to the central system controlling reproduction have not yet been clearly elucidated. Identification of this signals have remained elusive because of the large number of peripherally originating substances that can act centrally to modify neuronal activity [31].

Many researchers believe that leptin is the signal relaying the nutritional status information to the brain and thus allowing reproductive processes to proceed, to be initiated, or stalled [52, 61, 63, 66, 73]. One view is that leptin does not initiate puberty but acts as a permissive factor allowing pubertal maturation to proceed [63, 64]. Human studies indicate that leptin may play a 'facilitatory' role in the onset of puberty [14, 52]. And also in healthy adult and cyclic animals leptin serves as a metabolic signal to the reproductive system, informing it that sufficient fat stores are available to meet the caloric demands of reproduction. Metabolic

stress such as food restriction, metabolic wasting disease and severe exercise might be signaling the reproductive system via low circulating levels of leptin, and result in disruption or blockade of reproductive functioning [25, 29, 147, 212].
The second view suggests an active involvement in the early events of puberty onset which is supported by the notion that leptin triggers precocious vaginal opening in mice [6, 60, 76]. O*b/ob* female mice (`leptin knock-outs`) are infertile and remain in a constant prepubertal state in which ovarian and uterine weight, sex steroid hormone levels and pituitary gonadotropin secretion are depressed. Administration of recombinant leptin to female *ob/ob* mice completely restores gonadotropin secretion, secondary sex organ weight and fertility [25, 147]. Leptin treatment advanced sexual maturation in food-restricted animals, even in those with ad libitum access to the low-caloric feed [6, 25,63].

In order to be able to trigger puberty leptin must act centrally to induce luteinizing hormone (LH) release in immature animals. Leptin is capable to stimulate (GnRH and) LH release, not only in adult but also in prepubertal animals, at least partly due to a central action [76, 233]. Furthermore, LH was suppressed and the estrous cycle was disrupted in mature female rats after central infusion of anti-leptin serum [53]. Chronic icv infusion of leptin stimulated LH secretion in the food-restricted cow, and ewe [31]. Icv administration of leptin (0.01-1.0 µg) to peripubertal female rats stimulated LH secretion [76]. Leptin treatment directly stimulated basal LH secretion from anterior pituitary cells and GnRH release from hypothalamic tissue explants in gilts [31].

Leptin receptors are widely expressed in many CNS areas such as the hypothalamus. Within the hypothalamus the arcuate nucleus is the main target of leptin [181]. It appears that any effects of leptin on GnRH neurons are indirect because these neurons do not express leptin receptors [83, 102]: Leptin in the range 10^{-6} -10^{-12} M had no significant effect on GnRH release during first 30 min of incubation; but, during the second 30 min, the lower concentration (10^{-12} M) significantly increased GnRH release [158], which indicates that only sub-nanomolar concentrations of leptin stimulates GnRH release from the median eminence whereas higher leptin concentrations attenuate GnRH release.
These data indicate a central site of action of leptin affecting reproduction, and suggest that the stimulating effect is exerted over but a relatively narrow range of leptin concentration [52]. Treatment of hypothalamic explants from adult female rats with leptin increased GnRH

pulse amplitude without affecting pulse frequency [177]. Others claim that a specific and narrow range of leptin concentration is essential to maintain a normal reproductive function in both sexes and that any concentration below or above these thresholds might interfere in opposing ways with the functioning of the HPG axis [52].

Leptin receptors within the hypothalamus of both rodents and primates, are co-localised with POMC and NPY neurons [63, 83,102]. Leptin decreases NPY synthesis in the arcuate nucleus [60], and NPY inhibits LH release. It is hence suggested that leptin indirectly, by decreasing NPY, stimulates GnRH synthesis and release in to portal vessels from where it is carried to the anterior pituitary to stimulate FSH and LH release [158]. It is also suggested that serotonin may induce leptin effects on GnRH release since leptin receptors are localised on serotonergic neurons [83]. As serotonin has either stimulatory or inhibitory action on GnRH release, leptin might also stimulate GnRH via serotonergic neurons [158, 159].

The percentage of cells expressing leptin in the human pituitary (20-25%) is higher than in rat (5%) or mouse (7%) pituitaries [123]. Leptin's receptor in the pig was shown to be present in 20-25% of anterior pituitary cells and was expressed in almost all of the hormone-producing cell types: ACTH 70%, GH 21%, FSH 33%, LH 29%, TSH 32% [123]. Various studies suggest that leptin is involved in the regulation of the secretion of pituitary hormones, e.g. FSH, LH and GH [123, 165, 179]. Direct effects of leptin at the pituitary level, however, have been poorly recognized. Since both leptin and its receptor are expressed by the majority of pituitary cells, a paracrine or autocrine role of leptin in the pituitary is possible: Leptin was only slightly less effective to release LH than GnRH itself [233].

A strong association between leptin and LH release, with a lag time of 42-84 min has been found in women and the ultradian pattern of leptin was synchronous with LH and E_2 [3, 143]. Moreover, the nocturnal leptin peak was positively correlated to an LH pulse of longer duration, higher amplitude and larger area. The nocturnal synchronicity of LH and leptin was associated with E_2, suggesting a functional link between leptin and HPG axis [143]. Leptin treatment restored LH secretion and pubertal development in leptin-deficient patients [80]. Also, it has repeatedly been shown that leptin stimulates the synthesis and release of LH and FSH in different species [3, 53, 83, 170]. In another report icv infusion of leptin (10 μg) in ovariectomised female rats produced an increase in plasma LH level to 60% above the initial concentration [158]. In a recent study, treatment of hemi-pituitaries from proestrus-afternoon

rats with increasing doses of leptin caused a dose-dependent increase in LH release, possibly mediated by NO [75]. The response to leptin of the pituitary gland *in vitro* was higher when both E_2 and progesterone were present [75].

These data all suggest that leptin might act in the brain to regulate GnRH and LH secretion, that leptin is obligatory for fertility, and suggests that leptin may indeed serve as a physiological link between adipose tissue and reproductive functioning. It seems therefore that the normal onset of puberty reflects the activation of the neuroendocrine reproductive axis by leptin.

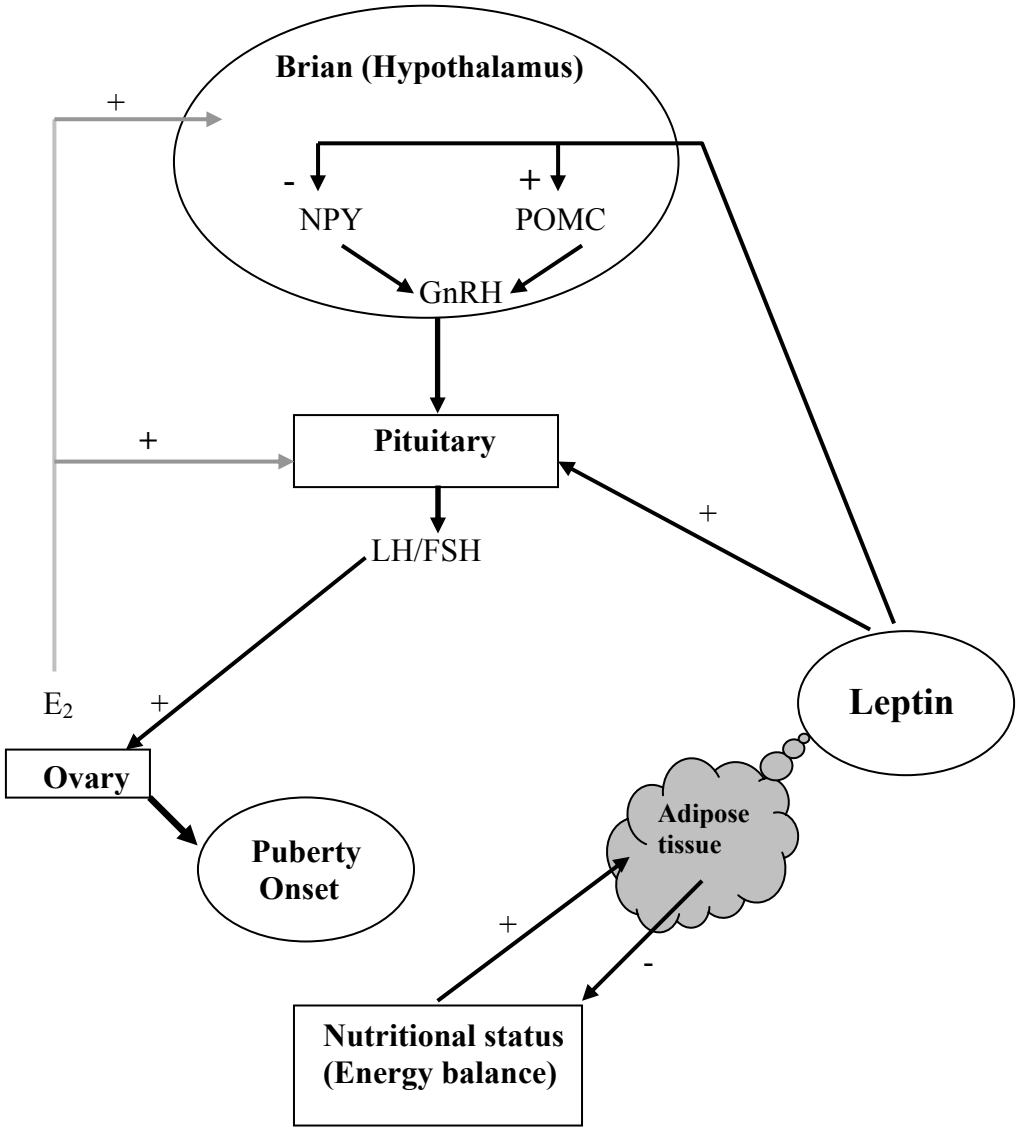

Figure 3: Involvement of leptin in the regulation of LH secretion and initiating of puberty. Leptin is secreted by adipose tissue in response to change in nutritional status (energy balance). Leptin inhibits NPY neurons and stimulates POMC neurons in the hypothalamus and thus indirectly stimulates GnRH neurons (see text) [modified from 31].

In contrast to the aforementioned however, reports exist claiming puberty onset is not dependent of leptin levels, at least in rodents. In adult female Zucker (*fa/fa*) rats (a rat strain lacking the leptin receptor) central leptin administration can decrease food intake and body weight gain in *ad libitum* fed lean and obese animals but the icv leptin treatment did not enhance reproductive maturation [91]. And, in female mice at15 to 20 days of age, i.e. before

the onset of puberty, leptin levels were found to decline and body weight was not found to be critical for the onset of puberty [49]. The reassessment of recent data about the role of leptin on puberty in mice and rats showed that leptin is not *the metabolic* trigger for puberty onset but is *one* among several permissive factors [64].

In seasonal mammals, annual cycles of food intake and adiposity are related to significant seasonal changes in plasma leptin levels and altered leptin gene expression in WAT [186]. During long photoperiods, leptin gene expression in WAT is up-regulated and its circulating concentration increases two- to four-fold in both sexes [186]. In Siberian hamsters, so-called `long-day' breeders, the natural seasonal decline in body weight, adipose tissue and plasma leptin levels are coincident with reduced gonadotropins secretion, the onset of gonadal regression and short-day induced infertility [186]. But, chronic leptin infusion (icv) was ineffective to modulate LH secretion for either short- or long-day housed animals [186]. Thus, it appears that pathways involved in the photoperiodic suppression of reproduction via melatonin are not responsive to leptin in seasonal mammals [186]. Collectively, there are significant species differences in the role of leptin in reproductive function not only among rodents but also between human and nonhuman primates.

5-6) Leptin: growth hormone, IGF-I, and puberty onset

Although leptin might signal to the HPG axis that the nutritional status is adequate for the onset of puberty, it probably cannot by itself trigger the changes in GnRH secretion to initiate puberty [73]. Leptin may therefore be one of a series of permissive factors involved in puberty onset, and not necessarily the *initiator* of puberty [52, 63, 147]. This makes it difficult to correlate the complex mechanism of puberty onset with the modifications of putative single signals such as leptin [63].

Growth hormone (GH) is the most abundant pituitary hormone in mammalian species both during development and in adult life. GH levels increase during puberty and are mainly responsible for the growth spurt seen in puberty. Despite the relatively high plasma GH levels observed, even in adulthood the precise role remains uncertain. Since a delayed or absent puberty is often associated with a GH-deficient or GH-resistant state, and as GH administration is shown to accelerate puberty, it is suggested that GH is required for the timing of sexual maturation [115]. GH may do so by activating the GnRH pulse generator

[32]. In contrast, in pigs GH treatment had no stimulatory effect on puberty, and the implantation of bovine GH in the median eminence of young female rats postponed puberty. GH may exert its inhibitory effect on the HPG axis at central sites and its stimulatory actions on pituitary gonadal function [115, 116]. Pituitary GH stimulates the production of hepatic IGF-I (insulin-like growth factor); GH and IGF-I act to stimulate mammary, ovarian, and uterine and/or oviduct function.

Apart from the pituitary, GH is also produced in the mammary gland, placenta, ovary and perhaps oviduct, and may act directly or via locally produced IGF-I to affect reproductive function [115]. GH may facilitate ovulation by increasing the sensitivity to gonadotropins and/or by reducing apoptosis in preovulatory follicles. This latter action of GH is thought to be mediated by IGF-I [65]. GH by itself also has numerous gonadotropic roles in female reproduction. These actions are thought to reflect endocrine roles of pituitary GH and to be complementary to the autocrine or paracrine roles of GH locally produced within the reproductive tissue. GH directly affects steroidogenesis, gametogenesis, and gonadal differentiation as well as gonadotropin secretion and responsiveness. GH may induce steroidogenesis directly or by potentiating gonadotropin action. In addition, GH may be permissive for gonadotropin-induced follicular development. It is suggested that a mini hypothalamic-hypophysial axis is also present in the reproductive tract of both sexes; GHRH and somatostatin are both synthesized in the gonads. Therefore, GH directly or indirectly via IGF-I regulates reproductive functions at all levels of the HPG axis [115, 116].

GH directly regulates adipocyte metabolism in domestic animals, rodents and man: GH stimulates lipolysis. Long-term GH treatment significantly reduces adiposity and increases lean body mass [155, 116]. The inverse relationship between adiposity and GH implies that adipose-derived factors might regulate GH secretion [105]. Leptin seems to be one of the factors regulating GH secretion: GH synthesis in rodents is impaired in leptin deficiency or leptin insensitivity states [8]. Several reports suggest that leptin regulates GH secretion in humans [70], sheep [152, 185], pigs [29], and rodents [53]. Recent data suggest that GH/IGF-I-induced changes in body composition in GH deficient patients may be affected by changes in the balance of free/bound leptin in the circulation [183]. Pulsatile GH secretion is markedly blunted during fasting, and restored by leptin replacement, while immunoneutralization of

leptin reduced GH secretion in fed rats [3]. Leptin and GH act via the same family of receptors coupled to the JAK-STAT pathway [3].

Under normal metabolic conditions leptin treatment stimulates GH secretion, and GH release is inhibited by a leptin anti-serum [53]. Central administration of leptin increases pituitary GH transcription, and increases hypothalamic GH-RH mRNA levels and reduces those of somatostatin mRNA [67, 209]. Also, in fed rats, icv infusion of leptin stimulated pituitary GH and hypothalamic GHRH mRNA expression, inhibited hypothalamic expression of somatostatin and NPY [105, 219]. This was also shown in hypophysectomised fasting rats [54]. *In vitro* an inhibitory effect of leptin on somatostatin transcription and secretion is shown [219].

Leptin receptors have been co-localized with GHRH and somatostatin, providing strong anatomical evidence for an interaction between leptin and somatotropic axis [102]. Somatostatin neurons in the sheep hypothalamus contain the leptin receptor indicating these neurons are direct targets for leptin [119]. The administration of anti-GHRH serum (500 µl, iv) completely blocked leptin-induced GH release in fasting rats, suggesting a role for GHRH and somatostatin as mediators of leptin-induced secretion [54]. *In vitro,* treatment with leptin for 24 h modifies both basal and GHRH stimulated GH secretion; but short-term exposure to leptin (1 h) had no effect on GH secretion [185]. Icv infusion of leptin anti-body in fed rats decreased the amplitude of pulsatile GH release, and leptin restored GH release in fasted animals [53, 105, 219].

It has been shown that NPY stimulates somatostatin and inhibits GH release [105]; so, leptin stimulation of GH is probably mediated by a down-regulation of NPY [219, 105], even it is suggested that any effect of leptin on GH secretion seems to be controlled by NPY [105].

In contrast, the effect of GH on leptin regulation is controversial: in rats, injection of GH (4 mg/kg/d) decreased plasma leptin levels [150]. Injection of recombinant porcine GH (4 mg/day, im) in castrated male pigs declined serum leptin levels [231]. GH treatment in human patients mainly decreased plasma leptin levels [78, 136, 156, 164, 184]. In prepubertal children given a GH stimulation test, leptin was inversely related to peak GH release, independent of body fat content [71, 105]. In both young and adult GH-deficient patients, 4 weeks of GH replacement did not change leptin levels [175]. GH treatment had no independent effect on either serum leptin or leptin gene expression [135]; and, GH treatment

(0.1 IU/kg/day) in obese children did not increase serum leptin levels [235]. GH infusion had no effect on leptin expression in hypophysectomised rats [40]; and, in fa/fa rats treated with GH leptin expression was inhibited, without any change in fat mass [122]. Also, GH infusion in mice (0.3 or 0.6 unit/animals) did not change the plasma leptin levels nor the expression of leptin gene in adipocytes [141].
But human patients treated with rhGH (0.2 IU/kg/d, sc) increased plasma leptin levels [118]. Infusion (sc) of GH in young castrated male cattle for 3 days also increased adipose tissue, leptin and IGF-I mRNA levels [114]. In GH deficiency in human, GH treatment (0.1 mg/kg) also enhanced serum leptin levels [96].
There is, therefore, little evidence that GH has a direct effect on leptin expression or secretion, although, via inhibition of hypothalamic NPY expression, leptin increases the tone of the GH axis but not vice versa [105]. It is not yet clear whether these discrepancies result from differences in the duration and intensity of GH treatment or exposure, or arise from species- and genotype-specific responses [144].

During puberty onset IGF-I is probably increased as a result of high GH levels. The initiation of female puberty is furthermore associated with an increasing expression of the IGF-I gene in the liver and with an elevated level of IGF-I in the peripheral circulation [178].

IGF-I is suggested to play a role in the initiation of puberty as an increase in nocturnal leptin, GH and IGF-I precedes an increase in nocturnal pulsatile LH secretion at the onset of puberty [206]. And IGF-I of peripheral origin is shown to contribute to the initiation of puberty in male rats by stimulating GnRH release [176]. Ventricular infusion (icv) of IGF-I accelerates the onset of puberty in juvenile female rats, and was shown to increase serum LH levels via GnRH [110]. The centrally administered IGF-I (2, 20, 200 ng/3 µl) induced a marked increase in LH secretion during early and late proestrus and estrus, and advanced puberty onset compared to control animals [110]. This increase in LH levels was suppressed by infusion of an antiserum against GnRH [110].
It has been shown that median eminence (ME) has high levels of IGF-I receptors suggesting that IGF-I plays an important role in this brain region [77, 109, 110]. The ME, being the target area for the GnRH nerve terminals might hence be a site of action for the peripherally derived IGF-I to activate GnRH/LH secretion, especially at puberty when hepatic IGF-I

production is elevated due to high GH levels. This increase in IGF-I receptors and its gene expression during proestrus occurred only in the region of the ME and not in other areas of the brain associated with reproduction [77, 109, 110].

The initiation of female puberty is furthermore associated with an increasing expression of the IGF-I gene in the liver and with an elevated level of IGF-I in the peripheral circulation [178]. IGF-I gene expression increased significantly in the liver on the morning of the first proestrus and followed by increased levels of serum IGF-I, which peaked during the afternoon of proestrus and were accompanied by increasing in serum LH, FSH, and E_2. So IGF-I seems to be a (metabolic?) signal capable of activating the GnRH/LH releasing system at the time of puberty.

Apart from GH/IGF-I, also an increase in nocturnal leptin precedes the increase in nocturnal pulsatile LH secretion heralding the onset of puberty [206]. GH treatment increases leptin mRNA abundance *in vitro*, but only in animals exhibiting a positive IGF-I response to GH; GH and IGF-I alone did not affect leptin secretion [114]. And after 3 days of GH treatment there is not only a significant up-regulation of IGF-I transcription in adipose tissue but also a significant increase in leptin mRNA abundance [114]. Hence, the actions of GH, IGF-I and leptin are amplified mutually as they control growth and the increase in muscle mass. The synergistic actions of these anabolic hormones also appear to be most significant also on puberty in man [157]. These findings suggest a synergistic role for leptin and the GH/IGF-I axis in regulating the pubertal initiation of the pulsatile GnRH/LH secretion.

Some studies however, report no effect of IGF-I infusion on puberty onset in female rats [99], or show an inhibitory action of IGF-I on the effects of GnRH [43]. Long-term infusion of rIGF-I (110 μg/kg/day, sc) in rhesus monkeys did not advance the age of menarche but did advance the age at first ovulation [227]. Whether IGF-I plays an inhibitory or stimulatory role on GnRH secretion remains unknown [110]; only chronic and central administration of IGF-I might affect puberty onset, whereas acute and peripheral infusion does not [178]. Accumulating reports suggest that IGF-I regulates reproductive development by acting at different levels of the hypothalamic-pituitary-gonadal axis [74, 138], and in several species, including humans, monkeys, rats, and mice, circulating IGF-I concentrations increase during puberty [74, 104, 206]. However, there is still controversy about the effects of IGF-I to induce

puberty onset, although the initiation of female puberty is associated with an increasing expression of the IGF-I gene in the liver inducing an elevated level of IGF-I in the peripheral circulation [178].
Taken together these data suggest that IGF-I may play an important role to induce puberty onset.
Overall, these findings suggest a role for leptin and GH/IGF-I in regulating the pubertal initiation of the pulsatile GnRH/LH secretion.

5-7) leptin: mechanisms of a central action

Puberty and puberty onset is a complex event, much more complicated than earlier thought. As with many biological systems, controversies and exceptions are not uncommon, particularly for permissive pathways such as leptin.
Also in postpubertal normal cycling females leptin possibly has a role in the integrative mechanisms that mediate the LH surge: Central infusion of a leptin anti-serum suppresses LH secretion and disrupts the estrous cycle in rats [185].
Leptin receptor mRNA has been localized both in central areas controlling reproductive function (e.g. the ventromedial and arcuate nucleus of hypothalamus), in the pituitary gland, and in the reproductive organs themselves (testis, ovary and uterus). Leptin could hence act at multiple sites in the reproductive system; with regard to a role in puberty onset a central site of action seems most likely [52, 113, 123].
The main hypothalamic targets for leptin are the paraventricular (PVN), arcuate (ARC), ventromedial (VMN) and dorsomedial (DMN) nuclei [92, 147, 188, 189]. The evidence for neuroendocrine effects of leptin on GnRH release is most convincing. The mechanism however, whereby leptin (directly) stimulates gonadotropin function is not yet known. Some of the proposed mechanisms by which leptin affects the HPG axis are summarized here:

1) The effects of leptin on hypothalamic GnRH release could be mediated by NPY. This neuropeptide is found in areas of the hypothalamus involved in food intake and neuroendocrine control. Contrary to leptin, NPY is a potent stimulator of food intake and an inhibitor of gonadotropin secretion. Leptin administration decreases NPY expression in the arcuate nucleus, and hence removes the NPY inhibition on GnRH release [92, 147,

188, 189]. Leptin receptors have been found on hypothalamic NPY neurons in mice and sheep [8].

2) It is possible that leptin regulates the secretion of GnRH via a number of neuronal satiety-related systems other than NPY, either inducing food intake such as melanin-concentrating hormone (MCH), pro-opiomelanocortin (POMC), and galanin (GAL), or decreasing food intake such as neurotensin (NT). Central injections of leptin decrease MCH, POMC and GAL gene expression and stimulate gene expression of NT. So it is possible that leptin's action on the onset of puberty may also be mediated, in part, via these neuronal systems which affect the nutrient status of the animal [61, 64, 66, 147, 233].
3) Glucose availability is a signal for the control of GnRH and may hence be a regulator of LH secretion [61, 88]. It is suggested that leptin might act by inducing or sensing changes in the (central) availability of glucose. Perhaps leptin acts centrally as or at a ‘glucosensor’ to stimulate neuronal glucose uptake by activation of a neuronal insulin-dependent glucose transporter (GLUT4?) [197]. Both leptin and glucose availability then serve as co-determinants for the timing of puberty [88]. High CNS levels of leptin are hence insufficient for a normal estrous cyclicity when fuel oxidation is inhibited.
4) Plasma concentrations of IGF-I increase notably during the onset of puberty in rodents, primates, sheep and cattle [88]. The data suggest that IGF-I may act centrally in the hypothalamus and/or pituitary to modulate gonadotropin secretion. The concomitant decrease in serum leptin and IGF-I concentrations during fasting suggests an association between IGF-I and leptin secretion [30]. By stimulating IGF-I, by triggering its secretion, leptin might affect the time of puberty onset [88]. Moreover and concurrent with the significant prepubertal elevation in nocturnal leptin secretion, also the nocturnal GH and IGF-I levels rise before the onset of puberty [206].

6) The scope of this thesis

Puberty onset depends on body size and weight, and the right metabolic cues rather than age. Numerous factors in the HPG are involved in the timing of the onset of puberty. It has frequently been shown that the nutritional status is obviously involved in this process: If the energy resources are insufficient, puberty onset is postponed. Many studies now have shown that leptin, mainly produced in adipose tissue, is a good candidate for the link between the nutritional status and the HPG-axis for the onset of puberty. But also the growth hormone-IGF-I axis (GH/IGF-I) is believed to affect the timing of puberty onset. Some reports even suggest that the effect of leptin on puberty onset might be exerted via the GH-IGF-I axis. So, we aimed to further elucidate the role of leptin in puberty onset and whether this role is exerted independently and directly on the HPG-axis, or indirectly via the GH-IGF-I axis.

On the basis of the aforementioned information, we postulate that leptin (directly or indirectly) plays an important role in the onset of puberty in mammals.

First, we intended to find out the link between leptin and the amount/percentage of body fat over the prepubertal period, and the link between leptin and puberty onset in our strain female rats (Wistar). Time of puberty onset was monitored by scoring the moment of vaginal opening (VO). The experiments did not intervene but addressed the events around puberty in this rat strain and extended the current knowledge of prepubertal physiology. Finally, we studied the effects of food-restriction (FR) on puberty onset, and how this treatment affects body fat, plasma leptin levels, and body temperature. We compared female rats fed *ad libitum* with FR animals which were pair-fed with 30% less energy. The low energy diet had the same vitamin and mineral content and an equal amount of protein as the control food but 30% of the carbohydrate and fat was replaced by cellulose. This is referred to as `FR-treatment` (Chapter 2).

Secondly, we used a model of puberty postponement due to energy restriction. If leptin were the signal for initiating the onset of puberty, it would trigger and advance puberty onset upon chronic intracerebroventricular (icv) and peripheral (sc) infusion in prepubertal female rats on a control or energy-restricted diet. Also, central immunoneutralization (icv) of leptin should postpone puberty onset (Chapter 3).

Thirdly and on the basis of our previous finding, we aimed to evaluate the effects of GH on the timing of puberty and also on leptin levels in prepubertal female rats. We centrally

administered recombinant human growth hormone (rhGH) to normal ad lib-fed or pair-fed FR female rats. We also centrally infused anti-rat GH or somatostatin (Som) in normal ad lib-fed or pair-fed female rats (Chapter 4).

Fourthly, according to our results (in particular Chapter 4), we intended to clarify the effects of IGF-I on puberty onset. To find out the role of leptin and the GH/IGF-I axis in regulating the timing of puberty onset, in prepubertal female rats either recombinant human IGF-I (rhIGF-I) or rat-IGF-I antibody was centrally infused (Chapter 5).

Finally, we summarized and briefly discussed the main conclusions of this thesis and a mechanism is proposed (on the basis of our finding) for puberty onset in mammals (Chapter 6).

Chapter 2

A signaling role for leptin in puberty onset in female rats?

Saeed Zeinoaldini, J.J.M Swarts, B.J.M. Van de Heijning

Abstract

In response to leptin signaling the nutritional status and the presence of an adequate amount of adipose tissue, the brain is thought to switch on the reproductive processes in general and puberty in particular.

A series of experiments was performed to study the link between whole body fat and plasma leptin levels on one hand, and the link between leptin and puberty onset on the other, in controls and food-restricted (FR) female Wistar rats of 21 d old.

A high positive correlation ($r = 0.776$) was found between body fat and prevailing plasma leptin levels, and between body fat and the time of puberty onset, i.e. vaginal opening (VO; $r = 0.691$, $P < 0.01$).

Blood samples collected every other day from age 22 to 42 d, at ZT 2, 6, and 12, showed the installation of a diurnal rhythm in leptin secretion with high levels at ZT 2 and 12 and low levels at ZT 6. Furthermore, we found leptin levels to increase over the pubertal period: on day 26 the leptin level was 2.06 ± 0.21 ng/ml, on day 28, 2.38 ± 0.35 ng/ml, on day 30, 2.73 ± 0.31 ng/ml, and on day 32, 3.09 ± 0.34 ng/ml at ZT 12, while the median age of VO was 30 d (95% confidence interval: 28.8 -31.2 d).

Compared to controls, food restriction delayed the time of VO considerably (median VO was 27 *vs.* 35 d). Body fat, plasma leptin levels and body temperature in the FR rats were significantly lower than in controls ($P<0.01$), but body fat was still positively correlated with leptin levels.

These findings support the notion that leptin could indeed serve as the link between the nutritional status and the reproductive axis in initiating puberty onset in rats.

Introduction

Puberty is the time in life when mature gametes are produced for the first time and sexual maturity is reached. Sex steroid production by the gonads is increased in response to the increasing secretion of gonadotropins from the anterior pituitary gland, which, in turn, is driven by an increased secretion of GnRH from the hypothalamus [131]. The hypothalamus rather than the pituitary and/or ovary is hence responsible for triggering puberty onset and ‘awaits’ a crucial cue to initiate a cascade of events. This cue might be a single factor or the

integration of multiple factors. An intriguing question in understanding puberty is how the brain ‘knows’ the body has reached the appropriate size and degree of maturity to start the high frequency GnRH secretion. The characterisation of these (metabolic) signals that linking growth and reproduction, and activate the dormant hypothalamus-pituitary-gonadal (HPG) axis at puberty is a pivotal challenge in reproductive biology [62].
Nutritional status has been shown to be involved in triggering puberty onset, and several hypotheses have been proposed in this respect. Kennedy [127, 128] proposed that body weight and food intake were initiating factors for puberty in rats, and demonstrated that the onset of puberty correlated with body weight rather than with age, while Frisch [93] proposed a ‘critical’ amount of fat to be crucial to switch on reproduction. More recently, availability and oxidation of metabolic fuels, and a critical level of a factor signaling this, were proposed to trigger puberty onset [88]. Both in the ‘critical fat’ and in the ‘metabolic fuel’ hypothesis peripheral signals are sensed centrally; the *nature* of these signals is still controversial [62].

Leptin, which is mainly produced in adipose tissue, serves as a peripheral signal to the brain resulting in a decreased food intake, an increased energy expenditure and a resistance to obesity [6, 8, 60]. Plasma leptin levels are positively correlated with adiposity in humans [14, 70, 146] and rodents [3]. Leptin thus signals the nutritional status and the presence of an adequate amount of adipose tissue from the periphery to the hypothalamus which in response switches on the reproduction process in general and puberty in particular [88, 147]. Receptors for leptin are present in the hypothalamus - pituitary axis [8, 9, 36, 152], enabling leptin to affect gonadotropin release via a pituitary and/or a hypothalamic site of action [233]. Exogenous leptin indeed increases serum LH levels in female mice, prevents the reduction of pulsatile LH secretion in fasted female rats [12, 170, 224], and increases GnRH pulse amplitude at all stages of the estrous cycle in rats [177]. Conversely, the administration of leptin antiserum into the third ventricle of female rats decreases LH pulsatility, disrupts cyclicity, and delays the onset of the prolactin surge [53]. However, leptin might not be an *acute* regulator of gonadotropin secretion, nor a primary stimulus of the reproductive axis, but merely act as a permissive factor also with respect to puberty [66].

In this study we describe the course of various parameters and features in female rats around puberty and the time of vaginal opening (VO). We try to find a link between leptin and body fat on one hand, and a link between leptin and puberty onset on the other. The

experiments did not intervene but served to describe the events around puberty in female rats, thereby partly confirming but also extending the current knowledge of pubertal physiology. Finally, we studied the effects of food-restriction (FR) on puberty onset, and how this treatment affects body fat, plasma leptin levels, and body temperature.
Altogether, the data obtained indicate a signaling role for leptin in puberty onset in female rats.

Materials and methods

Animals and experimental procedures

Wistar female rats, age 21 d and body weight (BW) 45 ± 2 g were obtained from Harlan Co. (Horst, the Netherlands). Rats were individually or group-housed in a room at 21.5ºC, 14/10 L/D regime, with drinking water *ad libitum*. Time of lights on (L phase) varied, but is referred to as ZT=0, all other procedures are relative to this time point. Animals were fed *ad libitum* a pelleted chow diet according to AIN guidelines.
One group of rats was food-restricted (FR) and was put on a diet with 30% less energy. The FR group was pair-fed to a control group (Con). The low energy diet had the same vitamin and mineral content and an equal amount of protein as the control food but 30% of the carbohydrate and fat was replaced by cellulose. The caloric content of the normal diet was 16 kJ/g and FR diet with 30% less energy contained about 10 kJ/g.
Body weight and food intake were measured once daily (at ZT 14) and vaginal opening (VO) was monitored two times per day (at ZT 6 and 14). In rats fitted with a temperature transponder (implanted subcutaneously under 5% Isoflurane anesthesia) body temperature was monitored twice daily (at ZT 6 and 14) from postnatal day 23 onward by means of a programmable scanner (BMDS®, USA). Blood samples were taken repeatedly via a tail nick [87] at various time points under Isoflurane (5%) anesthesia, or rats were bled prior to euthanasia (CO_2: O_2, 2:1) saturated with water [68] at the end of the experiment. Plasma was frozen and stored at -20ºC until assayed for rat leptin content. Rat's carcasses were stored at -20ºC to measure whole body fat.
In the same room at least two adult male rats of the same strain were housed with normal rat chow and water *ad libitum*, to provide a normal pheromonal environment. All animal experiments were approved by the Wageningen University Animal Ethical Committee.

Histology

10 rats were killed on the day of VO, and trunk blood was collected, ovaries were excised, and the number of corpora lutea was counted macroscopically. The ovaries were fixated in Bouin solution (15 ml picric acid, 5 ml formaldehyde 38% and 1 ml acetic acid 100%) for 24 h, and were dehydrated with a graded ethanol series, cleared with xylene, and embedded in paraffin. Sections of ovaries (5 µm) were hematoxylin-eosin stained and the presence of secondary corpora lutea was checked.

Fat analysis

To analyse the whole body fat, rat's carcasses were weighed, 300 ml water was added, and put in an autoclave at 130ºC for 10 h, whereupon the samples were homogenized two times. Samples were subsequently freeze-dried for 72 h to reach a constant dry weight. Fat analysis was carried out using the ether-extraction procedure. Briefly, the samples were put in a cottonfat-free tube, 160 ml petroleum ether was poured into the Soxhlett tool and the flasks containing the samples were attached. Extraction was carried out for 6 h. To dry the fat, upon evaporation of the ether, the flasks were placed overnight in a dessicator in a vacuum oven at 80ºC for 1.5 h, subsequently the flasks were cooled in a dessicator for 45 min. and weighed to calculate the whole body fat.

Radioimmunoassay (RIA)

To measure rat leptin levels, a commercial available kit was used (LINCO Research, USA). The intra-assay and inter-assay variations as determined using quality control results were 2.9% and 4.7%, respectively.

Statistics

VO data are expressed as median and 95% confidence intervals. The other data are expressed as means ± S.E.M. Pearson's correlation and linear regression were determined; results were analysed by one way ANOVA. A probability of $P < 0.05$ was considered significant.

Results

In a first 'inventory' experiment prepubertal female rats (n = 25) were monitored from postnatal day 25 to 45. Ten of them were killed on the day of VO and checked for the presence of primary corpora lutea in their ovaries evidencing ovulation. No secondary, old corpora lutea were found. The relative and absolute amount of fat, the leptin levels and body weight on VO day, and the number of corpora lutea are shown in Table 1.

n	VO age (day)	VO weight (g)	%Fat	Fat (g)	Leptin (ng/ml)	CL Left	CL Right	CL Sum
1	33	92	30.26	31.17	1.8	6	6	12
1	33	97	31.81	34.04	3	4	7	11
1	34	100	31.48	33.68	3.1	9	6	15
1	36	110	26.45	32.01	1.5	8	6	14
1	38	115	29.18	35.59	1.9	7	4	11
1	38	119	30.24	37.5	2.9	3	8	11
1	39	107	28.17	31.27	1.6	3	9	12
1	40	133	31.76	44.78	2.9	7	6	13
1	41	131	28.51	38.77	2.2	7	6	13
1	41	143	33.34	49.68	2.3	1	10	11

Table 1: Vaginal opening (VO) age and weight, fat percentage and absolute amount of fat and the number of corpora lutea of 10 rats.

From the other rats (n = 15) vaginal smears were taken daily and the estrous cycle was followed from the day of VO until postnatal day 45 when the animals were killed. Rats showed regular cyclicity with a 4-5 days cycle.

The incidence and the pattern of VO of all 25 rats in this experiment is shown in figure 1A and B. The median age of VO was 34 d (95% confidence interval: 32.5-35.5 d). The peak time of VO was 32-33 days (44% of the rats).

Figure 2 shows that rats with a higher body weight on postnatal day 30 show VO sooner than those with a lower weight on day 30 ($P<0.05$).

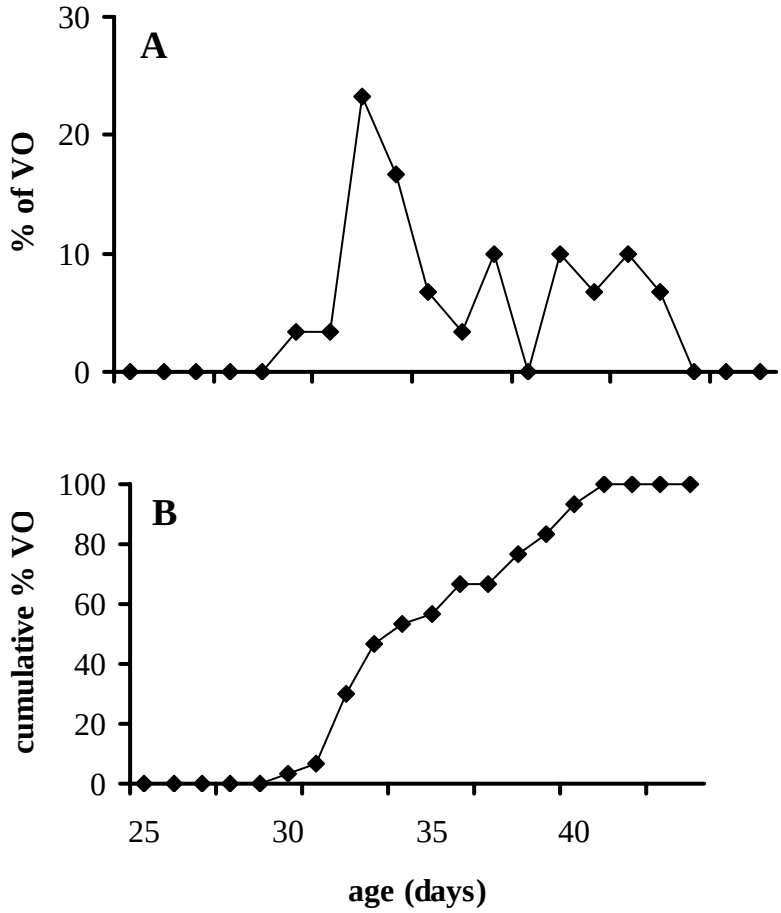

Figure 1:

A. The incidence of vaginal opening (VO), the median age of VO was 34 d (95% confidence interval: 32.5-35.5); n=25,

B. The cumulative percentage of VO.

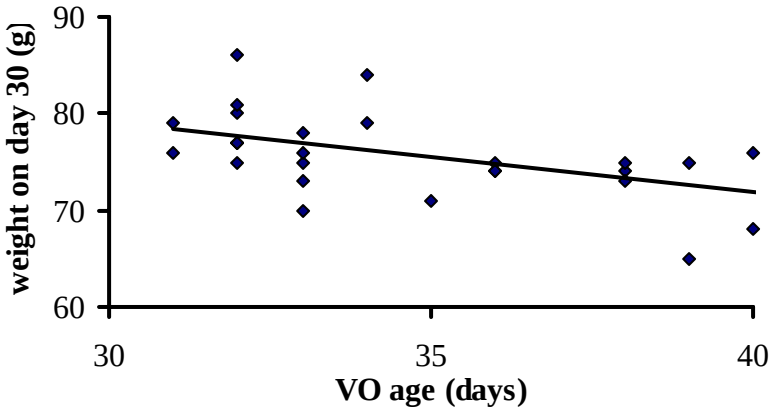

Figure 2: The correlation between vaginal opening (VO) age and the body weight on postnatal day 30 (n = 25); $R^2 = 0.273$ and r = - 0.522* (P<0.05).

The prevailing leptin levels in these animals show a strong positive correlation both between the relative (r = 0.776, P< 0.01; figure 3A), and the absolute amount of body fat and (r = 0.534, P< 0.05; figure 3B). The absolute amount of fat and the time of VO showed a positive correlation in these ten rats (figure 4; r = 0.691, P< 0.01).

In a separate experiment with 24 female rats we showed the prepubertal leptin secretion pattern to develop a diurnal rhythm with high levels at ZT 2 and ZT 12 and low levels at ZT 6 (figure 5). Furthermore, leptin levels are increasing over the prepubertal period to puberty onset: on postnatal day 26 leptin levels at ZT 12 were 2.06 ± 0.21, on day 28, 2.38 ± 0.35, on day 30, 2.73 ± 0.31, and on day 32, 3.09 ± 0.34 ng/ml (figure 5A). The VO pattern of this group of rats is shown in figure 5B. The median age of VO was 30 d (95% confidence interval: 28.8 -31.2 d).

Next, we studied in two separate experiments the effects of FR on the timing of puberty and compared normally fed rats with FR rats on a diet with 30% less energy. The FR effects on VO age, VO weight, and leptin levels on the day of VO are shown in Table 2. In the FR group, the leptin level is significantly lower than in the control group, and the day of VO is clearly postponed. VO weight is significantly higher than in the control group. Figure 6A shows the course of the body weight in the control (Con) and the FR rats.

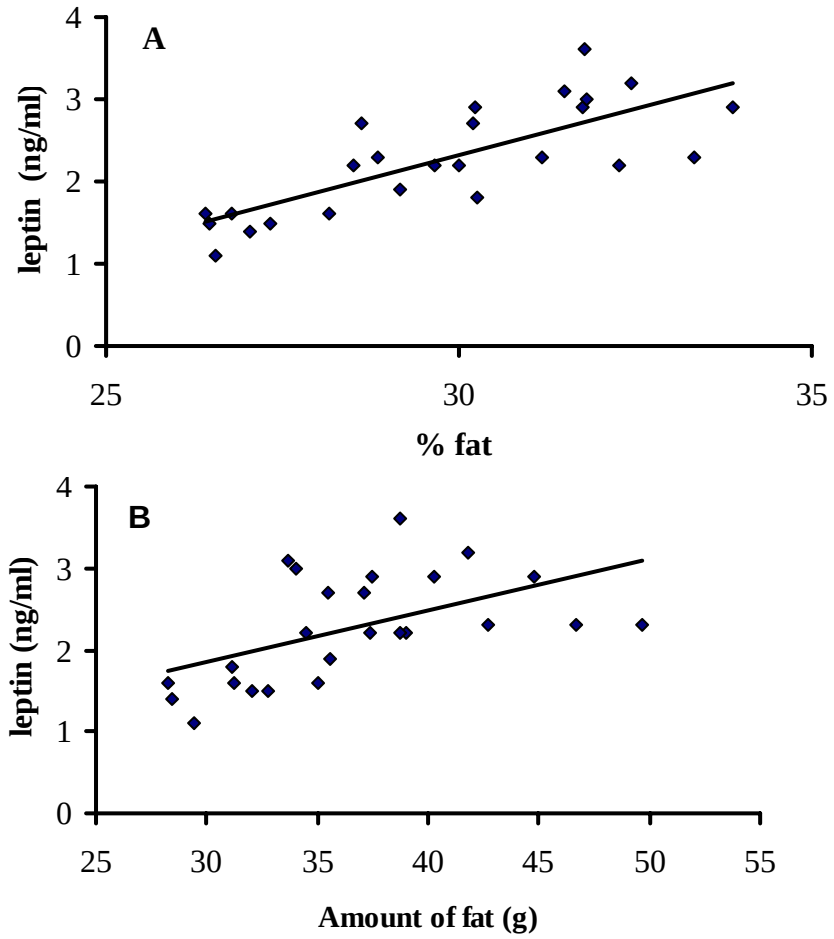

Figure 3:

A. The correlation between percentage of body fat and leptin levels at ZT 6 (n = 25); $R^2 = 0.603$ and r = 0.776 ** (P< 0.01);

B. The correlation between amount of fat and leptin levels at ZT 6 (n = 25); $R^2 = 0.285$ and r = 0.534 * (P< 0.05).

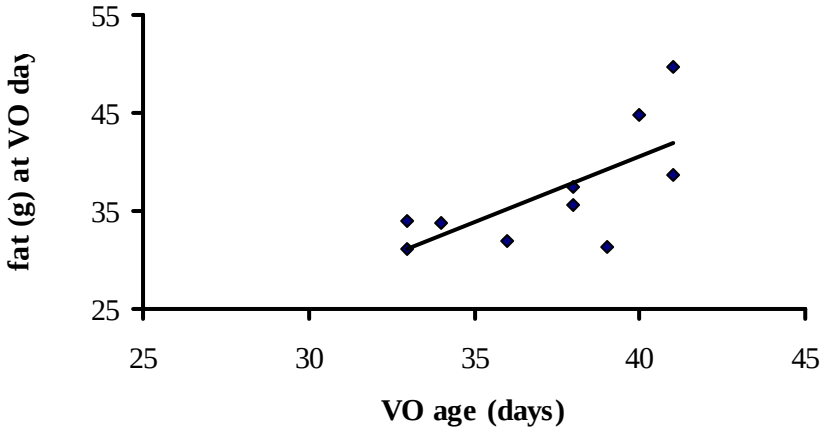

Figure 4: The correlation between time of vaginal opening (VO) and amount of fat at VO (n = 10); R^2 = 0.478 and r = 0.691* * (P< 0.01).

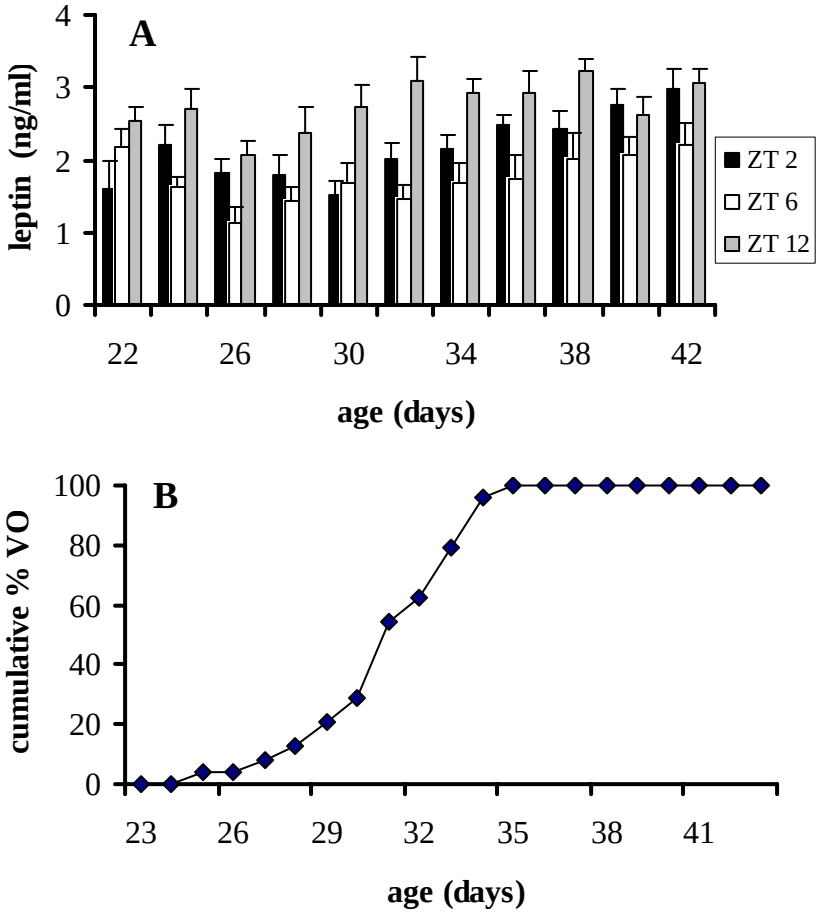

Figure 5:

A. The plasma leptin levels at ZT 2 = 09:00, ZT 6 = 13:00 and ZT 12 = 19:00 h (at each time point n = 8) in prepubertal female rats;

B. The cumulative percentage of VO (n =24).

	VO age (day)	95% Conf.interval	VO weight (g)	VO leptin level (ng/ml)	n
Con	27 [a]	25.9 – 28.1	85.4 ± 2.9 [a]	2.79 ± 0.31 [a]	12
FR*	35	28.2 – 41.8	103.2 ± 6.7	1.44 ± 0.17	12

Table 2:

The effects of food restriction on vaginal opening (VO) age, VO weight and leptin level on the day of VO (n =12); a designates significant difference ($P<0.05$). ***** Five rats in the FR group did not show VO by day 38.

Con: Control group, control food *ad libitum*

FR: Food restriction group, a diet with 30 % less energy (pair-fed to Con).

From postnatal day 25 onward the mean body weight in the FR rats is significantly lower than the control group ($P<0.01$). The FR treatment resulted in a delay in VO age as depicted in figure 6B. Five animals from the FR group do not yet have VO at the end of the experiment (day 38). The daily food intake does not differ between the two groups (data not shown) but the mean body temperature (the average at ZT 6 and 12) in FR rats is significantly lower than in the controls as shown in figure 9 ($P<0.05$). Body temperature was on average 0.5ºC higher at ZT 12 than at ZT 6. As shown in figure 7 and 8, the amount and percentage of body fat, as well as leptin levels in the FR rats are significantly lower than the controls at all time points ($P<0.01$).

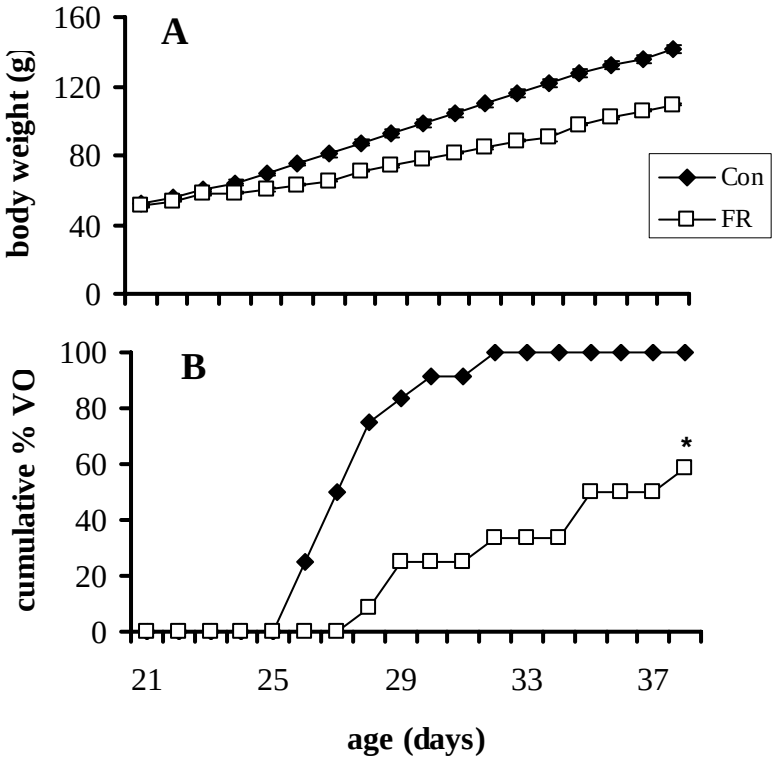

Figure 6:

A. The course of the body weight in the FR experiment (n = 18 in each group),

B. The cumulative percentage of animals showing VO in the control (Con) and the FR group ***** Five rats in the FR group did not show VO by day 38.

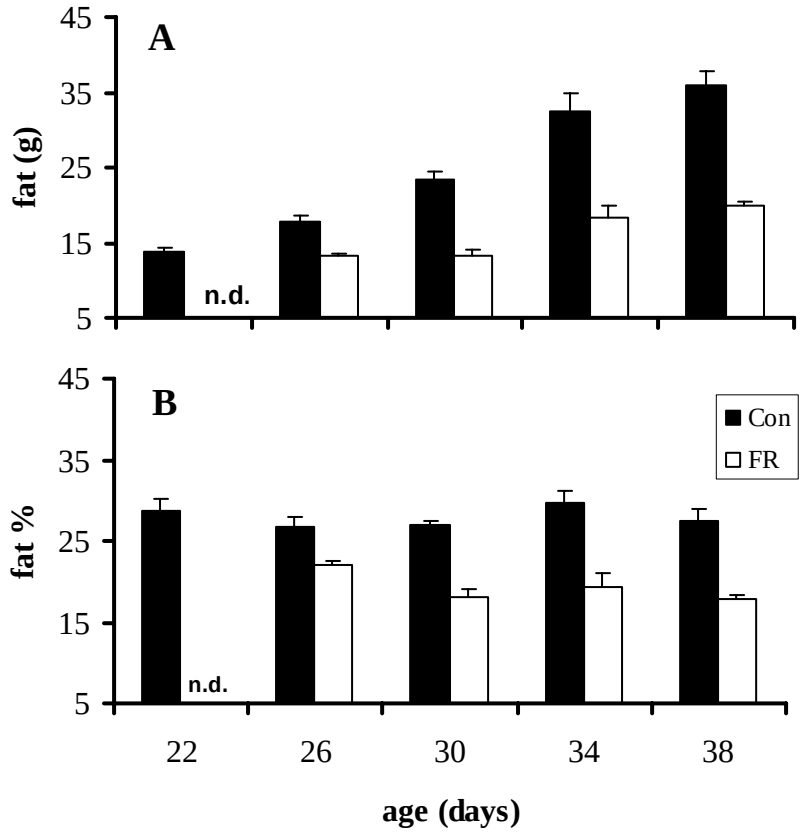

Figure 7: The absolute (A) and the relative (B) amount of body fat in control fed (Con) and FR animals (n = 4 at each time point in both groups); n.d. = not determined

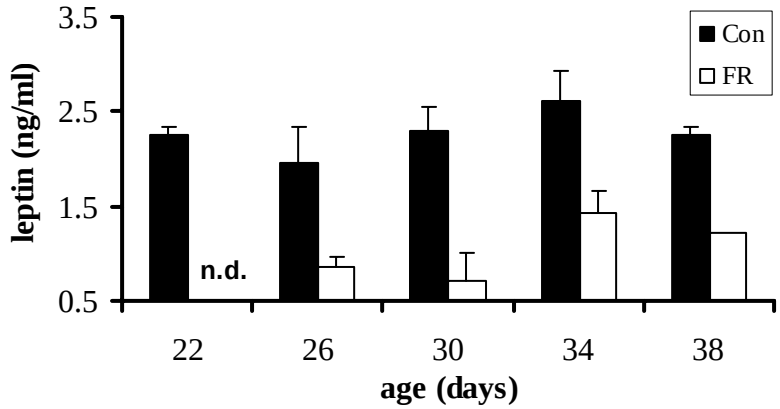

Figure 8: Plasma leptin levels at ZT 6 in controls (Con) and FR animals (n = 4 at each time point and in both groups); n.d. = not determined

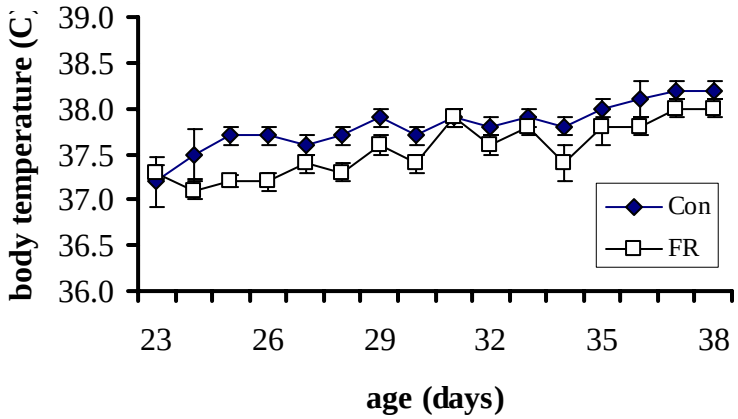

Figure 9: The mean daily body temperature (ºC) in controls (Con) and FR animals.

Discussion

One of the most important and contemporary issues in understanding puberty onset is determining how the brain is to know when the body is in the appropriate metabolic status and/or sufficient energy is stored (i.e. fat) to begin high frequency pulsatile GnRH secretion. Puberty onset is thought to be tightly coupled to growth and a positive sign in the somatic energy balance and metabolism; although the metabolic signal that could serve as the link between growth and reproduction is still unknown [62, 88, 93, 127, 128].

Corroborating the ideas of Kennedy and Frisch [93, 127, 128], a factor derived from adipose tissue is now thought to link 'fat' to reproduction: Leptin, synthesized and released from adipose tissue, is proposed to serve as the peripheral signal to the brain. Leptin receptors have been localized in various hypothalamic areas which are important in the regulation of both feeding and reproduction, and thus leptin relays the peripheral information to the CNS [81, 102, 147]. It supports the notion of leptin being even the 'trigger factor' to switch on the reproductive axis, i.e. initiate puberty [12, 60, 86, 88].
Injection of leptin (0.01- 10 µg) in the third ventricle induces the release of LH in prepubertal female rats [76], possibly via leptin receptors on GnRH neurons [46]. Since both leptin and its receptor are expressed by the majority of pituitary cells, a paracrine or autocrine role of leptin in the pituitary is also possible [123, 165, 179]. Treatment of hemi-pituitaries from proestrus-afternoon rats with increasing doses of leptin caused a dose-dependent increase in LH release, possibly mediated by NO [75]. The pituitary response to leptin in vitro was higher with estradiol and progesterone present [75].
These data suggest that leptin serves as an intermediate to link metabolism and reproduction, and that leptin might indeed trigger puberty onset.

We carried out a series of descriptive experiments listing changes in body fat and leptin levels in female rats around puberty. We found a strong positive correlation between individual body fat and plasma leptin levels (figure 3A and B), as has been previously shown in humans [14, 70, 146], and rodents [92]. Also, a positive correlation was found between the amount of fat and the time of VO indicating a critical level of fat is a crucial factor to trigger puberty onset in female rats. As shown in figure 7 the absolute, but not relative, body fat content increases with age, but this is not reflected in very much higher plasma leptin levels (figure 8).
The prepubertal rats with a higher weight on day 30 showed VO sooner than those with lower weight, indicating the weight at this age is a predictive value for the day of VO (figure 2); this result is in line with the *critical weight* hypothesis [127, 128].
Puberty onset was confirmed by showing corpora lutea in the ovaries of these animals (Table 1), indicating rats which showed VO, ovulated and could produce and release ova, and that puberty onset had taken place. The absolute amount of fat and the time of VO showed a positive correlation (figure 4; $r = 0.691$, $P < 0.01$).

In a separate experiment (figure 5) we showed the development of a diurnal rhythm in leptin secretion with high levels early (ZT 2) and late (ZT 12) in the light phase and with low levels at midday (ZT 6). Furthermore, we found that leptin levels gradually increase over the prepubertal period to puberty onset, especially at nadir time (ZT 6).

In rodents the level of leptin production is reported to vary with age: leptin is expressed during the prenatal period, and decreases rapidly after birth, followed by a transient increase in the neonatal period and a more steady increase in adults [3 , 8, 203]. In adult rats leptin displays a diurnal rhythm with a nocturnal rise reaching its peak levels between midnight and early morning and then declining to a nadir by midafternoon [212]. Our data (figure 5) confirm this and show the installation of the adult diurnal leptin rhythm to occur in the peripubertal period (from day 22 to 42) and to coincide with VO and puberty onset. The mechanism generating the diurnal pattern has not been elucidated, but it has been shown that the suprachiasmatic nucleus (SCN) has a direct control over the diurnal rhythm in plasma leptin levels in adult rats [124].

Some decades ago Kennedy showed that in female rats severe food restriction delayed VO for 10 days [127, 128]. Since then, it has been frequently shown that food restriction in many mammal species can cause postponement of puberty onset [50, 57, 79, 162]. Restriction to 50% of the normal daily food intake over 12 days decreased body weight by 20% and plasma leptin levels by 75% in female rats [217]. And feeding non-obese female rats at 80% of ad libitum intake completely inhibited the onset of puberty as no animals exhibited vaginal opening (VO) or became estrous yet at 38 d of age [63]. Furthermore, it has been shown that plasma leptin levels decrease with fasting in rodents and humans [12, 170]. Metabolic stress such as food restriction (FR), metabolic wasting disease and severe exercise result in negative signals or the absence of signals to the reproductive system (e.g. low plasma levels of leptin) leading to disruption or blockade of reproductive functioning [25, 29, 52, 147].

We indeed showed FR to delay puberty onset and VO timing, concomitant with a decrease in the body fat content, plasma leptin levels and mean body temperature. However, a strong positive correlation between body fat content and plasma leptin levels remained present. Although absolute amounts of body fat increased in the course of the experiment, the relative fat content (% dry weight) remained constant both in control fed and in FR rats (figure 7B). This indicates absolute and not relative fat content might serve as a signal for puberty onset.

All these effects culminate in a postponement of puberty onset due to the FR treatment as shown in figure 6B.

It is not clear if peripheral leptin levels change in a meaningful pattern over the prepubertal period because the growth-related changes found in circulating leptin differ greatly among species, due to for instance the sampling conditions, the great differences between the current assays for leptin [88]; and of course the substantial difference in the physiological action of leptin between rodents and primates [8, 9]. Some studies showed that in man leptin levels are elevated around puberty and that central precocious puberty in girls seems to be associated with a modest increase in serum leptin levels [61]. However, in monkeys the circulating leptin did not increase during puberty [88, 151]. For example, studies that determined leptin levels at bi-monthly intervals or cross-sectionally in monkeys failed to identify a leptin surge that coincided with the onset of puberty [8, 9]. But in mice, serum leptin concentrations peak during early development [12], and also in rats a progressive rise in peripheral leptin has been reported during puberty [100].

If a leptin surge is associated with puberty, it may be easily missed because continuous blood sampling around puberty is not easily achieved; particularly in female rats as continuously sampling of blood may seriously disturb the ongoing physiological processes. We sampled three groups of prepubertal female rats every other day at three time points during the light phase using the tail nick method, which, as can be derived from figure 5B, did not perturb the ongoing puberty onset and the VO pattern was comparable to the other experiments (figure 1B and 6B).

We showed that body fat content is proportional to body weight accrual in normally fed rats as relative fat content dose not change (figure 7). Individual body fat content may be positively correlated with leptin plasma levels, they do not with average body fat content nor do leptin levels increase proportionally to average body fat content (figure 7 and 8). Hence we conclude that although findings indicate that leptin could indeed serve as a functional link between the nutritional status and the reproductive axis, its secretory rhythm rather than absolute levels may be vital in initiating puberty onset.

ACKNOWLEDGMENTS

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Chapter 3

Chronic leptin infusion advances, but immunoneutralization of leptin postpones puberty onset in female rats

Saeed Zeinoaldini, J.J.M. Swarts, B.J.M. Van de Heijning

Abstract

Leptin is considered a good candidate to link the nutritional status and the gonadotropic axis. To find out if leptin is the signal to trigger the onset of puberty in female rats, we infused leptin (icv and sc) or immunoneutralized leptin in prepubertal rats.
We centrally (icv) and peripherally (sc) infused leptin (1 µg/day). We observed a marginal advancement of median VO age in normally fed rats (26 vs. 27 d) but only if leptin was applied centrally. In contrast, both centrally and peripherally infused leptin advanced VO age compared to controls in food-restricted rats (30 *vs*. 35 and 31 *vs.* 41 d, respectively).
Next, we centrally (icv) administered leptin antiserum (0.6 µg/day) to prepubertal female rats. Puberty onset was clearly postponed in ad libitum and pair-fed leptin-antiserum-infused animals compared to controls (33 and 31 *vs*. 28 d).
Our data clearly show that the negative effects of food restriction on puberty onset are counteracted or even normalized by the infusion of leptin. In normally fed animals centrally but not peripherally infused leptin advances, whereas immunoneutralization of central leptin postpones puberty onset in prepubertal female rats. We therefore conclude that central leptin might be crucial for initiating puberty in female rats.

Introduction

Puberty is a complex process as it is the transition from the juvenile into the adult, reproductive life phase. More than on age, puberty onset depends on body size and weight, and the right metabolic cues. Numerous factors in the hypothalamus-pituitary-gonadal axis (HPG) axis are involved.

The nutritional status is clearly involved in sexual maturation, as puberty onset is postponed in case of insufficient or inadequate energy resources, and gonadotropin secretion is impaired in nutritionally restricted animals and humans. In general, a positive correlation is observed between body fat, fertility and endocrine function, and it is hypothesized that various metabolic cues monitoring the energy balance signal the nutritional status between body (fat mass) and some CNS areas.

The adipose tissue-derived hormone leptin is a good candidate for the 'link' between the nutritional status and the HPG-axis with regard to puberty onset [6, 25, 63, 73], although the time, the site and mechanism of action still have to be determined. One view is that leptin does not initiate puberty but acts as a permissive factor allowing pubertal maturation to proceed [63, 64]. An active involvement in the early events of puberty onset however, is supported by the notion that leptin triggers precocious vaginal opening in mice [6, 60, 76]. To trigger puberty, leptin must act centrally to induce luteinizing hormone (LH) release in immature animals. Leptin is indeed capable to stimulate LH release, not only in prepubertal but also in adult animals, at least partly due to a central action [76, 233]. Consequently, central infusion of leptin-antiserum suppressed LH and disrupted the estrous cycle in mature female rats [53]. Leptin treatment advanced sexual maturation not only in feed-restricted animals, but also in case of ad libitum access to the feed [6, 25, 63]. These data all suggest that leptin is a prerequisite for fertility, acting in the brain to affect GnRH and LH secretion; leptin may indeed serve as a physiological link between adipose tissue and reproductive functioning [64].

In this study we explored the role of leptin in the timing of sexual maturation in a model of puberty postponement due to energy restriction. If leptin were the signal for initiating the onset of puberty, it would trigger and advance puberty onset upon chronic intracerebroventricular (icv) and subcutaneous (sc) infusion of leptin in female rats on a control or energy-restricted diet. Likewise, central immunoneutralization (icv) of leptin would thus postpone puberty onset.

Materials and methods

Animal procedures

Wistar female rats, age 21 d and body weight (BW) 45 ± 2 g, were obtained from Harlan Co. (Horst, the Netherlands). Rats were housed in a room at 21.5ºC, 14/10 L/D regime, and with drinking water *ad libitum*. Time of lights on (L phase) varied, but is referred to as ZT=0, and all other procedures are relative to this time point. Starting on postnatal day 22, animals were fed (at ZT 14) a pelleted chow diet according to AIN guidelines either *ad libitum* or pair-fed to an age-matched control group.

Groups of animals were food-restricted (FR) and were put on a diet with 30% less energy and pair-fed (PF) to the controls. The low energy diet had the same vitamin and mineral content

and an equal amount of protein as the control food but 30% of the carbohydrate and fat was replaced by cellulose. The caloric content of the normal diet was 16 kJ/g and FR diet with 30% less energy contained about 10 kJ/g. This is referred to as `FR-treatment`. Body weight and food intake were measured once daily and vaginal opening (VO) was monitored two times per day at ZT 6 and 14. The experiment ended on postnatal day 42, animals that did not show VO on this day were designated a VO time of 42 d in the statistical analysis. To measure leptin levels, blood samples were taken under anesthesia at ZT 6 on several days during the experiment and on the day of VO via a tail-nick [87]. Plasma was frozen at -20ºC until assayed for leptin content.

To ensure a proper pheromonal milieu at least two adult male rats were housed in the same room as the females. Males were on a normal chow feed and water *ad libitum*. All animal experiments were approved by the Wageningen University Animal Ethical Committee.

Peptides and antisera

Rat-recombinant leptin for icv infusion was purchased from Sigma (Sigma-Aldrich, Inc, USA). Leptin was dissolved in 0.2 µm-filtered 15 mM HCl. Then 0.2 µm-filtered 7.5 mM NaOH was added to a pH of approximately 5.2. Rat-recombinant leptin for sc infusion was purchased from Pepro Tech (USA), it was dissolved in sterile MilliQ water. Normal goat IgG and goat anti-rat leptin-antiserum (Lep-AS) (antigen affinity purified polyclonal antibody) were purchased from Pepro. Tech. (USA). Both lyophilized compounds were dissolved in sterile MilliQ water and were further diluted with 0.1% BSA. All solutions were further diluted with 0.1% BSA.

Intracerebroventricular (icv) and subcutaneous (sc) infusion

Alzet® osmotic minipumps model 2002 (Durect Co., USA; figure 10A) which delivered 12 µl/day for 14 days were filled with either saline, leptin (adjusted to a daily delivery of 1 µg), normal goat IgG or Lep-AS (the later two adjusted to a daily delivery of 0.6 µg). The filled minipumps (figure 10A) were put in sterile saline at 37ºC for 24 h.

At postnatal day 23 all animals were fitted with a stainless steel cannula positioned according to stereotaxical coordination into the lateral ventricle of the brain via a hole drilled in the skull 1.5 mm lateral, 1 mm posterior to bregma, and the cannula was fixed at a depth of 4.0 mm from the skull surface. The minipumps were subsequently implanted subcutaneously and connected to the lateral ventricle cannula.

Surgery was performed under O_2 /N_2O (1:1) and Isoflurane (5%) anesthesia. Pre- and post-operatively, the rats received analgesia for two days by an oral dose of ibuprofen (1 mg/rat) and their health condition was frequently checked.

The experiment ended on postnatal day 42: all rats were anesthetized (see above) and the cannula position was checked by injecting icv dye (Evans Blue). Only results of rats with the cannula correctly positioned in the lateral ventricle of the brain were included.

In case of subcutaneous (sc) infusion no brain surgery was performed.

Experimental groups

Groups of rats were either normally fed the control diet *ad libitum* (AL), pair-fed (PF) to a control group, or groups of rats were pair-fed the FR diet.

The control and FR groups (n =10) were infused either centrally (C) or peripherally (Sc) with either saline (Sal), leptin (Lep) or leptin-antiserum (Lep-AS). Goat IgG served as a blank for Lep-AS (figure 10B). Treatment groups are designated by an abbreviation of their treatment (see legends of Tables 1-4).

Group numbers smaller than 10 were due to either death during surgery (4 rats) or exclusion because of incorrect cannula position (10 rats).

Radioimmunoassay (RIA)

To measure rat leptin levels, a commercial available kit was used (LINCO Research, USA). Samples from one experiment were assayed in one run. The intra-assay and inter-assay variations as determined using quality control results were 3.2 % and 4.5%, respectively.

Statistical analysis

The median and 95% confidence interval were calculated for VO, and means ± SEM were calculated for body weight and leptin levels. One way ANOVA was used to test the effects of food restriction and leptin infusion, ANOVA was posthoc followed by Duncan's test. Kaplan-Meier analysis was used to test the effects on VO time. $P < 0.05$ was considered significant.

Results

Central leptin infusion (icv)

VO age was delayed due to the FR-treatment (FRSal *vs.* CSal), but this delay was smaller when the FR-treatment was accompanied by a leptin infusion (FRSal *vs.* FRLep). Leptin infusion in normally fed rats (CLep) only marginally advanced VO time (Table 1 and figure 1).

	VO age (day)	**95% Conf.interval**	**VO weight (g)**	**VO leptin level (ng/ml)**	**n**
CSal	27	26.5 – 28.1	74.9 ± 2.9 [a]	1.67 ± 0.19 [a]	9
CLep	26	24.8 – 27.2	58.3 ± 0.3 [b]	<0.1	7*
FRSal	35 [a]	33.5 – 36.5	90.3 ± 6.6 [c]	0.50 ± 0.23	10
FRLep	30 [b]	28.7 – 31.3	67.1 ± 4.3 [ab]	0.41 ± 0.13	7

Table 1:

The effects of food restriction and/or infusion of leptin (icv) on vaginal opening (VO) age, VO weight and leptin level on day of VO; a, b, c, ab designate significant differences. * Plasma leptin levels were all below detection level. **CSal:** Control group, control food *ad libitum* and icv saline;

CLep: Control food pair-fed to the CSal group with icv leptin (1 µg/day);

FRSal: Icv saline and a diet with 30% less energy (pair-fed to CSal);

FRLep: Diet with 30% less energy (pair-fed to CSal) with icv leptin (1 µg/day).

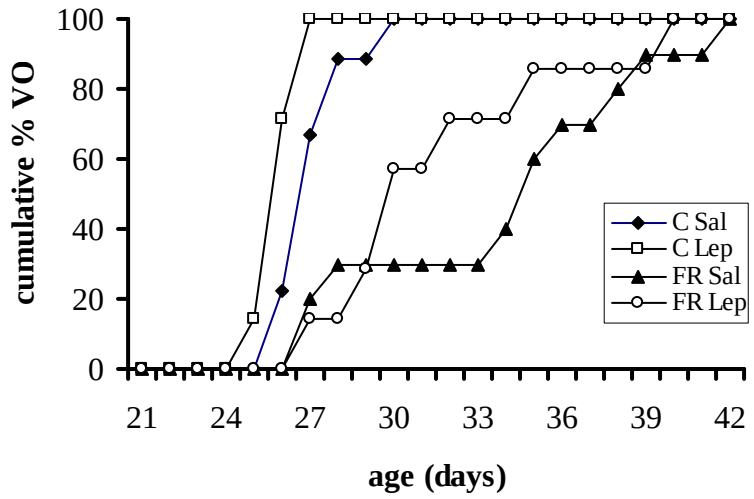


Figure 1: The cumulative percentage of animals showing vaginal opening (VO) in the experiment with icv leptin. See Table 1 for abbreviations.

From postnatal day 25 onward the body weight of the control group (CSal) was significantly higher than the other groups (figure 2A), and from postnatal day 31 onward the weight in the leptin-infused groups (CLep and FRLep) was significantly lower than in the FR saline-infused group (FRSal; $P<0.01$). The daily amount of consumed food of the control fed leptin-infused group (CLep) was significantly lower from postnatal day 24 onward ($P<0.01$; figure 2B). This was also true at some time points for the FR leptin-infused group (FRLep; $P<0.05$), but only when compared to the control group (CSal). The weight on VO day of the FRSal animals was significantly higher than in all other groups. Leptin treatment normalized VO weight in the FR group, but resulted in a decreased VO weight in the control fed rats (Table 1). Plasma leptin levels throughout the experiment were decreased compared to controls (Table 1 and figure 3), leptin levels in the control fed leptin-infused group (CLep) were hardly detectable. Leptin levels did not differ between the leptin-infused groups (i.e. CLep and FRLep) and the FR control group (FRSal) ($P<0.01$).

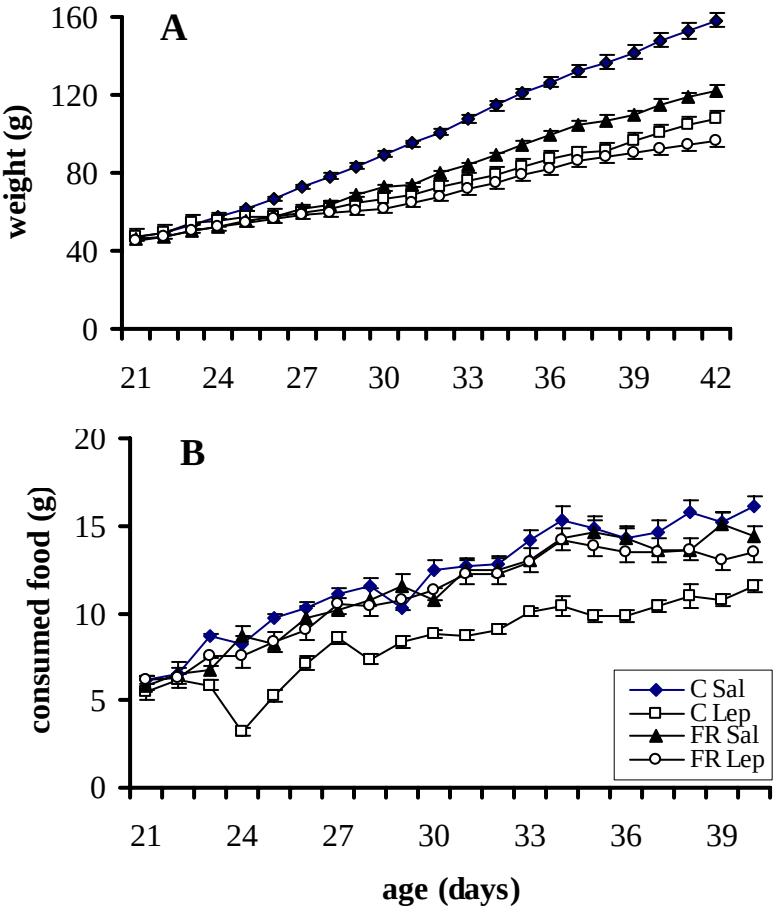


Figure 2:

A. The body weight in the experimental groups in the experiment with icv leptin;

B. The daily amount of consumed food in the experimental groups in the experiment with icv leptin.

See Table 1 for abbreviations.

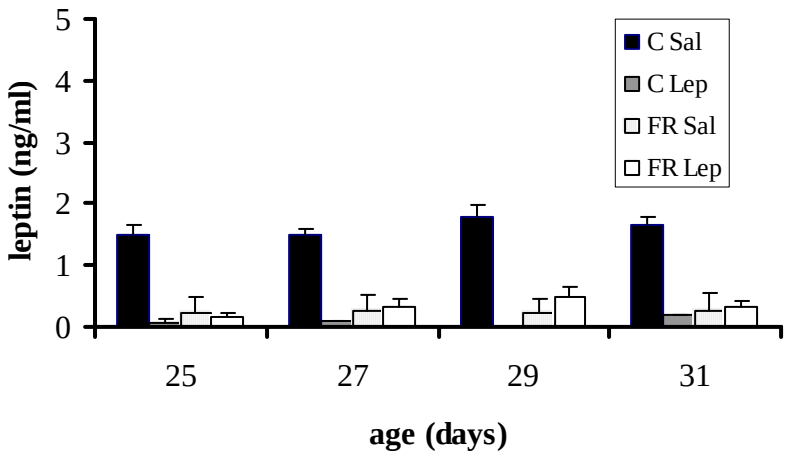


Figure 3: The plasma leptin levels in the experimental groups in the experiment with icv leptin. See Table 1 for abbreviations.

Peripheral leptin infusion (sc)

The FR-treatment caused 8 rats not to show VO yet on postnatal day 42 irrespective of the content of the sc infusion. Still, the FR-induced delay in VO age (FRScSal) was normalized by the sc-infused leptin (FRScLep), whereas in the normally fed rats (ScLep) VO age was not affected by the sc leptin infusion (Table 2 and figure 4).

Although daily food intake did not differ between the groups (figure 5B), a clear dichotomy became apparent from postnatal day 25 onward in the course of the body weight between the normally fed and FR treated rats (figure 5A), irrespective of the content of the sc infusion. The combining effects of leptin infusion on VO age and on the course of body weight, resulted in a higher weight on VO day in the FRScSal group only (Table 2). In ScLep animals the plasma leptin levels were significantly higher than controls from day 27 onward, including the day of VO ($P<0.05$). In contrast, in the FR animals the plasma leptin levels were significantly decreased throughout the experiment ($P<0.05$); but did not differ among the two FR groups (figure 6 and Table 2).

	VO age (day)	95% Conf.interval	VO weight (g)	VO leptin level (ng/ml)	n
ScSal	28	23.9 – 32.1	88.9 ± 5.6	1.60 ± 0.19	9
ScLep	29	27.6 – 30.4	85.8 ± 5.6	2.80 ± 0.40 [a]	10
FRScSal	41 [a]	38.2 – 43.8	107.4 ± 6.3 [a]	1.10 ± 0.10	10*
FRScLep	31	25.2 – 36.8	90.6 ± 9.3	1.50 ± 0.10	9 *

Table 2:

The effects of food restriction and/or infusion of leptin (sc) on vaginal opening (VO) age, weight and leptin level on VO; a designates significant difference.

* Five rats in the FRScSal and three in the FRScLep group did not show VO by day 42.

ScSal: Control group, control food *ad libitum* and sc saline;

ScLep: Control food pair-fed to the control group with sc leptin (1 µg/day);

FRScSal: Sc saline and a diet with 30% less energy (pair-fed to control);

FRScLep: Diet with 30% less energy (pair-fed to control) with sc leptin (1 µg/day).

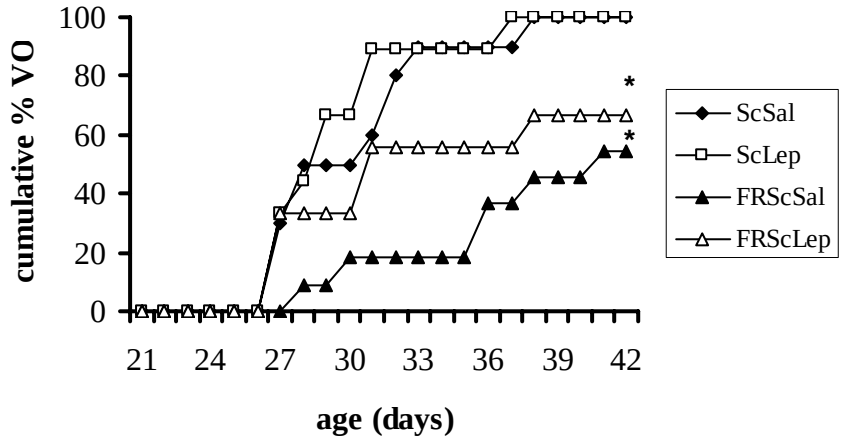


Figure 4: The cumulative percentage of animals showing vaginal opening (VO) in the experiment with sc leptin. See Table 2 for abbreviations.

***** Five rats in FRScSal and three in FRScLep group did not show VO by day 42.

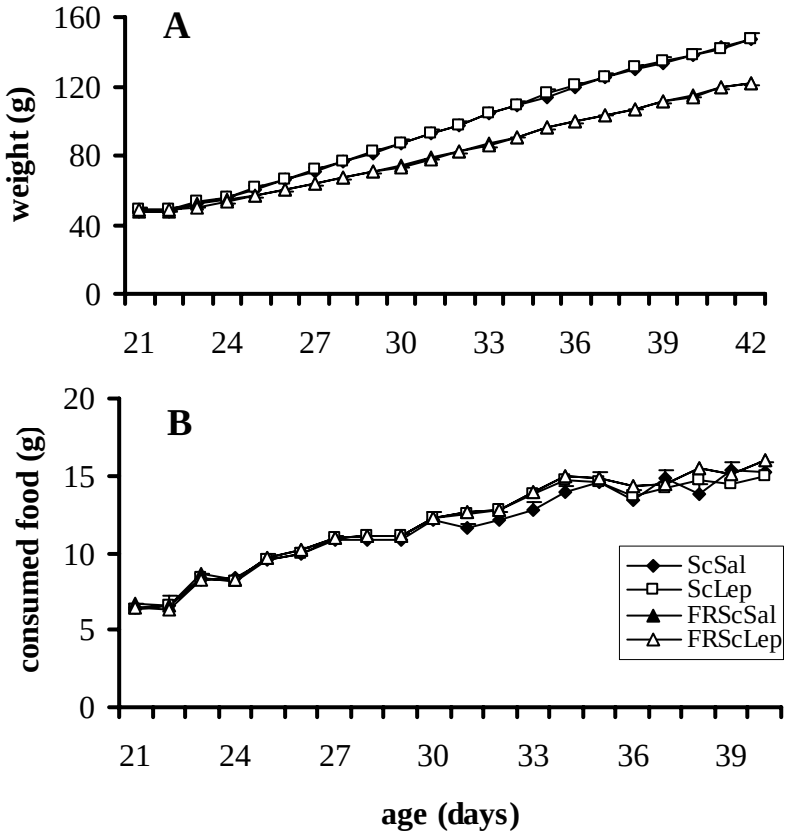


Figure 5:

A. The body weight in the experimental groups in the experiment with sc leptin;

B. The daily amount of consumed food in the experimental groups in the experiment with sc leptin.

See Table 2 for abbreviations.

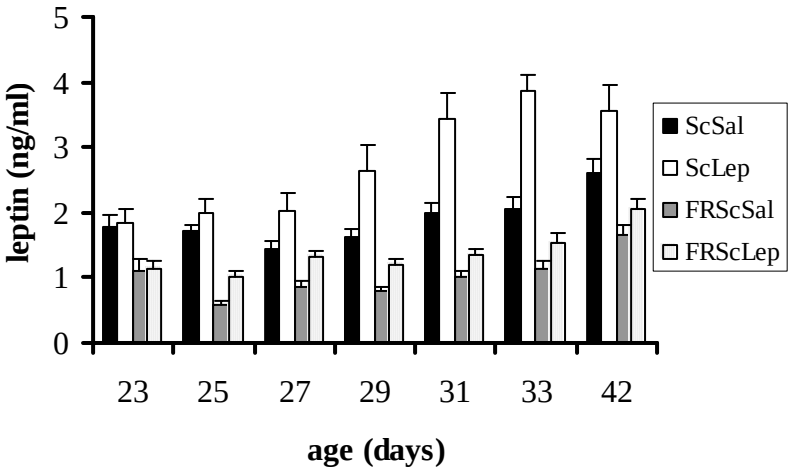


Figure 6: The plasma leptin levels in the experimental groups in the experiment with sc leptin. See Table 2 for abbreviations.

Central immunoneutralization of leptin (icv)

Compared to control animals (CBlank), age and the pattern of VO in the Lep-AS-infused groups (ALLep-AS and PFLep-AS) were postponed ($P<0.05$; figure 7 and Table 3). Daily food intake of the AL fed Lep-AS-infused animals (ALLep-AS) was higher from day 28 onward than the other two groups ($P<0.05$; figure 8B), but this did not result in a different body weight course (figure 8A). The delayed puberty onset occurred hence at a higher body weight in the two Lep-AS-infused groups (Table 3). Plasma leptin levels throughout the experiment did not differ significantly between the experimental groups (figure 9). However, in the pair-fed group (PFLep-AS) leptin levels became progressively lower, and differed on postnatal day 25 and 31 from the ad libitum fed groups (CBlank and CLep-AS).

	VO age (day)	95% Conf.interval	VO weight (g)	VO leptin level (ng/ml)	n
CBlank	28 [a]	25.4 – 30.6	83.4 ± 4.4 [a]	1.39 ± 0.18	7
AL Lep-AS	33	31.6 – 34.4	110.5 ± 4.4	1.68 ± 0.27	9
PF Lep-AS	31	29.1 – 33.9	101.3 ± 5.9	1.19 ± 0.22	9

Table 3: The effects of Lep-AS infusion on vaginal opening (VO) time, VO weight and plasma leptin levels at VO day; a designates significant difference.

CBlank: Control food *ad libitum* and icv normal goat-IgG (0.6 µg/day);

AL Lep-AS: Control food *ad libitum* and icv rat leptin antiserum (0.6 µg/day);

PF Lep-AS: Control diet pair-fed to CBlank, and icv rat leptin antiserum (0.6 µg/day).

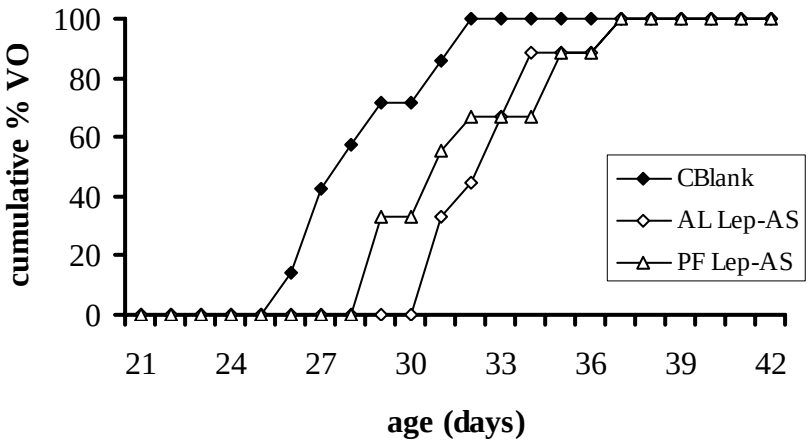


Figure 7: The cumulative percentage of animals showing vaginal opening in the experiment with icv Lep-AS. See Table 3 for the abbreviations.

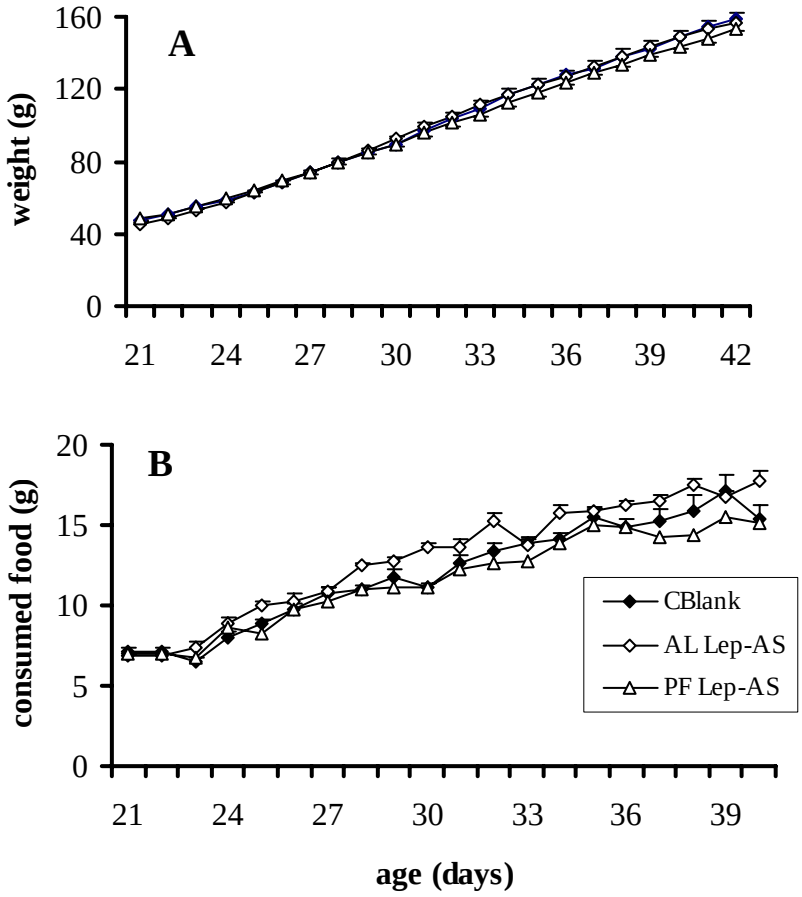


Figure 8:

A. The body weight in the experimental groups in the experiment with icv Lep-AS;

B. The daily amount of consumed food in the experimental groups in the experiment with icv Lep-AS.

See Table 3 for the abbreviations.

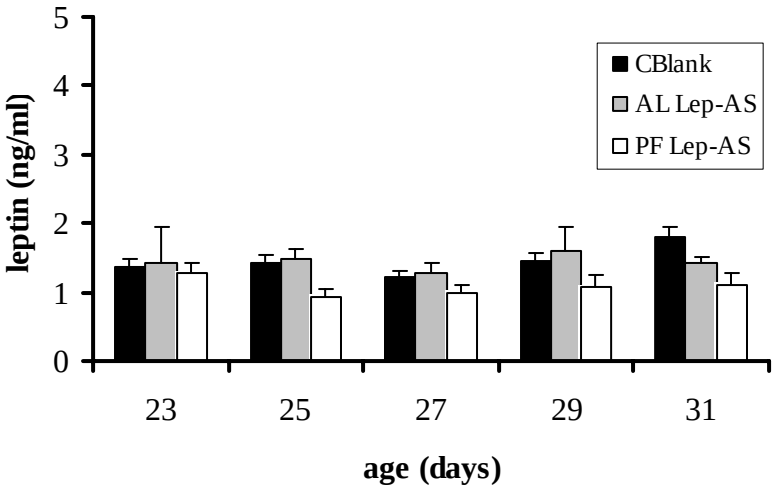
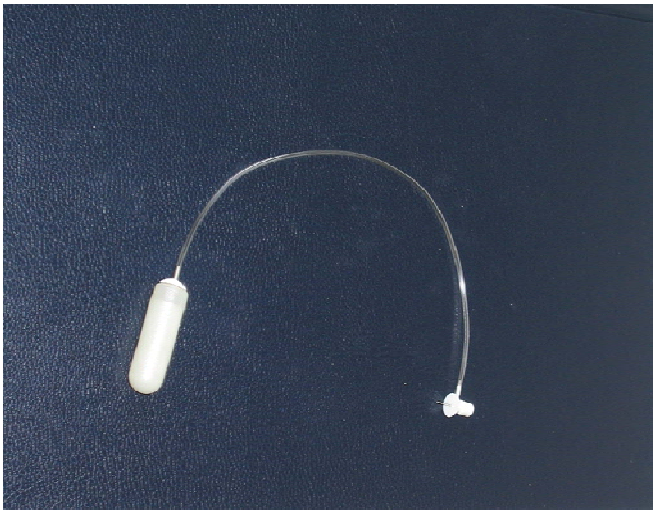


Figure 9: The plasma leptin levels in the experimental groups in the experiment with icv Lep-AS. See Table 3 for the abbreviations.

A.



B.

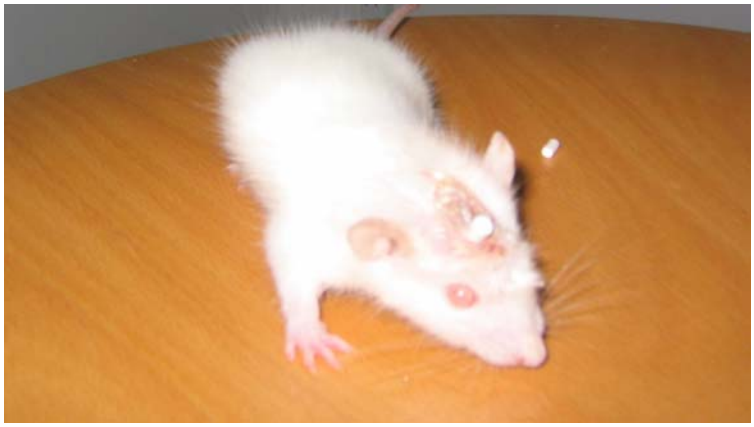


Figure 10: A filled osmotic minipumps (**A**) and a centrally-operated (icv) female rat including an osmotic minipumps (**B**).

Discussion

Puberty is characterized by the greatest sexual differentiation since fetal life and the most rapid rate of linear growth since infancy. It is the final stage of maturation of the hypothalamic-pituitary-gonadal (HPG) axis which is marked by changes in circulating level of gonadotropins and increased levels of sex steroids [5]. The increased gonadotropin secretion that promotes gonadal development during puberty is pituitary- driven by the enhanced activity of hypothalamic neurons producing gonadotropin releasing hormone (GnRH).

As leptin levels mirror the nutritional status, leptin is deemed a good candidate to link the nutritional status to the HPG-axis to trigger the onset of puberty [5, 8, 60]. Leptin receptors have been identified in the hypothalamus and on the gonadotropic cells of the anterior pituitary [81, 92, 161, 200]. Leptin directly and dose-dependently accelerates GnRH secretion (but not pulse amplitude) from arcuate nucleus (ARC) neurons both at the level of the cell bodies and at the axon terminals [140, 222, 223]. However, others find leptin to stimulate the GnRH-LH system in fasted animals only, and excess leptin to be without effect under well-fed conditions [73, 106, 108, 169, 224]. In the pituitary, leptin directly stimulates the release of LH and, to a lesser extent, FSH via nitric oxide synthesis [233].

Kennedy's initial observation that food restriction postpones puberty onset [127, 128] has been confirmed in many mammalian species [50, 79, 162]. Restriction of the daily food intake decreases body weight and plasma leptin levels in female rats [217] and inhibits the onset of puberty [63]. Likewise, genetically obese and leptin-deficient (*ob/ob*) mice are infertile, and show underdeveloped gonads with arrested gametogenesis, probably because of reduced hypothalamic GnRH content, low plasma gonadotropin and sex steroids levels [25, 60, 63]. Chronic leptin treatment (2 µg /g) restored fertility but reduced food intake and body weight in those animals. A critical plasma leptin level to trigger puberty was hypothesized [6].

We carried out a series of experiments on the effects of food restriction and infusion of leptin (icv and sc) and immunoneutralization of leptin (icv) on puberty onset in female rats. First, we used the FR model of delayed VO age, and centrally administered rat leptin to FR and control fed rats, and monitored puberty onset (i.e. VO age). The central administration of leptin was carried out by means of osmotic minipumps (figure 10A) which continuously infused leptin in the lateral brain ventricle for 14 d. Central leptin infusion not only restored the delay in puberty onset caused by food restriction (FRLep), but also advanced sexual maturation in the normally fed animals (CLep) (Table 1 and Figure 1). The leptin levels in the FR animals were again significantly lower than the controls, probably due to the lower amount of fat deposited to produce leptin. The plasma leptin levels in the pair-fed leptin-infused (CLep) animals were even below the detection limit of the assay which might be due to the negative feedback effects of the leptin infused in the brain. Moreover, plasma leptin levels did not differ between the saline- and leptin-infused groups, which indicates that centrally infused leptin did not cross the blood-brain-barrier or leak to the periphery. Gruaz et al. [100] also showed that the infusion of leptin in prepubertal female rats even with higher concentration (icv 10 µg /day) did not affect circulating plasma leptin levels. Consequently, the central leptin infusion might only indirectly interfere with the peripheral physiological processes and the observed puberty advancement in FR rats must be due to the centrally infused leptin.

We also peripherally (sc) infused rat leptin in another analogous experiment, again by means of osmotic minipumps. The sc infused leptin also restored the puberty onset postponement caused by FR (Table 2 and figure 4), and like the central infusion had a clear advancing effect on the pattern of VO, but was not able to completely restore the

postponement of VO time due to FR, as the VO pattern of FRScLep animals remained different from controls, some rats did not even show VO at 42 d. In spite of significantly higher plasma leptin levels in the ScLep compared to the control animals, both on VO day and over the experiment, the peripheral leptin infusion, in contrast to central infusion, had no advancing effect in ScLep animals (Table 2 and figure 4).

Leptin treatment (6.3 µg/g twice daily, ip) in female rats was reported to advance sexual maturation in pair-fed animals [63]; and, in female mice with ad libitum access to food [6, 25]. Furthermore, in prepubertal female rats severely food-restricted (36% of normal intake), a central infusion of leptin (icv 10 µg /day) restored the onset of puberty [100]. We centrally (icv) and peripherally (sc) infused a lower concentration of leptin (1 µg /day) that also advanced puberty onset in FR prepubertal female rats; probably because in our study the administration of leptin was continuous and chronic.

The body weight on VO day in the centrally and peripherally leptin-infused FR animals was significantly higher than in all other groups. As FR animals consumed a diet with 30% less energy they could not adequately store fat to produce enough plasma leptin for VO to occur at the same time as the control animals (Table 1 and 2). In the central leptin infusion experiment, the weight (on VO day and during the experiment) in the pair-fed leptin-infused (CLep) animals is significantly lower than the control animals (CSal), and also in both leptin-infused groups (CLep and FRLep) body weight is significantly lower than in the FR animals which reconfirms the effect of leptin in reducing body weight [5, 8, 9, 10, 60, 196]. In contrast, in case of peripherally infused leptin there is no significant difference between the body weight in the control animals and the ScLep group over the experiment. Also, the body weight in the FRScSal and FRScLep group is not significantly different, showing that peripheral infusion of leptin does not affect body weight (figure 5A).

In our study the food intake was decreased only by central but not peripheral leptin infusion (figure 2B and 5B): in the pair-fed leptin-infused animals (CLep) the daily amount of consumed food is significantly lower than the control group which reconfirms its frequently reported anorexic capacity. Leptin's anorexic effects might be mediated through the synthesis and release of neuropeptidergic effectors in specific areas of the brain [8, 9, 196]. One of the potent stimulators of feeding is NPY; NPY-containing neurons in the hypothalamus are located in the arcuate nucleus (ARC). An important interaction between leptin and NPY [8] is

suggested as leptin receptors and NPY are co-localized, enabling leptin to act on NPY synthesis and release to affect food intake [161, 191]. NPY synthesis in the ARC was indeed decreased by icv leptin injection, whereas leptin-induced inhibition of feeding was blocked by microinjection of NPY [133, 134]. Leptin administration thus removes the inhibitory action of NPY on pulsatile GnRH release [8, 9]. So, also in our study the central leptin infusion might have reduced food intake via NPY.

Finally, in an analogous experiment we centrally infused rat Lep-AS in female prepubertal rats on normal food *ad libitum* or pair-fed. The administration of Lep-AS by means of osmotic minipumps delayed the onset of puberty with some days (Table 3 and figure 7) but could not block it for a long time. Therefore, puberty onset was eventually initiated probably by other mechanisms in the brain. The central immunoneutralization of leptin did not change plasma leptin levels (Table 3 and figure 9). Indeed, Lep-AS infused in the brain is not likely to cross the blood-brain-barrier and leak to the periphery, to interfere with peripheral physiological processes involving leptin. The daily food intake was significantly increased in the *ad libitum* fed Lep-AS-infused animals, which confirms the negative effect of central leptin on food intake (the effect of leptin on NPY being removed).

There are some controversial reports stating leptin in some species is not involved in triggering puberty onset. In normally fed ovariectomised ewes, a three day icv infusion of leptin (20 µg /h) had no effect on the secretion of gonadotropins, prolactin, GH or cortisol, despite a highly significant reduction in the food intake that drove the animals into a negative energy balance [106]. Also, an increase in body fat and thus an increase in plasma leptin was not able to trigger puberty onset in female mice [49]. In another report, leptin treatment (6.3 µg/g twice daily, ip) could not completely reverse the impact a severe food restriction (70% of ad lib-feeding) had on the time of puberty onset in female rats [63]. It is likely that an extended period of exposure of the hypothalamus to a high level of leptin may result in the development of a central leptin resistance and a down regulation of central leptin receptors [176]. More importantly, these controversies in the leptin effects on puberty onset could be due to technical differences in the way and place of infusion, animal housing and lighting schedules, species differences, blood sampling techniques and feeding regimes.

We show a significant advancing effect of both centrally and peripherally infused leptin on VO time in prepubertal female rats. Also, the central immunoneutralization of leptin clearly

postponed puberty onset in these animals. So, we conclude that leptin is one of the crucial factors triggering puberty onset in female rats.

Grants

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Chapter 4

Bimodal effects of central infusion and immunoneutralization of growth hormone on the timing of puberty in the female rat

Saeed Zeinoaldini , J.J.M. Swarts, B.J.M. Van de Heijning

Abstract

Growth hormone (GH) affects reproductive function directly or indirectly (via IGF-I). Although GH levels increase during puberty, its role in puberty onset is still unclear. Recently, an interaction is suggested between leptin, as triggering factor of puberty, and GH. To evaluate the effects of GH on the timing of puberty as well as on leptin levels in prepubertal female rats, we centrally administered recombinant human GH (rhGH; 1 µg/day) to normally-fed or food-restricted (FR) rats. The infusion of rhGH postponed median puberty onset in normally fed rats compared to controls (34 vs. 27 d), but in FR animals rhGH significantly advanced median puberty onset (30 vs. 35 d). Plasma leptin levels were increased in rhGH-infused animals irrespective of their nutritional status.
Centrally infused rat-GH antiserum or somatostatin (Som) (0.6 µg/day) in pair-fed female rats advanced median puberty onset (25 vs. 28 d) but not in ad lib-fed animals. Plasma leptin levels were not significantly affected by the central infusions.
The effects of the centrally infused compounds appear to depend on the nutritional status imposed on the prepubertal animals. Furthermore, plasma leptin levels show no direct or predictive relation to the time of puberty onset. Although the data implicate an involvement of GH and leptin in puberty onset, the mechanism via which remains as yet unclear.

Introduction

Growth hormone (GH) secretion from the pituitary is regulated by the inhibiting neurohormone somatostatin (Som), and by the stimulating effects of GH-releasing hormone (GHRH). Apart from the pituitary, GH is also produced in the mammary gland, placenta, ovary and perhaps oviduct [115]. Hypophysial GH stimulates the production of hepatic insulin-like growth factor (IGF-I). Both GH (hypophysial or gonadal) and IGF-I (hepatic or locally produced) affect reproductive function: either directly or indirectly (via IGF-I) the functioning of mammary gland, ovary, uterus and/or oviduct is stimulated [115]. GH has numerous gonadotropic actions in female reproduction, reflecting endocrine roles of pituitary GH, complemented with autocrine or paracrine roles of GH locally produced within the reproductive tissue. GH directly stimulates steroidogenesis, gametogenesis, and gonadal

differentiation as well as gonadotropin secretion and responsiveness. In addition, GH may be permissive for gonadotropin-induced follicular development. In short, GH directly or indirectly (via IGF-I) regulates reproductive function at all levels of the hypothalamic-pituitary-gonadal (HPG) axis [115, 116].

The HPG axis becomes operational and active during puberty, the transient period from the juvenile to the adult life phase. GH levels increase during puberty and although the precise role in puberty onset is still uncertain, GH is believed to affect the time of puberty onset by activating the GnRH pulse generator [32]. The data in this regard, however, are highly controversial: the early administration (sc) of GH to normal, prepubertal, nonhuman primates or to children with a GH deficiency, did not consistently advance or delay the age of puberty onset, although the overall tempo of the pubertal processes was accelerated [126]. The implantation of GH in the median eminence, or the central infusion of GH in young female rats postponed puberty onset, reduced the ovarian LH receptor content and decreased the steroidogenic ovarian response [1, 2]. Hypersecretion of GH induced by infusing GHRH in humans (1 μg/kg), in contrast, accelerated puberty with normal prevailing concentrations of gonadotropins [126]. These studies suggest that changes in GH secretion do not provide direct cues for the normal time of puberty onset but as GH administration accelerates the puberty processes, GH still might play a permissive role in puberty and its onset.

The link of GH to puberty might be a metabolic cue in parallel to its gonadotropic actions, as GH is directly involved in lipid metabolism: GH stimulates lipolysis and increases lean body mass. Adiposity is regarded a trigger for puberty onset, and the adipose tissue-derived hormone leptin might hence be a good candidate for the link between the nutritional status and HPG-axis to trigger puberty onset [25, 63, 73]. Leptin treatment stimulates GH secretion, and pulsatile GH secretion is restored by leptin replacement, while immunoneutralization of leptin reduced GH secretion in rats [3]. Leptin receptors have been shown on GHRH and somatostatin neurons, providing strong anatomical evidence for a direct effect of leptin on the somatotropic axis [102]. Overall, these data suggest that there is an interaction between leptin and GH which levels both rise during puberty. Leptin might trigger puberty onset by stimulating GH release, or is the reverse true?

We studied the effects of GH on the timing of puberty and on leptin levels in prepubertal female rats, in the model of the delayed puberty onset due to food-restriction (FR). We

centrally administered recombinant human growth hormone (rhGH) to normally fed and FR prepubertal female rats. The infusion of rhGH postponed puberty onset in normally fed rats, but in the FR animals it significantly advanced puberty onset.

Next, we centrally infused anti-rat GH or somatostatin (Som) in normal ad lib-fed or pair-fed female rats. Central infusion of rat-GH antiserum or Som in the pair-fed animals advanced puberty onset but not in the ad lib-fed animals.

Materials and methods

Animals

Wistar female rats were obtained from Harlan Co. (Horst, the Netherlands) on postnatal day 21, weighing 45 ± 2 g. Rats were housed in a room at 21.5ºC, 14/10 L/D regime (lights on at 06:00 h = ZT 0), and with drinking water *ad libitum*. On postnatal day 22, animals were fed (at ZT 14) a pelleted chow diet according to AIN guidelines either ad libitum or pair-fed to an age-matched control group. Groups of animals were food-restricted (FR) and received a diet with 30% less energy. The 30% less energy diet had the same vitamin and mineral content and an equal amount of protein as the control food, but 30% of the carbohydrate and fat content was replaced by cellulose. The caloric content of the normal diet was 16 kJ/g and FR diet with 30% less energy contained 10 kJ/g. In the same room at least two adult male rats of the same strain were housed with food and water *ad libitum*, to provide a normal pheromonal environment.

All animal experiments were approved by the Wageningen University Animal Ethical Committee.

Antisera and peptide preparation

Recombinant human GH (rhGH; Zomacton), obtained from Ferring, (Hoofdorp, The Netherlands) dissolved 1 mg/ml in sterile saline (0.9% NaCl) was used. Normal goat IgG was purchased from Pepro. Tech. (USA), goat recombinant anti-rat GH (antigen affinity purified polyclonal antibody) was purchased from DSL (USA), and the somatostatin analog Octreotide was obtained from Novartis (Sandostatine®, Novartis Pharma B.V., The Netherlands). All

lyophilized compounds were dissolved in sterile MilliQ water or sterile saline (0.9% NaCl) and were further diluted with 0.1% BSA.

Intracerebroventricular (icv) surgery

Alzet osmotic minipumps model 2002 (Durect Co., USA) which delivered 12 µl/day for 14 days, were filled with either rhGH (adjusted to a daily delivery of 1 µg), normal goat IgG, goat anti-rat GH or somatostatin (all adjusted to a daily delivery of 0.6 µg). Saline-filled minipumps served as control. The filled minipumps were put in sterile saline at 37ºC for 24h. At postnatal day 23, all animals were fitted with a stainless steel cannula positioned according to stereotaxical coordinates into the lateral ventricle of the brain via a hole drilled in the skull 1.5 mm lateral, 1 mm posterior to bregma, and the cannula was fixed at a depth of 4.0 mm from the skull surface. The minipumps were subsequently implanted subcutaneously and connected to the lateral ventricle cannula.

Surgery was performed under O_2 /N_2O (1:1) and Isoflurane (5%) anesthesia. Pre- and post-operatively, the rats received analgesia for two days by an oral dose of Ibuprofen® (1 mg/rat) and their health condition was frequently checked.

Experimental groups

GH infusion study

Four groups of 10 prepubertal female rats (age 21 days) were individually housed: A control group got normal diet *ad libitum* and was infused icv saline (CSal). The group CGH got control food pair-fed to the CSal group and received icv rhGH (1 µg/day). The two other groups were food-restricted (FR) and were infused either saline (FRSal) or rhGH (FRGH).

GH antiserum (GH-AS) or somatostatin infusion study

Five groups of 10 prepubertal female rats (age 21 days) were individually housed: A control group got food *ad libitum* and icv normal goat- IgG (0.6 µg/day) as a blank (CBlank). Two groups of animals received an icv infusion of GH antiserum (0.6 µg/day) and either food ad libitum (GH-AS AL) or were pair-fed to CBlank (GH-AS PF).

Two other groups were fed likewise but were infused somatostatin (0.6 µg/day): Som AL and Som PF.

Experimental procedure

In both studies the consumed food and body weight were measured daily and vaginal opening (VO) was monitored two times per day at ZT 6 and at ZT 14. To determine the plasma leptin levels, blood samples were taken under anesthesia (see above) at ZT 6 via a tail-nick [87] on days (23), 25, 27, 29, 31 and on the day of VO. Plasma was frozen at -20ºC.

The experiment ended on postnatal day 42; animals that did not show VO at this age were designated a VO time of 42 days in the statistical analysis. On day 42 all rats were anesthetized (see above) and the cannula position was checked by injecting dye (Evans Blue). Only results of rats with the cannula positioned correctly in the lateral ventricle of the brain were included. In the GH infusion study, one rat from the control group (CSal) died during surgery and, two rats in the FRGH group were excluded because of incorrect cannula position. Also in the GH antiserum experiment, one rat from the CBlank group died during surgery and three rats were excluded because of wrong cannula position, one from the CBlank group and two rats from the GH-AS AL group.

Radioimmunoassay (RIA)

To measure rat leptin levels a commercially available kit was used (LINCO Research, USA). The intra- and inter-assay variations as determined using quality control results were 1.5% and 3.2%, respectively. No crossreactivity was found between rhGH and rat leptin.

Statistical analysis

The median was determined for the time of VO, and means ± SEM were calculated for body weight and leptin levels. One way ANOVA was used to test the effects of food restriction and GH/GH-AS infusion; posthoc ANOVA was followed by Duncan's or Student-Newman-Keuls' test (SNK). Kaplan-Meier analysis was used to test the effects on VO time. $P < 0.05$ was considered significant.

Results

GH infusion

VO age and pattern are significantly delayed in the food-restricted control group (FRSal), and to the same extent in the pair-fed rhGH-infused animals (CGH; Table 1 and figure 1). The time of VO in the FR rhGH-infused group (FRGH) is significantly advanced (and normalized) compared to the FR controls (FRSal), but not compared to the normally fed rhGH-infused group (CGH *vs.* FRGH).

	VO age (day)	**95% Conf.interval**	**VO weight (g)**	**VO leptin level (ng/ml)**	**n**
CSal	27 [a]	26.3 - 27.7	74.9 ± 2.9 [a]	1.67 ± 0.19 [a]	9
FRSal	35 [b]	33.5 - 36.5	90.3 ± 6.6 [b]	0.50 ± 0.23 [b]	10
CGH	34 [bc]	29.5 - 38.6	98.5 ± 7.9 [c]	8.29 ± 1.35 [c]	10*
FRGH	30 [ac]	25.8 - 34.2	79.5 ± 7.1 [ab]	1.85 ± 0.49 [a]	8 *

Table 1:

The effects of rhGH infusion and food restriction on vaginal opening (VO) age, body weight and leptin level on the day of VO; a, b, c, designate significant difference;

* Two rats in each group did not show VO by postnatal day 42.

CSal: Control group, control food (ad libitum) and icv saline;

FRSal: Icv saline and a diet with 30% less energy (pair-fed to CSal);

CGH: Control food (pair-fed to CSal) and icv rhGH (1 µg/day);

FRGH: Diet with 30% less energy (pair-fed to CSal) and icv rhGH (1 µg/day).

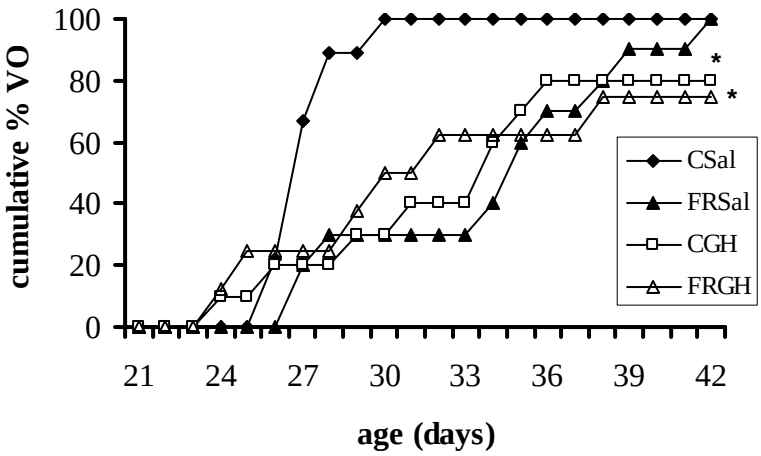

Figure 1: The cumulative percentage of animals showing vaginal opening (VO) in rhGH-infusion experiment, ***** Two rats in each group did not show VO by postnatal day 42; abbreviations as in Table 1.

The weight on VO day in all treatment groups is higher than controls (CSal). As was the case with VO time, also the weight on VO day in the FR rhGH-infused group (FRGH) was back to control values (CSal).

Food restriction decreases plasma leptin levels (CSal *vs.* FRSal), which are gradually normalized upon rhGH infusion during the FR-treatment (CSal *vs.* FRGH), but significantly higher at all time points in the pair-fed rhGH-infused animals on control food (CGH; Table 1 and figure 3).

The delay in VO pattern due to FR-treatment is only partially restored upon rhGH infusion (FRSal *vs.* FRGH), as 2 of the 8 animals in the FRGH group did not yet show VO at postnatal day 42 when the experiment ended. Also in the CGH group 2 animals did not yet show VO at day 42.

In spite of an equal daily food intake (figure 2B), the body weight (BW) is negatively affected by the FR-treatment, and in addition by the icv infusion of rhGH: From day 30 the BW of the control animals (CSal) is significantly higher than all other groups and the BW of the FR rhGH-infused group (FRGH) is significantly lower than its control group (FRSal).

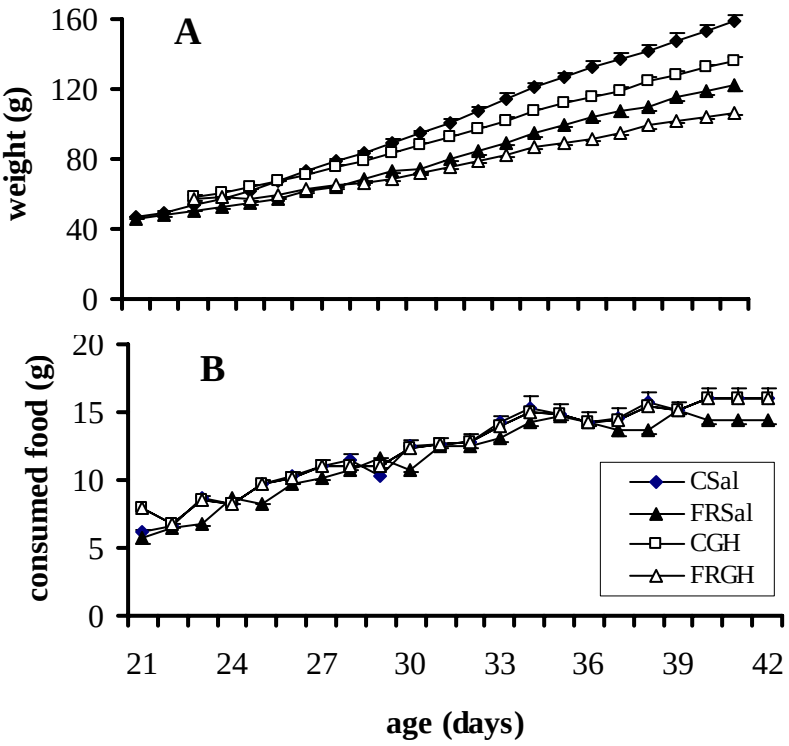

Figure 2:

A. The body weight course in the GH-infusion experiment;

B. The daily amount of consumed food in the GH-infusion experiment; abbreviations as in Table 1.

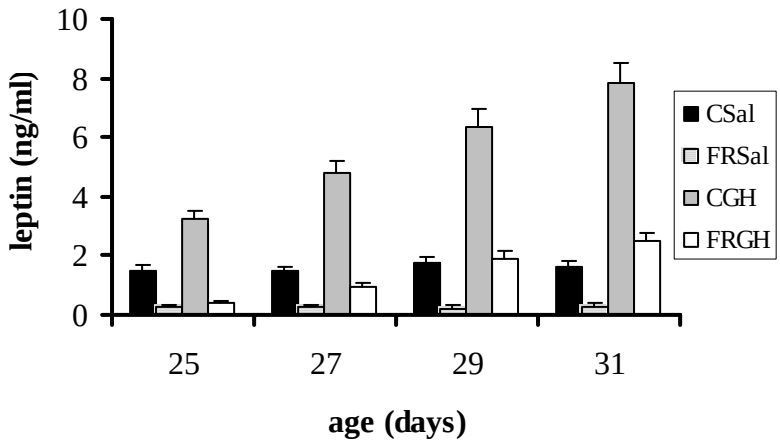

Figure 3: The peripubertal plasma leptin levels at ZT 6 in the GH-infusion experiment; abbreviations as in Table 1.

GH-AS or somatostatin infusion

Only infusion of GH-AS to pair-fed animals (GH-AS PF) significantly advanced puberty onset resulting in a lower BW on VO day (Table 2 and figure 4). No effect was found of the somatostatin infusion (Table 2 and figure 6).

Plasma leptin levels did not differ significantly among the control group (CBlank) and any treatment group (Table 2 and figure 8).

Central infusion of GH-AS or somatostatin did not affect body weight: From an initial weight of about 45 g animals grew to about 150 -160 g at postnatal day 42 in all groups (figure 5A and 7A).

The daily food intake was not affected by the somatostatin infusion (figure 7B), but was significantly increased due to the infusion of GH-AS in the ad lib-fed animals (GH-AS AL).

	VO age (day)	95% Conf.interval	VO weight (g)	VO leptin level (ng/ml)	n
CBlank	28	25.4 – 30.3	83.4 ± 4.1	1.39 ± 0.18	8
GH-AS AL	28	25.2 – 30.8	85.9 ± 4.4	1.40 ± 0.17	8
GH-AS PF	25 [a]	24.2 – 27.7	72.0 ± 2.6 [a]	1.40 ± 0.22	10
Som AL	28	26.5 – 29.5	85.6 ± 3.2	1.95 ± 0.26	10
Som PF	27	25.5 – 28.5	76.9 ± 2.9	1.64 ± 0.08	10

Table 2:

The effects of GH-AS or somatostatin infusion on vaginal opening (VO) age, body weight and leptin level on the day of VO; a, designates significant difference.

CBlank: Control food (ad libitum) and icv normal goat IgG (0.6 µg/day);

GH-AS AL: Control food (ad libitum) and icv GH-AS (0.6 µg/day);

GH-AS PF: Control food (pair-fed to CBlank) and icv GH-AS (0.6 µg/day);

Som AL: Control food (ad libitum) and icv somatostatin (0.6 µg/day);

Som PF: Control food (pair-fed to CBlank) and icv somatostatin (0.6 µg/day).

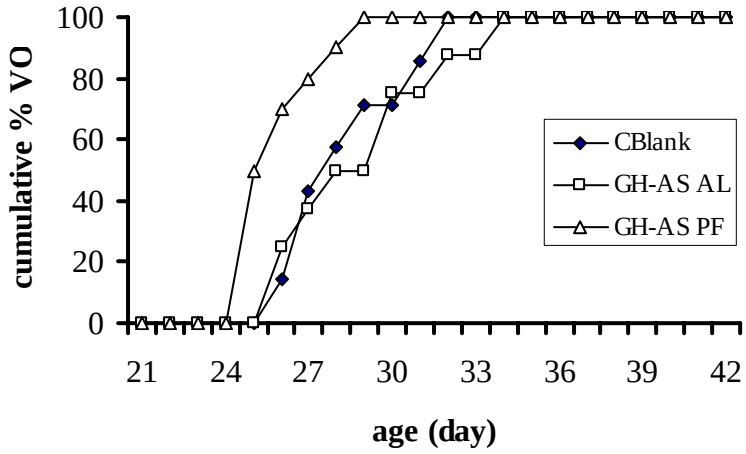

Figure 4: The cumulative percentage of animals showing vaginal opening (VO) in the GH-AS-infusion experiment; abbreviations as in Table 2.

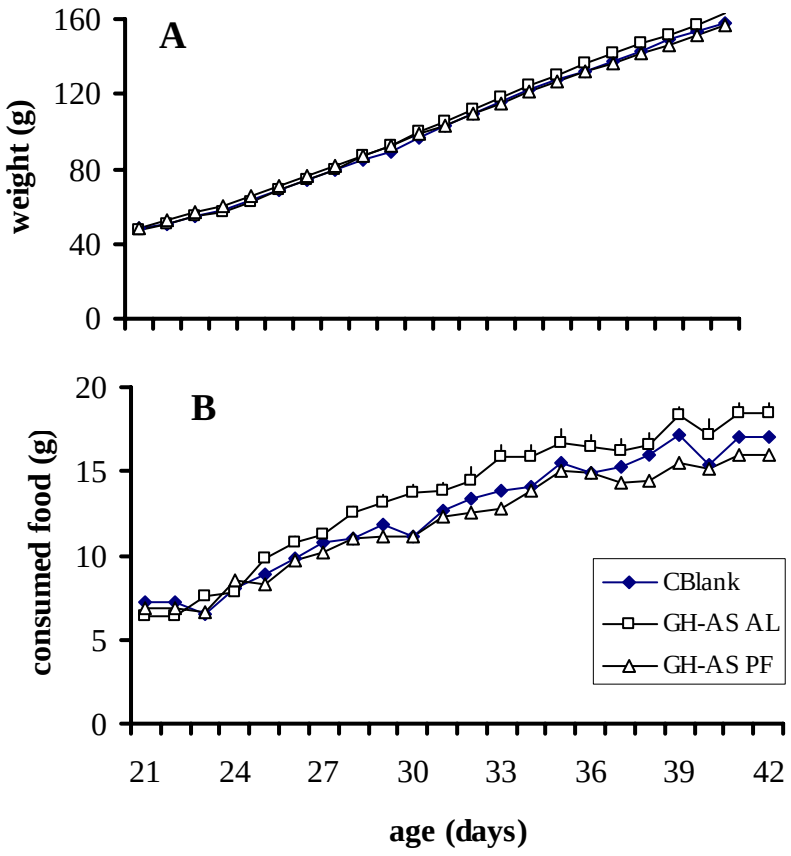

Figure 5:

A. The body weight course in the in the GH-AS-infusion experiment;

B. The daily amount of consumed food in the GH-AS-infusion experiment; abbreviations as in Table 2.

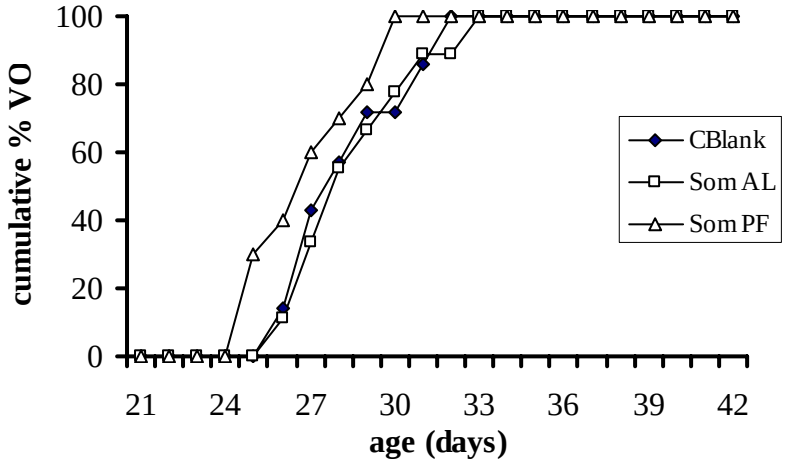

Figure 6: The cumulative percentage of animals showing vaginal opening (VO) in the somatostatin-infusion experiment; abbreviations as in Table 2.

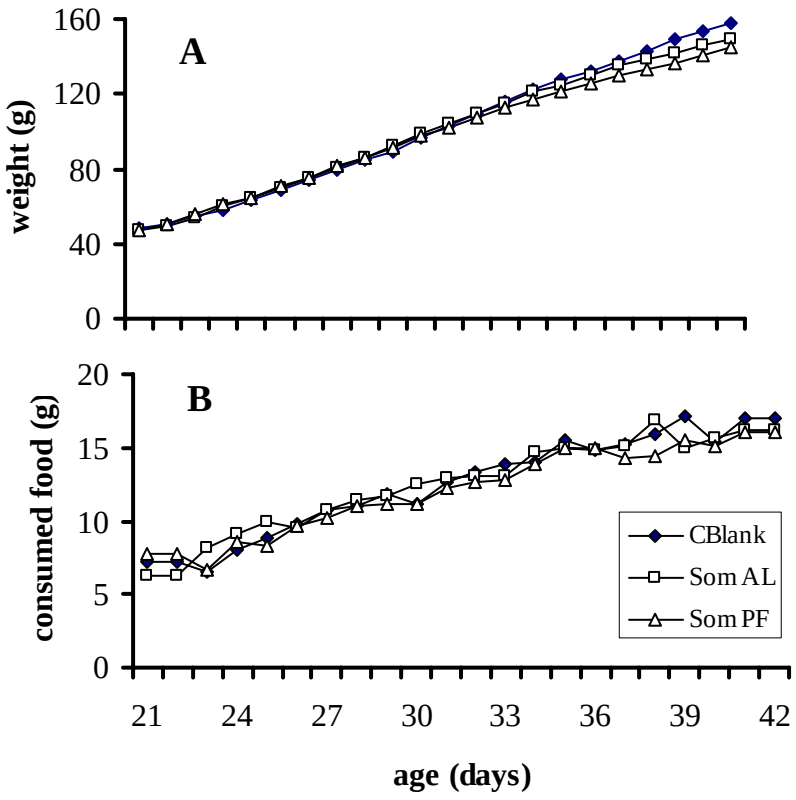

Figure 7:

A. The body weight course in the in the somatostatin-infusion experiment;

B. The daily amount of consumed food in the somatostatin-infusion experiment; abbreviations as in Table 2.

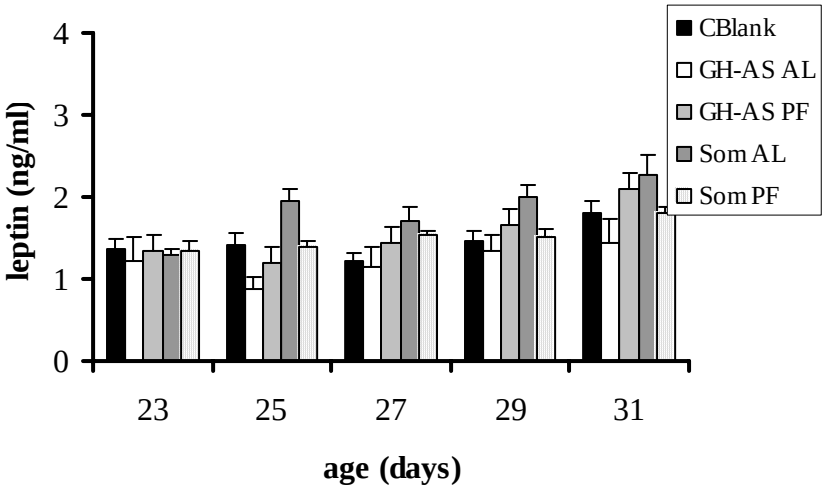

Figure 8: The plasma leptin levels at ZT 6 in the GH-AS-infusion experiment and the somatostatin-infusion experiment; abbreviations as in Table 2.

Discussion

In order to further elucidate the role of GH in puberty onset we manipulated the central levels of GH by infusing either recombinant human growth hormone (rhGH), rat- GH antiserum (GH-AS) or somatostatin. We used the model of the FR rat to delay puberty onset as also described in detail elsewhere and we monitored puberty onset by scoring the moment of vaginal opening (VO) [50, 79, 162, 217, this book Chapter 2]. Indeed, puberty onset was delayed in the FR group (CSal vs. FRSal) and plasma leptin levels were decreased (Table 1 and figure 1).

We centrally administered rhGH to pair-fed animals. The central administration was by means of osmotic minipumps which continuously for 14 d infused rhGH in the lateral ventricle. The infusion of rhGH postponed puberty onset in pair-fed animals on normal food (CGH) but advanced puberty onset in the FR animals (FRGH) (figure 1 and Table 1). Plasma leptin levels in the FRGH group were normalized and significantly higher, both on VO day and during the experiment, than in the FR control group (FRSal). Central leptin infusion in FR female rats normalized and restored puberty onset [100, Chapter 3]. Thus, in the FR rats infused with rhGH (FRGH), the restored time of puberty onset complies with and might be due to the normalized leptin levels (figure 3). On the other hand, the plasma leptin levels in

the pair-fed rhGH-infused animals (CGH) were five times higher than in control animals, rose in time, and were significantly higher than all other groups (Table 1 and figure 3). But despite these high plasma leptin levels, infusion of rhGH delayed puberty onset (CGH vs. CSal). Note that the high plasma leptin levels detected are not due to crossreactivity of centrally infused rhGH that possibly might have leaked to the periphery.

It has been shown that peripheral leptin might cross the blood-brain-barrier (BBB) and enter the brain by a saturable transport system employing the short form of the leptin receptor (LepRa) [21-24, see also Chapter one]. Such a saturable transport system provides a mechanism by which plasma leptin might exert an effect in the brain [21-24]. The hypothalamus, relatively leaky to leptin from the periphery, contains the arcuate nucleus (Arc), where leptin is thought to inhibit feeding by inhibiting NPY neurons and to promote reproductive processes by stimulating GnRH terminals [181, 226].

However, a prolonged exposure of the hypothalamus to (exogenously infused) leptin may cause the development of a central leptin resistance [181]. A direct impairment of leptin transport across the BBB (up to 76% less efficient) has been shown to be the cause of obesity in rats, mice and humans [20]. Also in obese individuals, it has been reported that very high plasma leptin concentrations are also associated with impaired reproductive function due to leptin resistance [211], and furthermore hyperleptinemia is shown to be negatively correlated with gonadotropin secretion in girls [44]. Leptin resistance might thus be one possibility why in the normal diet pair-fed animals (CGH) the very high levels of plasma leptin were not able to induce puberty onset in this group, and a delay was observed. In the FR GH-infused rats (FRGH) the more modestly increased plasma leptin levels advanced time of VO possibly due to a restored leptin transport across the BBB.

Besides a leptin resistance, it is possible that the central infusion of rhGH modified the secretion of somatostatin (Som) in the hypothalamus. Som-neurons are widely distributed throughout the brain [187]. Som cells located in the periventricular nucleus of the hypothalamus (PeVN) projecting to the median eminence (ME) are mainly responsible for the regulation of pituitary GH secretion [39]. In animals overexpressing GH, the amount of Som mRNA and the number of Som neurons in the PeVN was increased; while, in GH-deficient animals the amount of Som mRNA and the number of Som neurons was decreased [117]. Also, the distribution of GnRH neurons in several hypothalamic areas was changed in animals

overexpressing GH [195]. An interaction might exist between Som neurons and the GnRH system as recently it was shown that icv infusion of a Som analog caused a significant attenuation of the estradiol-induced LH surge in adult female rats partly by reducing GnRH cell activation [216]. It is, therefore, possible that also in our study the central infusion of rhGH, might stimulate Som neurons and increase Som levels in the hypothalamus and thus indirectly modulate the GnRH/LH axis resulting in a postponed puberty onset in CGH animals.

Apart from food-restriction also infusion of rhGH significantly reduced BW accrual over the experiment (figure 2A), in spite of an equal daily food intake (figure 2B). GH promotes lipolysis and lean BW gain. Hence growth and weight increase is the predominantly the result of lean mass deposit, and fat deposit is minimal. We did not determine whole body fat content but predict these levels to be low in the 'skinny' FR groups. As the FR diet contained the same amount of proteins as the control diet but 30% less carbohydrate and fat, replaced by cellulose, bulk food intake of the animals was also equal (figure 2B). The very high prevailing plasma leptin levels in the GH-infused animals apparently did not affect appetite and satiety. The control GH-infused (CGH) animals have a lower BW (and hence less adipose tissue) but a higher plasma leptin level, indicating that the centrally infused GH induces leptin release. But by what mechanism GH causes the high plasma leptin levels is as yet unclear.

In an analogous and complementary experiment, we tried to block the action of central endogenous GH by centrally infusing GH-AS or somatostatin in normal ad lib-fed or pair-fed female rats. Both treatments are supposed to decrease central GH levels. In order to detect any anti-appetite or anti-satiety effects of the infused components, antiserum against rat GH or the Som analogue Octreotide was infused in animals pair-fed (PF) to the controls and in animals with ad libitum access (AL) to the food.

Central infusion of GH-AS or somatostatin advanced puberty onset in the pair-fed animals only (Table 2, figures 4 and 6), in congruence with the results of central infusion of rhGH which also advanced puberty onset in FR animals only (FRGH) but not in normally fed animals. The observed advancement in time of puberty onset was not accompanied by significant differences in plasma leptin levels over the experiment (Table 2 and figure 8), although plasma leptin levels in the Som-infused groups were somewhat elevated. Indeed, based on our findings with GH infusion one would expect decreased plasma leptin levels and

a postponement of puberty onset by infusing GH-AS or Som. In contrast, we found no effect or an (slight) advancement of puberty onset accompanied by no or marginally increased leptin plasma levels.

Also, central infusion of GH-AS or somatostatin did not induce changes in body weight in these animals (figure 5A and 7A). VO weight was lower in both PF groups but reached significance in the GH-AS infused group only and not in the Som-infused animals (Table 2). Immunoneutralization of GH resulted in a higher food intake in the AL group (figure 5B). Hence, the PF group can be regarded as marginally food restricted. This somehow caused an advanced puberty onset and a lower VO weight (Table 2 and figure 4), although leptin levels were similar to the control group (CBlank, Table 2 and figure 8).

The reports on the effects of GH on puberty onset in different species are highly controversial: the central infusion of GH in female rats reduced the ovarian LH receptor content, decreased the steroidogenic ovarian response and postponed puberty onset [1, 2]. Infusion of GH-AS in female rats decreased the GnRH-mediated LH secretion, suggesting that GH might also facilitate GnRH actions on LH secretion. Similarly, puberty was advanced in transgenic female mice that overexpress GH [55].

GH treatment in children with a GH deficiency also delayed the age of puberty onset, or had no effect [126]. Injections (sc) of GH failed to affect the age of puberty in beef and dairy heifers [55] and in sheep [207, 208], suggesting that in these species changes in GH secretion do not provide direct clues for the normal time of puberty onset [207, 208].

We show that the infusion of rhGH in the FR animals (FRGH) significantly advanced puberty onset, indicating GH might stimulate puberty onset (by increasing circulating leptin levels) but only when animals are challenged with suboptimal conditions such as food restriction.

The effects of GH infusion on plasma leptin levels also vary among species: in rats injection of GH (4 mg/kg/d) decreased plasma leptin levels [150], but GH infusion had no effect on leptin expression in hypophysectomised rats [40]; recombinant porcine GH (4 mg/day, im) reduced serum leptin levels in castrated male pigs [231]; GH treatment of human patients generally decreased plasma leptin levels [78, 184], or leptin was inversely related to peak GH release [102]; in mice GH infusion (0.3 or 0.6 IU/animal) changed neither plasma leptin levels nor leptin gene expression in adipocytes [141]. In contrast, treatment of GH-deficient human patients with rhGH (0.2 IU/kg/d, sc) increased plasma leptin levels [85, 118].

It is not clear whether these discrepancies result from differences in the duration and intensity of GH treatment or exposure, or arise from species and genotype-specific responses [144]. There is, therefore, little evidence that GH has a direct effect on leptin expression or secretion.

We found plasma leptin levels in rhGH-infused animals (CGH) to be five times higher than control animals and to increase as GH infusion proceeds. What is the source of this leptin? It is now well-established that leptin is mainly produced by white adipose tissue. Central infusion of rhGH might activate some indirect and unknown (neurotransmitters or neural) pathway to affect adipose tissue to produce the high level of plasma leptin in both normally fed and FR animals (CGH and FRGH). Changes in leptin upon treatment with GH, independent of fat mass and without any changes in fat content have been previously reported in children, but gave no clue with regard to the mechanism behind it [71].

In summary, the central infusion of rhGH advanced puberty onset in FR female rats but not in the normal pair-fed group; also, central infusion of GH-AS or somatostatin slightly advanced puberty in the pair-fed animals. We assume that leptin is involved in these effects, although the mechanism via which is as yet unclear.

ACKNOWLEDGMENTS

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Chapter 5

Exogenous but not endogenous insulin-like growth factor I (IGF-I) postpones puberty onset in the female rat

Saeed Zeinoaldini, J.J.M. Swarts, B.J.M. Van de Heijning

Abstract

Due to its actions on and presence in GnRH neurons, central but also peripherally-derived IGF-I is suggested to play a role in the initiation of puberty. IGF-I directly affects GnRH synthesis and release, and has been shown to be involved in the processes leading to puberty initiation. Possible intermediates in these effects of IGF-I on puberty might be the adiposity signaling hormone leptin and growth hormone (GH). These three proteins might act individually or synergistally to initiate puberty.

To investigate the role of IGF-I in this possible cascade of effects, we studied the effects on puberty onset and plasma leptin levels of administration or immunoneutralization of IGF-I in prepubertal female rats by centrally and chronically infusing either IGF-I or IGF-antiserum.

Central infusion of IGF-I significantly delayed puberty onset in the ad libitum fed state only and not in 30% feed restricted (FR) animals. FR-treatment decreased plasma leptin levels which were normalized in FR-treated animals infused with IGF-I. Plasma leptin levels in control fed animals were not affected by the IGF-I infusion.

Immunoneutralization of endogenous IGF-I by centrally infused IGF-antiserum had no effect on the time of puberty onset, but enhanced prevailing plasma leptin levels, particularly in animals pair-fed to the controls.

Centrally added IGF-I postpones puberty onset to the same extend as FR treatment does, and does so irrespective of prevailing plasma leptin levels. Centrally present endogenous IGF-I does not seem to be involved in puberty onset although it attenuates peripheral leptin levels.

Introduction

Gonadotropin-releasing hormone (GnRH) is the key regulator of the reproductive system, directly regulating the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) which both play a crucial role in the initiation of reproduction, i.e. in puberty onset. Biosynthesis and release of GnRH are under complex excitatory and inhibitory control by a number of neurotransmitters and paracrine factors, among which possibly also metabolic signals of peripheral origin. A number of peripherally originating substances are likely candidates as they are able to centrally influence reproductive processes. Among them is insulin-like growth factor I (IGF-I), a peripheral mediator mainly produced and released by

the liver induced by growth hormone (GH). In addition, IGF-I is also synthesized within the hypothalamus and the pituitary gland [109].

IGF-I is suggested to play a role in the initiation of puberty: The majority of GnRH cell bodies are immunopositive for IGF-I, especially around puberty when plasma levels of IGF-I are increased due to an elevated hepatic IGF-I gene expression and production induced by high GH levels [110, 163, 178, 227]. In prepubertal rats IGF-I of peripheral origin directly acts on GnRH neurons and affects GnRH synthesis and release [74, 109,178]. Absence of IGF-I secretion delayed normal maturation in male mice [56], whereas ventricular infusion (icv) of IGF-I accelerated the onset of puberty in juvenile female rats, and was shown to increase serum LH levels during early and late proestrus and during estrus [110]. This stimulated LH release due to IGF-I, could be blocked by immunoneutralization of GnRH [110].

IGF-I receptors are widely expressed in the central nervous system, e.g. in the hypothalamus [237]. Also the median eminence (ME), being the target area for the GnRH nerve terminals, contains high concentrations of the IGF-I receptor and might hence be an important site of action for (peripherally or centrally derived) IGF-I to stimulate GnRH/LH secretion [77, 109, 110, 163, 178].

In conclusion, IGF-I seems to be a signal capable of activating the GnRH system at the time of puberty; IGF-I can modify the GnRH synthesis at the hypothalamic level as well as affect the GnRH action on the pituitary gonadotrophs.

Apart from GH/IGF-I, also an increase in nocturnal leptin levels precedes the rise in nocturnal pulsatile LH secretion heralding the onset of puberty [206]. GH treatment increases leptin mRNA abundance *in vitro*, but only in animals exhibiting a positive IGF-I response to GH; GH and IGF-I alone did not affect leptin secretion [114]. And after 3 days of GH treatment (sc) there is not only a significant up-regulation of IGF-I transcription in adipose tissue but also a significant increase in leptin mRNA abundance [114]. Hence, the actions of GH, IGF-I and leptin are amplified mutually as they control growth and the accrual in muscle mass. These findings suggest a synergistic role for leptin and the GH/IGF-I axis in regulating the pubertal initiation of the pulsatile GnRH/LH secretion.

Some studies, however, report no effect of IGF-I infusion on puberty onset in female rats [99], or even show an inhibitory action of IGF-I on GnRH secretion [43]. Whether IGF-I plays an inhibitory or stimulatory role on GnRH secretion remains unknown [110].

In order to clarify the effects of IGF-I on puberty onset and to find out the role of leptin and the GH/IGF-I axis in regulating the time of puberty onset, in prepubertal female rats either recombinant human IGF-I (rhIGF-I) or rat-IGF-I antibody was infused centrally and chronically. Time of puberty onset was monitored by scoring the moment of vaginal opening (VO). At various time points blood samples were taken to determine plasma leptin levels.

Materials and methods

Animals

Wistar female rats were obtained on postnatal day 21 weighing 45 ± 2 g from Harlan Co. (Horst, the Netherlands). Rats were housed in a room at 21.5º C, 14/10 L/D regime (lights on at 06:00 h = ZT 0), and with drinking water *ad libitum*. Two adult male rats also were housed with female rats. On postnatal day 22, animals were fed (at ZT 14) a pelletd chow diet according to AIN guidelines either ad libitum or pair-fed to an age-matched control group. Groups of animals were food-restricted (FR) and received a diet with 30% less energy. The 30% less energy diet had the same vitamin and mineral content and an equal amount of protein as the control food, but 30% of the carbohydrate and fat content was replaced by cellulose. The caloric content of the normal diet was 16 kJ/g and FR diet with 30% less energy contained 10 kJ/g. In the same room at least two adult male rats of the same strain were housed with food and water *ad libitum*, to provide normal pheromonal environment. All animal experiments were approved by the Wageningen University Animal Ethical Committee.

Antisera and peptide preparation

Recombinant human IGF-I (rhIGF-I), purchased from DSL (USA) was dissolved in acetic acid (10 mM) and was further diluted with 0.1% BSA. Normal goat IgG was purchased from Pepro. Tech. (USA); goat rat-IGF-I antiserum (IGF-AS) (antigen affinity purified polyclonal antibody) was purchased from DSL (USA). All lyophilized antisera were dissolved in sterile MilliQ water and then further diluted with 0.1% BSA.

Intracerebroventricular (icv) surgery

Alzet osmotic minipumps model 2002 (Durect Co., USA) which delivered 12 µl/day for 14 days, were filled with either saline, rhIGF-I (adjusted to a daily delivery of 1 µg), normal goat IgG or goat anti-rat IGF-I (the later two adjusted to a daily delivery of 0.6 µg). The filled minipumps were put in sterile saline at 37ºC for 24 h.

At postnatal day 23 all animals were fitted with a stainless steel cannula positioned according to stereotaxical coordination into the lateral ventricle of the brain via a hole drilled in the skull 1.5 mm lateral, 1 mm posterior to bregma, and the cannula was fixed at a depth of 4.0 mm from the skull surface. The minipumps were subsequently implanted subcutaneously and connected to the lateral ventricle cannula.

Surgery was performed under O_2 /N_2O (1:1) and Isoflurane (5%) anesthesia. Pre- and post-operatively, the rats received analgesia for two days by an oral dose of Ibuprofen® (1 mg/rat) and their health condition was frequently checked.

Experimental groups

IGF-I infusion study

Four groups of 10 prepubertal female rats (age 21 days) were individually housed: A control group which got normal diet *ad libitum* and was infused icv saline (CSal); a group FRSal with icv saline and pair-fed to CSal a diet to 30% less energy; a group CIGF with control food pair-fed to the CSal group with icv rhIGF-I (1 µg/day); and a group FRIGF with 30% less energy food pair-fed to the CSal group and also with icv human recombinant IGF-I (1 µg/day).

IGF-I antiserum (IGF-AS) infusion study

Three groups of 10 prepubertal female rats (21 days old) were individually housed: A control group which got food *ad libitum* and icv normal goat-IgG (0.6 µg/day) as a blank (CBlank); two groups received rat IGF-I antiserum (0.6 µg/day) and either food ad libitum (AL) or pair-fed to CBlank (PF).

Experimental procedure

The consumed food and body weight were measured daily and vaginal opening (VO) was monitored two times per day at ZT 6 (12:00 h) and at ZT 14 (20:00 h). To determine the

plasma leptin levels, blood samples were taken under anesthesia (see above) at ZT 6 via tail-nick [87] on days (23), 25, 27, 29, 31 and on the day of VO. Plasma was frozen at -20ºC.

The experiment ended on postnatal day 42; animals that did not show VO on this day were designated a VO time of 42 days in the statistical analysis. On day 42 all rats were anesthetised (see above) and the cannula position was checked by injecting dye (Evans Blue). Only results of rats with the cannula positioned correctly in the lateral ventricle of the brain were included. In the IGF-I infusion study, one rat died during surgery in the control group (CSal), and because of incorrect cannula position, one rat in the CIGF group was excluded. Also, in the IGF-AS experiment, one rat in the control group died (CBlank) during surgery, and in addition one rat in each group had an incorrectly positioned cannula and had to be excluded.

Radioimmunoassay (RIA)

To measure rat leptin levels a commercially available kit was used (LINCO Research, USA). The intra-assay and inter-assay variations as determined using quality control results were 2.2 and 5.7 %, respectively. No crossreactivity was found between rhIGF-I and rat leptin.

Statistical analysis

The median was calculated for the time of VO, and means ± SEM were calculated for body weight and leptin levels. One way ANOVA was used to test the effects of food restriction and IGF-I/IGF-AS-I infusion; posthoc ANOVA was followed by Duncan's or Student-Newman-Keuls' test (SNK). Kaplan-Meier analysis was used to test the effects on VO time. $P < 0.05$ was considered significant.

Results

IGF-I infusion

Puberty onset was equally postponed due to the FR treatment and/or the central infusion of rhIGF (Table 1 and figure 1). In the food-restricted IGF-infused group (FRIGF) three rats (i.e. 33%) did not even show VO on postnatal day 42. As a result, the weight on VO day is significantly higher in the treatment groups (Table 1). Food restricted animals show a decreased plasma leptin level compared to the normally fed controls, irrespective of IGF-

infusion (Table 1 and figure 3). IGF infusion appears to stimulate leptin levels in the FR treated animals but can not normalize them (FRIGF *vs.* CSal).

	VO age (day)	**95% Conf.interval**	**VO weight (g)**	**VO leptin level (ng/ml)**	**n**
CSal	27 [a]	26.3 - 27.7	74.9 ± 2.9 [a]	1.67 ± 0.19 [a]	9
FRSal	35	33.5 - 36.5	90.3 ± 6.6	0.50 **±** 0.23 [b]	10
CIGF	32	29.1 - 34.9	95.9 ± 8.4	1.30 ± 0.10	9
FRIGF	35	29.3 - 42.8	97.0 ± 6.8	0.80 ± 0.09	10*

Table 1:

The effects of rhIGF-I infusion on vaginal opening (VO) age, VO weight and leptin level on VO day; a and b designate significant differences. ***** 3/10 did not show VO on day 42.

CSal: Control group, control food (ad libitum*)* and saline;

FRSal: Icv saline and a diet with 30% less energy (pair-fed to CSal);

CIGF: Control food pair-fed to the CSal group with icv rhIGF-I (1 µg/day);

FRIGF: Diet with 30% less energy (pair-fed to CSal) with icv rhIGF-I (1 µg/day).

In spite of an equal bulk food intake (figure 2B), the 30% less energy-containing food results in a lower weight curve irrespective of IGF-infusion which does not seem to affect body weight (figure 2A).

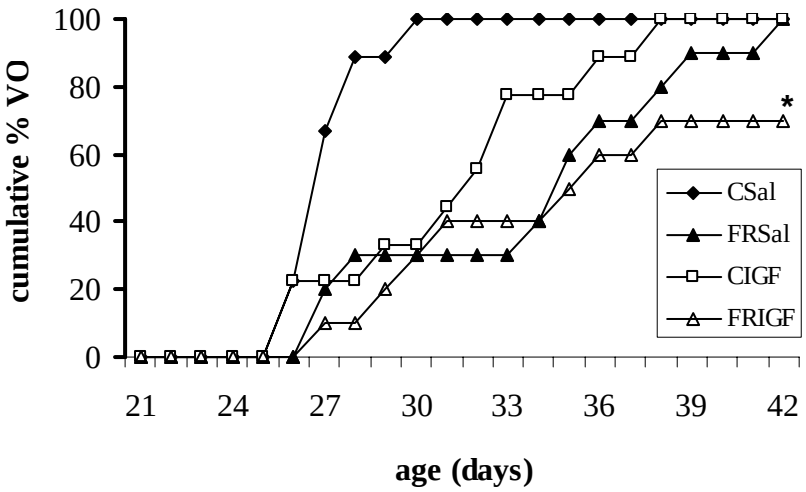

Figure 1: The cumulative percentage of animals showing vaginal opening (VO) in the rhIGF-I-infusion experiment. See Table 1 for abbreviations. ***** 3/10 did not show VO on day 42.

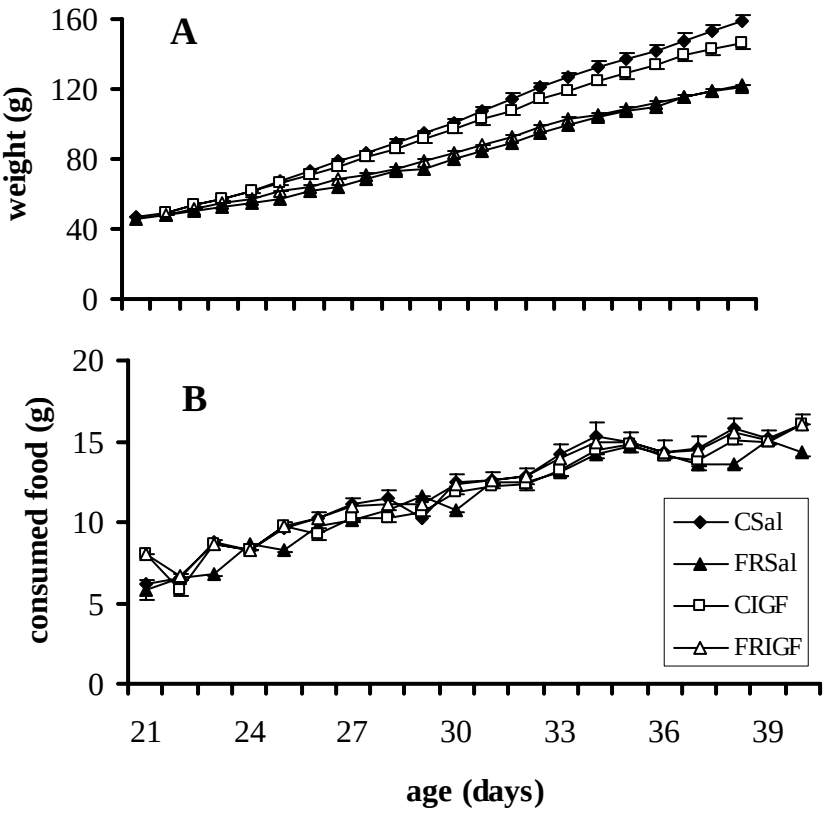

Figure 2:

A. The body weight curve in the rhIGF-I-infusion experiment;

B. The daily amount of consumed food in the rhIGF-I-infusion experiment. See Table 1 for abbreviations.

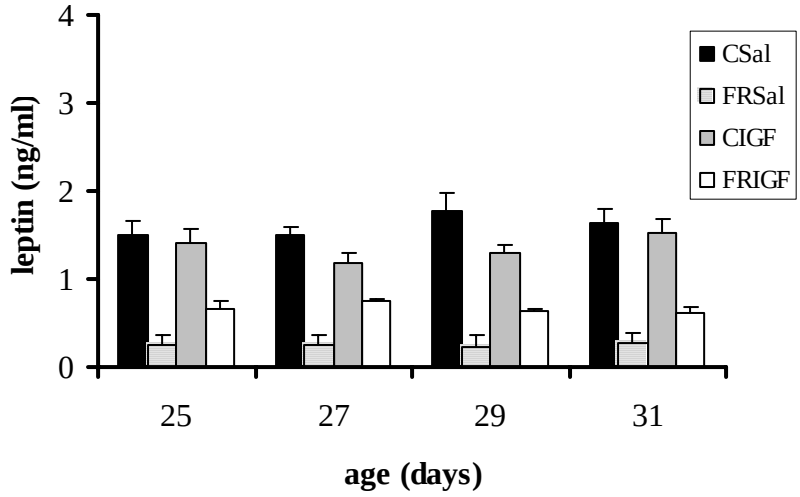

Figure 3: The plasma leptin levels at ZT 6 in the rhIGF-I-infusion experiment.
See Table 1 for abbreviations.

IGF-I immunoneutralization

The central infusion of rat IGF-AS did not affect puberty onset, although plasma leptin levels in both IGF-AS-infused animal groups (AL and PF) are significantly increased (Table 2, figure 4 and 6). Plasma leptin levels in the PF group are even higher than in the AL animals from postnatal day 27 onward (Table 2 and figure 6).

Immunoneutralization of IGF did not interfere with the course of the body weight (figure 5A) nor did it affect daily food intake (figure 5B).

	VO age (day)	95% Conf.interval	VO weight (g)	VO leptin level (ng/ml)	n
C Blank	28	25.4 – 30.3	83.4 ± 4.1	1.39 ± 0.18 [a]	8
IGF-AS AL	29	28.2 – 29.8	86.4 ± 4.6	2.26 ± 0.26 [b]	9
IGF-AS PF	29	28.4 – 29.6	90.0 ± 6.4	3.81 ± 0.36 [c]	9

Table 2:

The effects of IGF-AS infusion on vaginal opening (VO) age, VO weight and leptin level on VO day; a, b, and c designate significant differences.

CBlank: Control group with normal food *ad libitum* and icv normal goat-IgG (0.6 µg/day);

IGF-AS AL: Control food *ad libitum* and icv IGF-AS (0.6 µg/day);

IGF-AS PF: Control food pair-fed to CBlank and icv IGF-AS (0.6 µg/day).

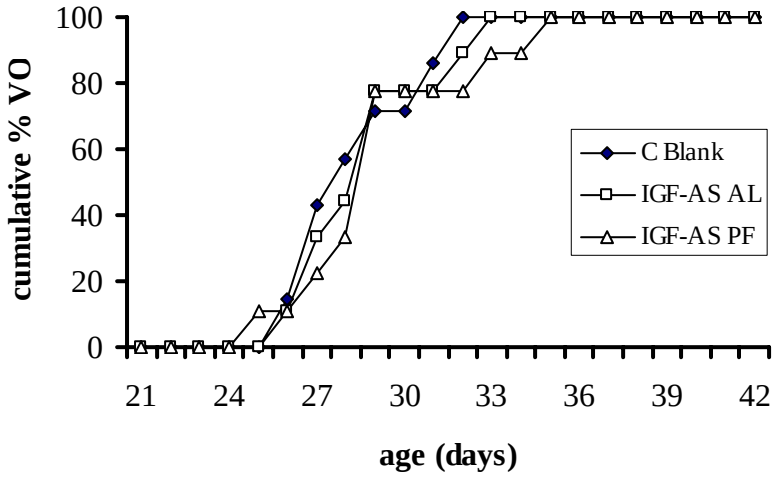

Figure 4: The cumulative percentage of animals showing vaginal opening (VO) in the IGF-AS-infused experiment. Abbreviations as in Table 2.

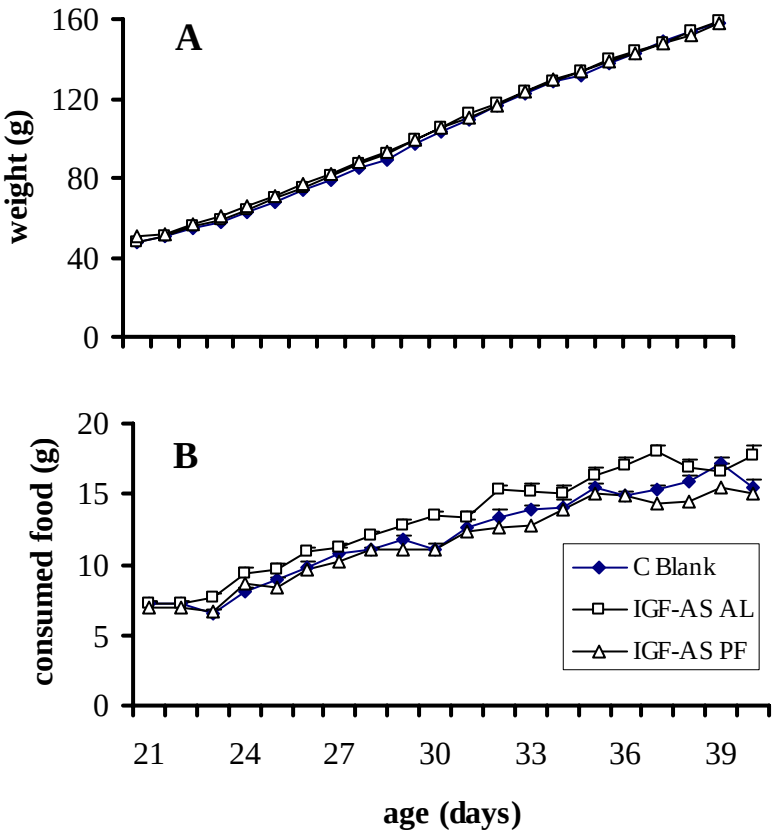

Figure 5:

A. The daily weight course in the IGF-AS-infusion experiment;

B. The daily amount of consumed food in the IGF-AS-infusion experiment. Abbreviations as in Table 2.

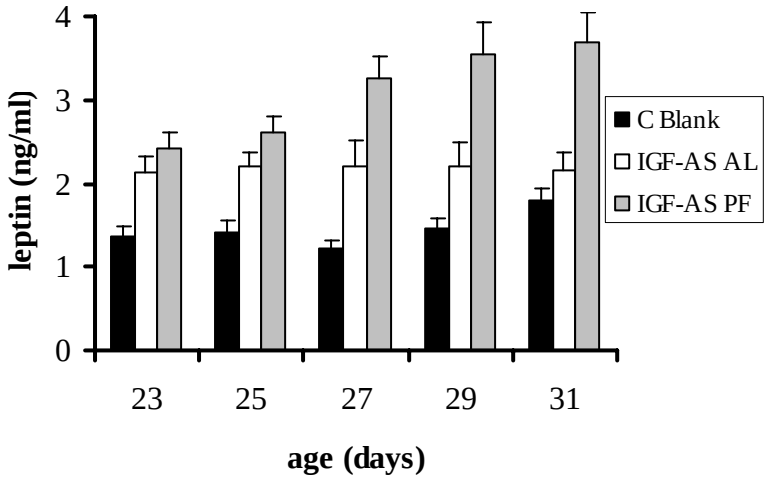

Figure 6: The plasma leptin levels at ZT 6 in the IGF-AS-infusion experiment.

Abbreviations as in Table 2.

Discussion

Accumulating reports suggest that IGF-I regulates reproductive development by acting at different levels of the hypothalamic-pituitary-gonadal axis [74, 138]. In several species, including humans, monkeys, rats, and mice [74, 104, 206], female puberty is associated with a GH-induced increase in the expression of IGF-I in the liver on the morning of the first proestrus resulting in increased levels of serum IGF-I, which peak during proestrus afternoon accompanied by increases in serum LH, FSH, and E_2 [56, 110, 178]. Both peripherally and centrally derived IGF-I can accelerate the onset of puberty in juvenile rats, via a stimulation of the GnRH release [110]. In spite of this, it is not clear whether IGF-I can induce puberty, although the GH/IGF-I axis is believed to play a role in the initiation of puberty.

We studied the effects of food restriction in combination with the central infusion of either recombinant human IGF-I (rhIGF-I) or rat IGF-I antiserum (IGF-I-AS) on puberty onset in female rats.

Food restriction (FR) (i.e. 30% less energy pair fed to controls) postponed puberty onset from a median age of 27 to 35 days, as was reported earlier [50, 79, 162, 217]. Centrally administered rhIGF-I equally postponed puberty onset, indicating central infusion of rhIGF delayed puberty onset independent of the dietary energy intake. Also, VO day in the FRSal group did not significantly differ from the FRIGF group showing that central infusion of rhIGF did not extra affect puberty onset in FR animals. Although in the FR group (FRIGF) three out ten rats (i.e. 33 %) did not yet show VO on postnatal day 42, IGF-I infusion in FR animals did not appear to induce an extra delay of puberty onset (see figure 1 and Table 1). Of course we do not know at what time the three animals in the FRIGF group would show VO as the experiment was ended at postnatal day 42.

In normally fed IGF-infused animals (CIGF) plasma leptin levels on VO day were lower than in control rats, but leptin levels did not differ over the experiment due to the IGF-I infusion. Plasma leptin levels are significantly higher in the FRIGF groups than in the FRSal animals, not on VO day (Table 1) but over the experiment (figure 3), and it appears that central infusion of rhIGF stimulates leptin release. The higher levels of leptin in the FRIGF group were not sufficient to affect body weight, daily food intake or to normalize VO age (Table 1, figure 2 and 3).

It has been shown that administration (sc) of rhIGF to adult male rats (1 mg/day) or to male hypophysectomised adult rats (300 µg/day) reduced fat mass, suppressed leptin mRNA and serum leptin concentration [40]. In contrast, our data indicate that the central infusion of rhIGF only marginally affected plasma leptin levels (in the control animals) or significantly increased plasma leptin levels in FR rats (figure 3).

Previous studies report no effect of IGF-I infusion (2 or 4 µg/day) on puberty onset in female rats [98, 99]. One possibility for the discrepancy between our study and others [77, 109, 110] is that we (1 µg/day) and others [99; 2 or 4 µg/day] centrally, chronically and continuously for 14 days infused IGF-I in a relatively high concentration, while others injected a single, acute and low dose of IGF-I (e.g. [110]: 2, 20, 200 ng/3 µl); even by infusing 2 ng of IGF-I puberty was significantly advanced and LH levels increased.

Our data show that puberty onset was not affected by centrally infusing IGF-AS in female rats and that all animals showed VO already on day 35 (Table 2 and figure 4). Also, the central infusion of IGF-AS did not change the daily food intake or body weight (figure 5) indicating IGF-I is not able to affect body weight as was also shown for male rats [178]. Animals in the IGF-AS AL group could compensate the negative effects of IGF-AS on food intake, which was not possible for the pair-fed animals (IGF-AS PF), but such effects were too small to cause significant food intake changes (AL *vs.* PF). It has already been shown that central and chronic administration of IGF-AS in male rats reduced testicular weight, decreased pituitary LH content, serum testosterone, and delayed puberty which related to a decrease in the frequency of GnRH pulses [178].

Immunoneutralization of endogenous IGF-I increased plasma leptin levels in both IGF-AS treated groups, even more so in the PF group than in the AL group (figure 6). It is not clear whether the source of the leptin released due to the IGF-AS infusion was adipose tissue or liver. It is possible that the central infusion of IGF-AS might disinhibit some unknown neurotransmitters and/or neural pathways in the hypothalamus to stimulate adipose tissue to increase leptin production. Endogenous IGF-I in the brain would attenuate leptin release via this way.

Our data show that central infusion of insulin like growth factor I (IGF-I) postpones puberty onset both in food-restricted and in normal ad lib-fed female rats and does so independent of circulating leptin levels. The central immunoneutralization of endogenous

IGF-I by the infusion of anti-rat IGF-I however, did not influence puberty onset in these animals.

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Chapter 6

Summary and general Discussion

Puberty is the time of life when mature gametes are produced for the first time and the reproductive process is initiated: The production of sex steroids by the gonads increases, in response to a higher secretion of gonadotropin from the anterior pituitary gland, which, in turn, is being driven by an increased secretion of GnRH from the hypothalamus. Perhaps one of the most important and contemporary issues in understanding puberty is determining *how* the brain *'knows'* the body has reached the appropriate size and degree of maturity to begin high-frequency GnRH secretion. The (metabolic) signal(s) that could serve as molecular link(s) between growth and reproduction remain(s) to be determined.

Leptin signals the nutritional status and the presence of an adequate amount of loaded adipose tissue, as a long-term resource of energy, to the brain which in response switches on the reproduction process (i.e. puberty onset) [7-12, 60, 66, 88, 196, 212].
It was indeed found that leptin treatment can advance reproductive maturation in both undernourished and well-fed animals [6, 11, 25, 52, 86]. Both the *ob/ob* female mice (leptin knock-outs) and the *db/db* mouse (leptin's receptor knock-outs) are infertile and remain in a constant prepubertal state with reduced ovarian and uterine weight, sex steroid hormone levels and pituitary gonadotropin secretion. Administration of recombinant leptin to female *ob/ob* mice completely restores gonadotropin secretion, secondary sex organ weight and fertility [7-12, 25, 147].

The present thesis provides further evidence that leptin, mainly the product of adipose tissue, is an important and crucial signal between the nutritional status and the brain to trigger puberty onset. In addition, we suggest that there is a functional interaction between growth hormone (GH) and leptin to initiate puberty in female rats.
In this chapter we intend to:
I. Summarize the main findings of our experiments,
II. Briefly discuss the effects of leptin on food intake and the regulation of body weight, III. Propose a general mechanism of puberty onset incorporating our findings,
IV. Discuss the interaction between leptin and the GH/IGF-I axis on puberty onset.

6-1. Summary of findings

To investigate the implications and the effects of leptin on puberty onset, basic information on the reproductive function, diurnal plasma leptin levels, the secretion pattern of leptin and the relationship between leptin and fat in prepubertal female Wistar rat is required. First, we therefore carried out some descriptive and basic studies. Time of puberty onset was monitored by scoring the moment of vaginal opening (VO). The experiments did not intervene but served to address the events around puberty in this strain, thereby partly confirming but also extending the current knowledge of prepubertal physiology. The data showed a strong positive correlation between the percentage of body fat and leptin levels, and showed leptin is increasing over the prepubertal period. Then, we aimed to find out the effects of food-restriction (FR).The amount and percentage of body fat, plasma leptin levels and body temperature in the FR rats were significantly lower than the controls throughout the experiment. The data emphasised that leptin could indeed serve as a functional link between the nutritional status and the reproductive axis in initiating puberty onset. Together, the data obtained indicate a signaling role for leptin in puberty onset in female rats (Chapter 2).

It has been shown that food restriction can cause postponement of puberty onset [50, 57, 79, 162 and this book Chapter 2]. We also applied this paradigm, and compared normal female rats fed *ad libitum* with food-restricted (FR) animals which were pair-fed with 30% less energy. In the FR group, the plasma leptin levels at VO day were significantly lower than controls and puberty onset was clearly postponed.

We used FR rats as a model for delayed puberty onset, and centrally (icv) or peripherally (sc) administered leptin, or we centrally (icv) immunoneutralized leptin. Instead of discrete injections (icv or sc), we administered these compounds via a continuous infusion (icv or sc) using implanted osmotic minipumps delivering 0.5 µl/h for 14 d. In this way, a steady level of compound delivery is guaranteed avoiding major fluctuations in concentration. We chose arbitrarily a dose of 1 µg/day (i.e. 42 ng/h) which is considering its normal plasma levels and estimated half-life, a physiological dose for this hormone.

Central leptin infusion not only restored the delay in puberty onset caused by food restriction but also advanced sexual maturation in the normally fed animals. The plasma leptin levels in the FR animals were again significantly lower than the controls. The plasma leptin levels in the pair-fed leptin-infused animals were even below the detection level of the assay which

might be due to the negative feedback of leptin-infused in the brain. Moreover, plasma leptin levels did not differ between the saline and leptin-infused groups, which indicates that centrally infused leptin did not cross the blood-brain-barrier or leak to the periphery (Chapter 3).

Then, we peripherally (sc) infused leptin to FR and normal female rats to compare its effect on puberty onset with central infusion. Like the central infusion, also the peripheral leptin infusion advanced puberty onset in the FR animals. So, we showed an advancing effect of both centrally and peripherally infused leptin on puberty onset in prepubertal female rats.

As can be expected from the foregoing, the central immunoneutralization of leptin clearly postponed puberty onset in these animals. But the central immunoneutralization of leptin did never affect the plasma leptin levels. We therefore conclude that leptin is one of the crucial factors triggering puberty onset in female rats (Chapter 3).

Growth hormone (GH) is believed to be involved in puberty onset. In the FR rat model system we therefore centrally (icv) infused GH. The infusion of GH postponed puberty onset in normally fed rats, but advanced puberty onset in FR animals. Also, the plasma leptin levels in the GH-infused animals were significantly higher than their controls and increased as GH infusion proceeded.

We then aimed to block the action of central endogenous GH by the central immunoneutralization (icv) of GH or by centrally infusing somatostatin. Central infusion of GH antiserum (AS) advanced puberty onset in the pair-fed animals but not in the ad lib-fed animals. Also the central infusion of somatostatin worked likewise in both groups. These findings match with the results of the central infusion of GH which also advanced puberty onset in FR animals only. The observed advancement in puberty onset was not accompanied by any difference in plasma leptin levels over the experiment, although plasma leptin levels in the somatostatin-infused groups were elevated (Chapter 4).

Both central and peripherally-derived IGF-I is thought to play a role in the initiation of puberty. According to our previous results (Chapter 4) and the data in the literature, we aimed to evaluate the effects of IGF-I on puberty onset and to find out the role of leptin and the GH/IGF-I axis in regulating the time of puberty onset in prepubertal female rats, by centrally infusing IGF-I. Central infusion of IGF-I significantly postponed puberty onset in the ad libitum fed animals. FR treatment decreased plasma leptin levels but levels were increased in FR-

treated animals infused with IGF-I. Plasma leptin levels in the control fed animals were not affected by the IGF-I infusion.
Again we intended to block the action of central endogenous IGF-I by the central immunoneutralization (icv) of IGF-I. Although immunoneutralization of endogenous IGF-I enhanced prevailing plasma leptin levels, there was no effect on the timing of puberty. Centrally-present endogenous IGF-I does not appear to be involved in puberty onset although it seems to inhibit leptin secretion (Chapter 5).

6-2. Leptin: food intake and body weight

In our study food intake was decreased only by central but not peripheral leptin infusion: in the pair-fed leptin-infused (icv) animals the consumed food was significantly lower than the control group which confirmed the negative effect of central leptin on food intake. Congruently, food intake was significantly increased upon the central immunoneutralization of leptin which again confirmed the negative effect of leptin on food intake.

Centrally infused leptin may reduce food intake as it may directly affect neurotransmitter and/or neuropeptide systems controlling food intake in the brain [8, 9, 19, 142, 196]. NPY is one of the important stimulators of feeding in the brain. NPY-containing neurons in the hypothalamus are located in the arcuate nucleus (ARC). Neurons synthesizing NPY in the ARC are highly responsive to states of energy deficiency and a higher metabolic demand. Their expression is stimulated by food deprivation, increased exercise, cold and pregnancy. Evidence from several studies suggests an important interaction between leptin and NPY [8, 9, 19, 31, 181]. It is suggested that the ARC is a principal monitor of leptin signaling in the brain as the ARC accounts for the majority (about 75%) of leptin-responsive cells in the hypothalamus [31, 42, 142, 167, 181, 226]. Consequently, the central leptin infusion and immunoneutralization of leptin might render its effects for a great deal via NPY neurons in the ARC (Chapter 2).

Leptin treatment has been shown to cause a dose-dependent loss of body weight (BW) and fat depots, and an increase in energy metabolism [113, 165]. We indeed found that the BW in centrally leptin-infused animals was significantly lower than in control animals which confirms former data on the central effect of leptin in reducing BW [7-12, 60, 92, 196]. But,

peripherally infused leptin did not affect BW. Along with the observed reduction in food intake, centrally infused leptin also leads to lipolysis and fatty acid oxidation, resulting in a reduction in adipose tissue mass and hence in a reduced BW [8, 9, 92].
In normal physiology, leptin expression and serum levels decrease of course in response to weight loss [73, 86, 170], in this way supporting the survival of the individual by sparing its reserves and sustaining food intake.

6-3. Leptin: proposed mechanism for puberty onset

The neurobiological mechanisms that synchronise the activity of the diffusely distributed GnRH neurons and thereby generate the pulsatile discharge of GnRH, are not yet completely determined. The hypothalamic GnRH pulse generator becomes operational long before the onset of puberty [139, 180]. GnRH release is affected by the negative feedback of steroid hormones at the level of the hypothalamus. Steroid hormones negatively feedback and thus decrease the frequency of the pulsatile GnRH release and hence the LH release from the pituitary [131, 139].
One of the important and ongoing features for puberty onset is a *critical weight* or a ´critical amount of fat´ which recently has been modified into 'a critical level of metabolic signals', suggesting an immature female will not ovulate (for the first time) until she has accumulated a critical amount of fat (or a critical level of metabolic signals) relative to her lean body mass [88, 196, 226].
When energy is scarce, the physiological mechanisms prioritise those processes that ensure the survival of the individual. Many physiological processes (e.g. digestion, thermogenesis) have priority over reproduction. In fact, reproduction has a very low priority in physiology, and because reproductive processes are energetically expensive they are likely to be postponed when the basic requirements and the individual survival is in jeopardy [66, 88, 113, 212, 196, 226]. In limited energy conditions (e.g. food restriction), the reproductive axis function, and in particular GnRH neurons are 'energetically challenged' and GnRH secretion is shut down. An important question therefore is, *how* the brain *'knows'* when the (stored) energy is sufficient to initiate reproduction (i.e. puberty onset). According to the ´critical level of metabolic signals´ hypothesis the brain should be able to read, interpret and integrate a wide range of signals that describe the animal's nutritional status and its immediate

environment, and to make appropriate adjustments in food intake, energy expenditure and metabolism as a result of the information received through these signals [226].

On the basis of our findings (in particular Chapter 3 and 4) and accumulated data in the literature over the last ten years, we believe that leptin is an important link between the nutritional status (i.e. adipose tissue as long-term source of energy) and the brain (specifically GnRH neurons) to initiate puberty. We, like others [6, 60, 63] showed that even in normally fed rodents, leptin administration advanced the onset of puberty (Chapter 3). Infusion of leptin, either centrally (icv) or peripherally (sc), partially restored the timing of puberty in FR rats (Chapter 3) and mice [63]. Also, we show that the infusion of GH in the FR animals significantly advanced puberty onset compared to FR saline-infused animals, indicating GH might stimulate puberty onset (by increasing circulating leptin levels) when animals are challenged with suboptimal conditions such as food restriction (Chapter 4). Overall, these finding suggest that leptin is one of the crucial factors to trigger puberty onset.

The mechanism via which leptin triggers puberty onset is not yet fully understood. Leptin has been shown to stimulate GnRH secretion from the basal hypothalamus [31, 73, 233]. Leptin might stimulate GnRH neurons *directly* [31, 73], but more likely *indirectly* by affecting various other neurons in the hypothalamus as GnRH neurons themselves do not express leptin receptors [84, 102]. Leptin receptors are widely expressed within the hypothalamus of both rodents and primates, and are co-localised with NPY [8, 36, 63, 84, 102]. As already mentioned (paragraph 6.2), NPY-neurons are located in the ARC of the hypothalamus, and are involved in food intake and leptin signaling. Leptin decreases NPY synthesis in the ARC [36, 63, 73], and NPY inhibits LH release. Thus leptin indirectly, by decreasing NPY, stimulates GnRH synthesis and release into the portal vessels from where it is carried to the anterior pituitary to stimulate FSH and LH release (figure 1) [158, 159].

Apart from neurons expressing NPY, also neurons expressing pro-opiomelanocortin (POMC) might be involved in the conveyance of the leptin signal to GnRH neurons, as POMC-neurons make a direct synaptic contact with GnRH neurons [73]. A major proportion of the POMC neurons in the hypothalamus expresses leptin receptors and are thus direct targets of circulating leptin. POMC neurons are believed to be key mediators of the central leptin action [36, 102]: POMC cells respond very rapidly to leptin, and at the same time as POMC cells are

activated by leptin, NPY cells are inhibited [3, 36]. Hence, also via activation of POMC neurons leptin might indirectly regulate GnRH release [36, 66, 73].

A third player in this field might be somatostatin (Som). Som neurons are widely distributed throughout the brain [187], but Som cells located in the periventricular nucleus of the hypothalamus (PeVN) project to the median eminence (ME) and are mainly responsible for the regulation of pituitary GH secretion [39]. It has been suggested that a possible interaction exists between the Som neurons and the GnRH system at the level of the hypothalamus. It has been recently shown that the icv infusion of a Som analog caused a significant attenuation of the E_2-induced LH surge in adult female rats and it appears that Som inhibits the LH surge partly by reducing GnRH cell activation [216].

In normally fed rats, icv infusion of leptin stimulated pituitary GH and hypothalamic GHRH gene expression, but inhibited hypothalamic expression of somatostatin and NPY [105, 219]. This was also shown in hypophysectomised fasting rats [54]. Leptin, therefore, stimulates GHRH neurons and inhibits somatostatin neurons, thus indirectly stimulating GnRH synthesis and release (figure 1).

Finally and fourth, since both leptin and its receptor are expressed by the majority of the anterior pituitary cells, a paracrine or autocrine role of leptin in the pituitary is also possible [76, 123, 233, 234]. Leptin stimulates hence LH release, at least partly, also at the level of the pituitary in prepubertal animals (figure 1).

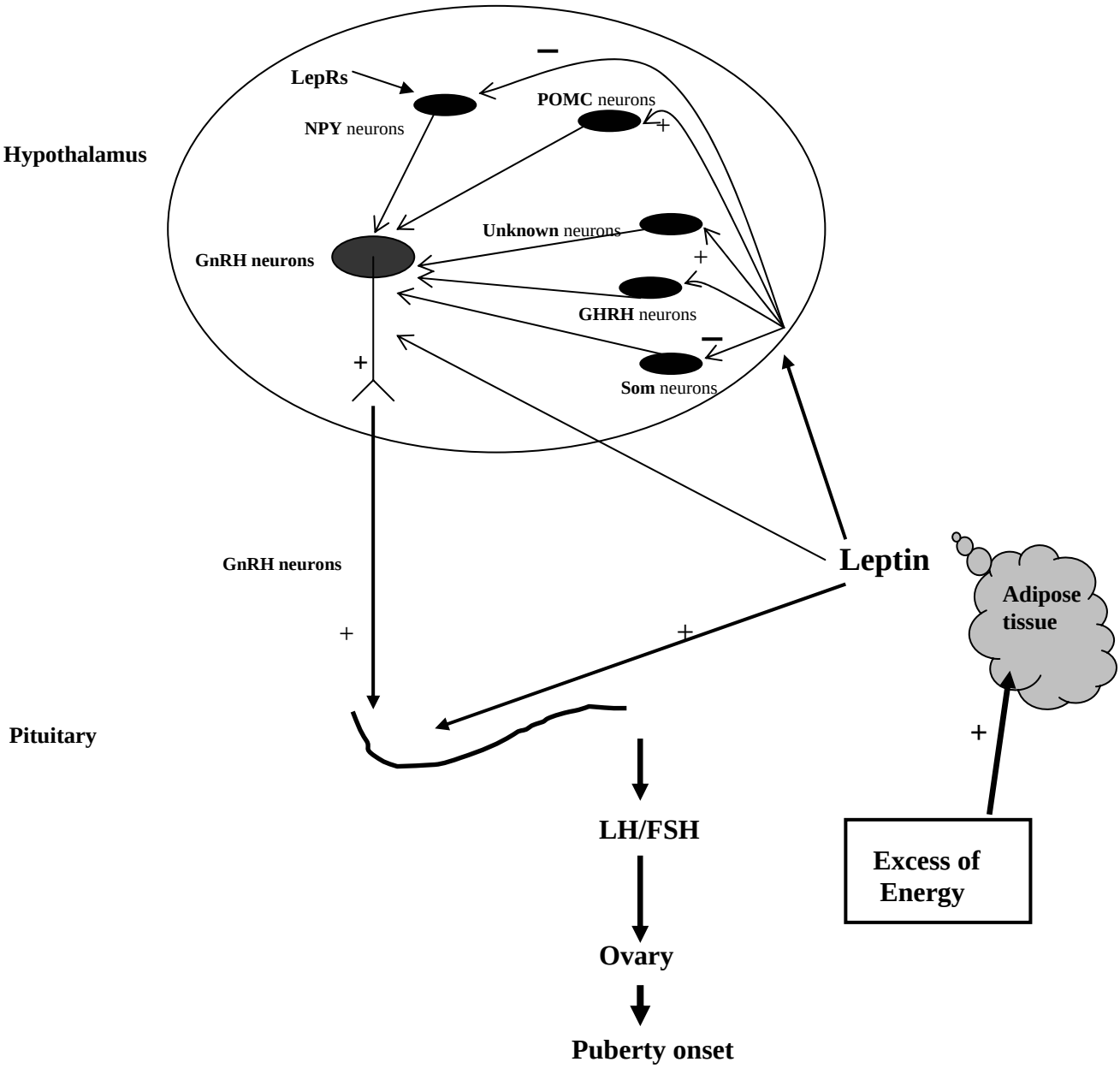

Figure 1: Proposed involvement of leptin in the neuroendocrine regulation of GnRH neurons, GnRH/LH secretion and puberty onset (see text for abbreviations and description).

6-4. Leptin: GH/IGF-I axis interaction and puberty onset

GH is believed to affect the time of puberty onset by activating the GnRH pulse generator [32, 33]. Although changes in GH secretion do not provide direct cues for the normal time of puberty onset, GH still might play a permissive role in puberty and its onset [115, 116].

Leptin treatment stimulates GH secretion, and pulsatile GH secretion is restored by leptin replacement, while immunoneutralization of leptin reduced GH secretion in rats [3]. Central administration of leptin affects gene expression in the somatotropic axis: it increases pituitary GH mRNA, and enhances hypothalamic GHRH mRNA levels but reduces those of somatostatin mRNA [27, 67, 209]. Leptin receptors have been shown on GHRH and somatostatin neurons, providing strong anatomical evidence for a direct effect of leptin on the somatotropic axis [102]. Overall, these data suggest that there is an interaction between leptin and GH; levels of both hormones rise during puberty.

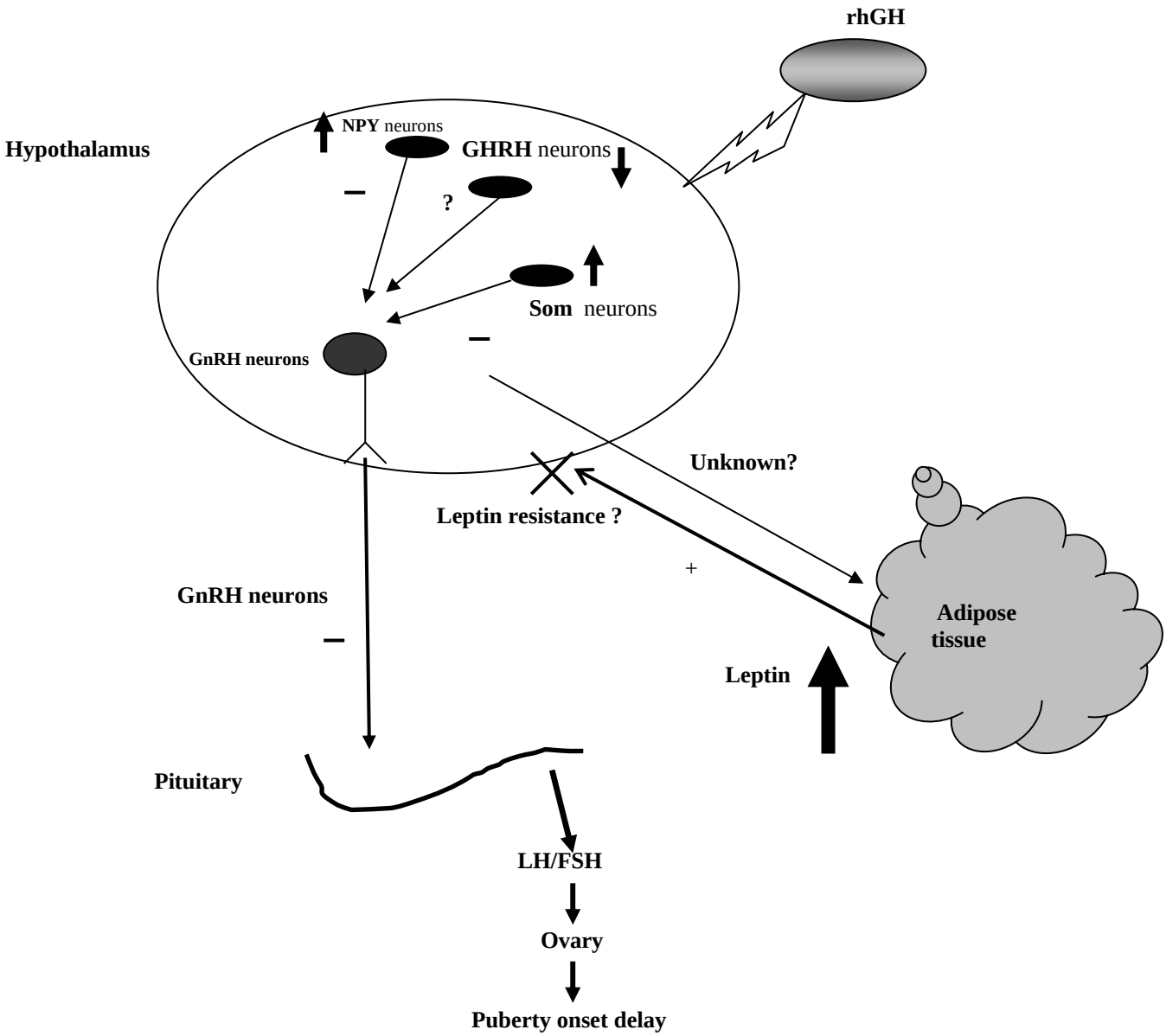

Figure 2:
I: rhGH-infused normally fed rats with leptin resistance

Vice versa, the effects of GH infusion on plasma leptin levels vary among species: in rats, injection (sc) of GH (4 mg/kg/d) decreased plasma leptin levels [150], but GH infusion had no effect on leptin expression in hypophysectomised rats [40]. GH treatment of human patients decreased plasma leptin levels [78, 136, 156, 164, 184]. In contrast, treatment of GH-deficient human patients with GH (0.2 IU/kg/d, sc) increased plasma leptin levels [15, 85, 96, 118].

We tested the effect of GH on leptin secretion and puberty onset in female rats. We showed that the central infusion of GH delayed puberty onset in normally fed rats (figure 2, I), but advanced puberty onset in FR animals (figure 2, II).

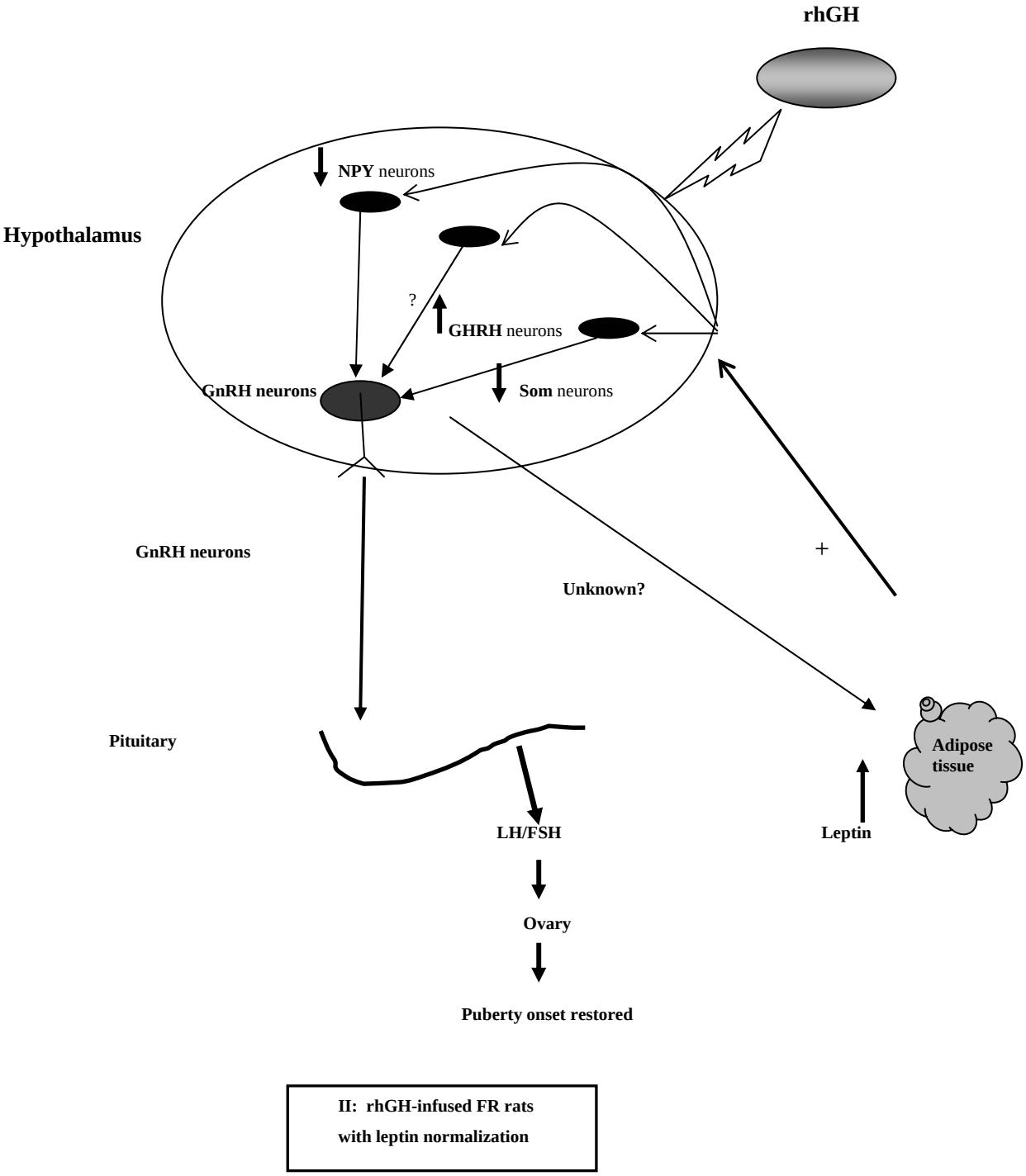

Figure 2: Proposed involvement of leptin and GH axis interaction and puberty onset in the neuroendocrine regulation of GnRH neurons, GnRH/LH secretion and puberty onset in both rhGH-infused normally fed (**I**) and FR (**II**) rats (see text for abbreviations and description).

Plasma leptin levels in the GH-infused animals were raised and increased in time. We already showed that centrally (icv) or peripherally (sc) administered leptin also restored the puberty onset postponement caused by food restriction. Also, we showed that central immunoneutralization of leptin delayed puberty onset (Chapter 3). Taken together, on the basis of these findings and the data in the literature, we suggest that the normalisation of puberty onset in the GH-infused animals was due to an indirect effect of leptin.

What is the source of this leptin? Leptin is mainly produced by white adipose tissue, the central infusion of GH might via some indirect and yet unidentified (neurotransmitters or neural) pathway prompt adipose tissue to produce the high levels of leptin. Changes in leptin levels upon treatment with GH were shown to be independent of fat mass and did not induce any changes in fat content [71, 120, 121].

IGF-I is suggested to have a role in the puberty onset as IGF-I directly affects GnRH synthesis and release [74, 109, 110]. Indirectly the LH release is thus stimulated by IGF-I. The majority of GnRH cell bodies are immunopositive for IGF-I receptors and also the median eminence (ME), being the target area for the GnRH nerve terminals, has high concentrations of the IGF-I receptor and might hence be an important site of action for (peripherally or centrally derived) IGF-I to stimulate the GnRH/LH secretion [77, 109, 110, 163, 178].

Central infusion of IGF-I significantly postponed puberty onset in ad libitum fed animals without changes in the plasma leptin levels, indicating central infusion of IGF-I delayed puberty onset independent of leptin.

Plasma leptin levels were normalized in the FR-treated animals infused with IGF-I over the experiment but had no effect on the time of puberty. Immunoneutralization of endogenous IGF-I by centrally infused IGF-antiserum had no effect on the timing of puberty onset although it attenuated peripheral leptin levels (Chapter 5). We assumed that the effects of IGF-I might be independent of leptin or work via an as yet unknown interaction between IGF-I and leptin.

In conclusion, our findings provide further evidence that leptin, a hormone produced mainly by adipose tissue, is an important and crucial signal between the bodily nutritional status and the brain (i.e. the hypothalamus) to trigger puberty onset. Furthermore, we suggest that there is a functional interaction between growth hormone (GH) and leptin to initiate puberty in female rats.

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Samenvatting

De puberteit is de levensfase waarin het kind, het juvenile dier, volwassen wordt en de geslachtsrijpheid bereikt wordt. Het reproductieve proces wordt gestart:de geslachtsorganen gaan rijpe gameten en sex steroïden produceren, hiertoe aangezet door de hypofyse die in verhoogde mate gonadotropines (LH en FSH) gaat afgeven, op haar beurt hiertoe aangezet door de hypothalamus die steeds meer gonadotroop hormoon (GnRH) gaat afgeven.
Tal van hormonaal gestuurde groei-processen worden aangezwengeld en in verhoogde snelheid (de zgn. ‘groei-spurt’) uitgevoerd tot de adulte proporties van het dier worden bereikt.

Maar hoe weet nu de hypothalamus (en het brein i.h.a.) wanneer de afgifte van GnRH opgestart moet worden? Hoe weet het brein dat het lichaam de adequate afmetingen heeft bereikt en van een voldoende grote vetreserve is voorzien, om een dracht (of zwangerschap) te volbrengen? Welke (metabole) signalen kunnen deze informatie overdragen naar het brein en als ‘link’ tussen groei en reproductie dienen?
Mogelijk zou het hormoon leptine deze rol kunnen vervullen:
Dit hormoon wordt afgegeven door vetcellen naar rato van de hoeveelheid vet die hierin ligt opgeslagen. Leptine is (net als insuline) een belangrijk verzadigingshormoon en betrokken bij de regulatie van de voedselopname. Een positieve energiebalans gaat dan ook samen met een hoog leptine gehalte in het plasma.

Behandeling met leptine kan inderdaad puberteits-processen versnellen, zelfs in ondervoede dieren/kinderen in een negatieve energie-balans. Transgene dieren die geen leptine kunnen maken (*ob/ob*) of die de leptine-receptor missen (*db/db*) zijn dan ook onvruchtbaar en blijvend in een prepuberale toestand; toediening van leptine aan dergelijke (*ob/ob*) dieren induceert evenwel de puberteit en maakt ze vruchtbaar.
In dit proefschrift wordt verder bewijs geleverd voor een belangrijke rol van leptine in het initiëren van de puberteit. Ook wordt bewijs geleverd voor een functionele interactie hierin van leptine met groeihormonen (GH en IGF-1).
Hiertoe werden diverse experimenten met vrouwelijke prepuberale ratten uitgevoerd en beschreven. De bevindingen zullen nu kort worden besproken en toegelicht.

Puberteit in de vrouwelijke rat

Allereerst is basale informatie verzameld over het puberteitsproces in de rat: prepuberale vrouwelijke ratten werden gevolgd vanaf levensdag 22 (de dag na spenen) tot dag 42. In deze periode maken zij de puberteit door en start hun eerste oestrische cyclus. In de rat gaat dit gepaard met het verbreken van een soortement van ‘maagdenvlies’ ofwel de ‘vaginale opening’ (VO) hetgeen uitwendig zichtbaar is; op deze manier kan het tijdstip van puberteit dus vastgesteld worden.

Naast het tijdstip van VO werden voedselopname en gewichtstoename gevolgd en middels de bloedmonsteringstechniek van de ‘tail-nick’, een sneetje in de staart, werden ook de plasma-spiegels van leptine in kaart gebracht. Voorts werden op diverse tijdstippen dieren gedood en werd hun vetpercentage vastgesteld (totaal lichaamsvet).
Een sterke correlatie tussen het vetpercentage en de circulerende concentraties leptine in het plasma werd gevonden. Ook correleerde het tijdstip van VO met het vetgehalte van de dieren. De gemiddelde leptine-concentratie nam toe in de prepuberale periode en de leptine-spiegels gaan een diurnaal ritme (nadir op ZT 6) vertonen naarmate het tijdstip van puberteit dichterbij komt. Voorts bleek dat het gewicht van de dieren een voorspellende waarde had voor het tijdstip van VO: hoe zwaarder de dieren, hoe eerder VO.

FR-model

Een negatieve energiebalans remt de puberteitsprocessen en stelt het moment van geslachtrijpheid uit. Dit mechanisme werd aangewend om een ‘model van uitgestelde puberteit’ te ontwikkelen. Prepuberale dieren werden voer-beperkt (‘feed restricted’, FR) door ze voer te geven met 30% (massa%) minder energie. In vergelijking met controle-voer werd 30% van de koolhydraten- en vet-inhoud van het voer vervangen door inert cellulose; de bulk voer-opname bleef aldus gelijk en de dieren werden op deze wijze enkel energie-beperkt. Voer-beperkte, FR-dieren kregen evenveel voer als de controle dieren de dag tevoren gegeten hadden: ‘pair-feeding’.

Dit model van ‘feed restriction’ (FR) liet normaal groeiende dieren zien die echter veel minder vet aanzetten, een lager leptine-gehalte in hun plasma hadden, en een lagere lichaamstemperatuur hadden; het moment van VO in de FR-groep lag ongeveer een week later dan in de controle-dieren.

Leptine-effecten

In dit model van uitgestelde puberteit is vervolgens geprobeerd om het VO-uitstel teniet te doen door leptine toe te dienen. Door middel van onderhuids geïmplanteerd osmotische minipompjes werd hiertoe chronisch (van dag 22 tot dag 42) en continue (0.5 μl/h) leptine toegediend (1 μg/dag) in een lateraal hersenventrikel (centraal) of onderhuids (perifeer).
In normaal gevoede dieren had het leptine-infuus slechts een marginaal effect en beïnvloedde het tijdstip van VO nauwelijks. In de FR-groep was het tijdstip van VO uiteraard verlaat t.o.v. de controle-groep. In deze FR-dieren gaf het leptine-infuus een significante *vervroeging* van het tijdstip van VO te zien, ongeacht langs welke weg het toegediend werd (centraal of perifeer). Leptine lijkt dus de puberteitsprocessen te versnellen.
Het centraal geïnfundeerde leptine bereikte de periferie niet, getuige de plasma leptine-levels die zeer laag waren en bleven gedurende de infusie-periode. Perifeer toegediend leptine verhoogde de plasma-waarden van leptine echter wel, zoals verwacht. Kennelijk kan leptine wel van perifeer naar centraal maar niet of moeilijker andersom.
Centraal toegediend leptine verlaagde de voedselopname in de pair-fed groep op controle dieet en dientengevolge bleef ook de gewichtsontwikkeling achter in deze groep. In het experiment waarin leptine perifeer werd toegediend werd geen effect waargenomen op de voedselopname of de gewichtsontwikkeling.
Hoewel de voedselopname in de pair-fed FR-groepen niet achterbleef bij de controle-groepen lag de curve van hun gewichtsontwikkeling (door de lagere energie-inname) wel onder die van de controles.

De rol van leptine in de initiatie van de puberteit werd vervolgens onderzocht in een experiment waarin het centrale leptine werd uitgeschakeld door het te neutraliseren met een antiserum tegen leptine. Het antiserum werd centraal en op dezelfde manier toegediend als het leptine-infuus zoals hierboven beschreven; als controle werd een IgG-oplossing gebruikt.
De effecten van het neutraliseren van het centrale leptine waren conform de verwachtingen: In dieren die het leptine-antiserum toegediend kregen werd het tijdstip van VO *uitgesteld* met 3-5 dagen. Het leptine-antiserum had geen effect op de plasma-waarden van leptine, maar remde de verzadiging licht en induceerde een hogere voedsel-opname in de groep dieren met *ad libitum* toegang tot voer.

Groeihormonen en leptine

Ook groeimediatoren zoals groeihormoon (GH) en insulin-like growth factor 1 (IFG-1) lijken betrokken bij de puberteit en de initiatie ervan, mogelijk in samenwerking met leptine. Daarom werd het voornoemde FR-model van uitgestelde VO ook gebruikt in twee experimenten waarin deze groeimediatoren centraal werden geïnfundeerd (eveneens met de minipompjes zoals hierboven beschreven).
GH en IGF-1 werden eerst geïnfundeerd in normaal gevoede dieren:
Infusie van GH stelde het tijdstip van VO uit terwijl het de plasma-waarden van leptine enorm stimuleerde. Ook een infuus van IGF-1 in het hersenventrikel leidde tot een later tijdstip waarop de dieren VO vertoonden, maar het IGF-1 infuus beïnvloedde de plasma-leptine spiegels niet.
In het FR-model gaven GH en IFG-1 infusie tegenstrijdige effecten te zien: GH-infusie in FR-dieren verkortte in lichte mate het tijdstip van VO t.o.v. de FR-controle dieren, terwijl het IGF-1 infuus in dit geval geen duidelijk effect liet zien (het tijdstip van VO werd niet duidelijk vertraagd of versneld).
Evenals in de normaal gevoede dieren stimuleerde het GH-infuus de leptine plasmaspiegels in de FR-dieren, zij het in veel lichtere mate. Ook in de FR-dieren geïnfundeerd met IGF-1 werd een toename in leptine plasmalevels waargenomen, dit i.t.t. de normaal gevoede dieren.
De centrale infusies (van GH danwel IGF-1) of FR hadden geen effect op de voedselopname maar zorgden wel voor effecten in de gewichtsontwikkeling van de verschillende diergroepen waarbij GH (en IGF-1) en/of FR een negatief effect hadden t.o.v. de controle groep.

Het centraal aanwezige GH werd op twee manieren in een experiment onderdrukt: enerzijds via immunoneutralizatie door infusie van antiserum tegen GH, en anderzijds door een infuus van een somatostatine-analoog (Octreotide®).
Immunoneutralizatie van centraal GH, en in mindere mate de toediening van somatostatine, vervroegde het tijdstip van VO in dieren pair-fed met de controles; deze bevinding is tegengesteld aan de gevonden effecten van GH-infusie en bevestigt deze hiermee. In *ad libitum* gevoede dieren heeft de infusie van het GH-antiserum, dan wel somatostatine echter geen effect op het tijdstip van VO.

Noch de infusie van GH-antiserum, noch infusie van somatostatine heeft een significant effect op de circulerende concentratie leptine. Immunoneutralizatie van centraal GH i.t.t. inhibitie van centrale GH-levels door somatostatine, onderdrukt de verzadigingsmechanismen waardoor de *ad libitum* gevoede dieren meer aten dan de controle dieren. Desondanks verlopen de gewichtscurves van alle groepen vrijwel identiek.

Immunoneutralizatie van centraal IGF-1 laat weinig effect zien op het tijdstip van VO, hoewel het de plasmaspiegels van leptine significant verhoogt m.n. in de pair-fed dieren. Hierdoor lijken de *ad libitum* gevoede dieren meer te gaan eten; dit resulteert evenwel niet in een afwijkende curve in de gewichtsontwikkeling binnen de duur van het experiment (tot dag 42).

Discussie

Leptine lijkt dus een cruciale rol te spelen in de initiatie van puberteitsverschijnselen. Deze effecten van leptine worden verondersteld op centraal, hersenniveau te werken.
Tijdens de groei (van dag 22 naar 38) vormt zich steeds meer vet, en neemt het leptinegehalte in het plasma toe. Het vetpercentage wordt gehandhaafd op ongeveer 30%.
Gaandeweg (van dag 22 naar het tijdstip van puberteit) stelt zich een diurnaal ritme in wat leptine plasmawaarden betreft, waarbij de piek in de donkerfase ligt en het dal in de lichtfase; voor een nachtdier als de rat is dit niet vreemd.

Leptine toediening (alleen centraal) remt de voedselopname, waarschijnlijk omdat de dieren hierdoor eerder verzadigd zijn. Hoewel als gevolg hiervan de gewichtsontwikkeling achterblijft bij de controlegroep wordt het moment van VO niet verlaat maar zelfs licht vervroegd in dieren die centraal dan wel perifeer leptine toegediend krijgen.
De verzadigingseffecten van leptine werken uiteraard centraal, vandaar dat perifere toediening geen effect liet zien. Het perifeer toegediende leptine laat echter net als het centraal toegediende leptine eveneens een klein positief effect op het tijdstip van VO zien. Dit is een indicatie dat leptine in voldoende mate van perifeer naar centraal kan worden getransporteerd maar niet andersom. Er zijn inderdaad aanwijzingen voor transporters die dit mogelijk maken.

Voedselbeperking door energie in voer te vervangen door cellulose maar het eiwitgehalte in het dieet gelijk te houden, laat een verlaagde gewichtsontwikkeling zien die echter nog wel groei laat zien. De verlaging in de dagelijkse gewichtstoename moet op het conto geschreven worden van minder vetafzet hetgeen uit de vetbepalingen ook naar voren komt. Percentueel en absoluut bevatten de voerbeperkte dieren minder vet.
Dientengevolge zijn ook hun leptine plasmaspiegels verlaagd. De vertraagde puberteit, met als indicatie het verlate tijdstip van VO, kan uitgelegd worden als veroorzaakt door dit verlaagde leptine-level.

De waargenomen verlaagde gemiddelde lichaamstemperatuur in voerbeperkte dieren t.o.v. controles kan uitgelegd worden als een aanpassing aan de lagere energie-intake in deze dieren of kan een gevolg zijn van een lager isolatie-vermogen door de verminderde vetafzet in deze dieren waardoor deze moeilijker warmte kunnen vasthouden.
Ondanks de lage leptine-levels in de voerbeperkte dieren vertonen deze dieren toch op een gegeven moment VO en start hun puberteit. Door leptine toe te dienen kan dit moment wat worden vervroegd nog: leptine werkt derhalve positief hierop. Leptine is evenwel niet de enige factor die bepaalt dat puberteitsprocessen gestart worden. Het zou weliswaar de ‘trigger’ hiervan kunnen zijn, maar de fysiologie gebruikt een andere factor hiervoor (groeihormonen?) of start autonoom (rijpheid van het brein?) wanneer het leptine-signaal uitblijft of inadequaat is. Dieren deficiënt in leptine of leptine-receptoren blijven evenwel in een prepuberale status ‘steken’, hetgeen een cruciale rol als ‘puberty trigger’ voor leptine weer wel ondersteunt. In het FR-diermodel circuleert nog wel leptine, zij het weinig.
Ook de experimenten waarin het centrale leptine (geheel of deels) werd uitgeschakeld door antiserum ondersteunen eveneens de trigger-rol van leptine. In deze dieren was de aanvoer vanuit de periferie intact en onverstoord, het leptine werd echter continue weggevangen door de toegediende antilichamen.

De link van leptine met de groeimediatoren GH en IGF-1 is niet geheel helder, mede omdat er verschillende effecten gevonden werden in normaal gevoede en voerbeperkte dieren.
Ook de effecten van immunoneutralizatie van deze groeimediatoren waren niet altijd complementair aan de infusie-experimenten.

Als meest in het oog springend is het effect dat GH infusie heeft: het tijdstip van VO wordt verlaat terwijl de leptine plasma-levels enorm worden gestimuleerd. Langs welke weg dit laatste wordt bewerkstelligd door het centraal toegediende GH is onbekend, evenals waarom het tijdstip van VO hierdoor juist wordt vertraagd. De geringere gewichtstoename die waargenomen wordt doet vermoeden dat als gevolg van GH er minder vetaanzet plaatsvindt, maar vetbepalingen ontbreken om dit te bevestigen. Ook induceert het hoge centrale GH level mogelijk diabetogene effecten (lipolyse, hoge plasma vetzuur-levels) die in een later tijdstip van VO resulteren.

Opvallend was voorts dat verlaging van centrale GH-levels (door antiserum of somatostatine infusie) alleen in pair-fed dieren een vervroeging van het tijdstip van VO liet zien en geen effect ressorteerde in dieren die *ad libitum* toegang tot voer hadden. Een goede verklaring hiervoor is moeilijk; opvallend is wel dat de richting van het effect van het antiserum en somatostatine dezelfde kant (vervroeging VO tijdstip) op gaat als infusie van GH in FR-dieren, maar de andere kant op als GH-infusie in normaal gevoede dieren.

IGF-1 infusie laat in normaal gevoede dieren dezelfde effecten zien als GH, i.e. een verlaat tijdstip van VO, dit is op zich logisch daar de metabole effecten van IGF-1 overeenkomen met die van GH. De andere effecten van IGF-1 infusie en immunoneutralizatie zijn lastig te verklaren of te interpreteren. De rol van IGF-1 in de sturing en start van puberteitsprocessen lijkt derhalve niet zo groot. Maar onduidelijk blijft waarom toediening van IGF-1 antiserum de plasma leptine-waardes in hoge mate kan stimuleren zonder enig effect te laten zien op het tijdstip van VO of de voedselopname.

Conclusies

De bevindingen in dit proefschrift, hoewel niet allemaal eenduidig en te verklaren, voeren meer bewijs aan voor een rol van leptine als ‘trigger’ van de puberteit bij vrouwelijke ratten. Een functionele interactie is hierin aanwezig tussen leptine en groeimediatoren, hoe deze relatie precies ligt is evenwel op dit moment nog niet duidelijk.

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"Take knowledge from cradle to the grave"

(Prophet of Islam, Mohammad PBUH)

I am deeply grateful to Great God for keeping me healthy and making me able to successfully finish this work. Also, this work would never have been possible without the contribution of the other people around me.

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Saeed Zeinoaldini

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List of publications

Abstracts

Saeed Zeinoaldini, J.J.M. Swarts, B.J.M. Van de Heijning, D. Van der Heide (2004). Intracerebroventricular (icv) infusion of leptin and its effect on puberty onset in female rats. PhD-retreat 2004, Nijmegen, The Netherlands. p 46

Saeed Zeinoaldini, J.J.M. Swarts, B.J.M. Van de Heijning, D. Van der Heide (2004). Intracerebroventricular (icv) infusion of leptin and its effect on puberty onset in female rats. 37 th Annual meeting of Society for the Study of Reproduction (SSR), Vancouver, British Columbia, Canada. p 234

Saeed Zeinoaldini, J.J.M. Swarts, B.J.M. Van de Heijning, D. Van der Heide (2004). A signaling role for leptin in puberty onset in the female rats. 12 th International Congress of Endocrinology, Lisbon, Portugal. p 235

Saeed Zeinoaldini, J.J.M. Swarts, B.J.M. Van de Heijning, D. Van der Heide (2005). Chronic leptin infusion advances but immunoneutralization of leptin postpones puberty onset in female rats. 6th Puberty Conference, Evian, France.

Full papers

Saeed Zeinoaldini, J.J.M. Swarts, B.J.M. Van de Heijning (2004). A signaling role for leptin in puberty onset in the female rats? *Submitted*

Saeed Zeinoaldini, J.J.M. Swarts, B.J.M. Van de Heijning (2004). Chronic leptin infusion advances, but immunoneutralization of leptin postpones puberty onset in female rats. *Submitted*

Saeed Zeinoaldini, J.J.M. Swarts, B.J.M. Van de Heijning (2004). Bimodal effects of central infusion and immunoneutralization of growth hormone on the timing of puberty in the female rat. *Submitted*

Saeed Zeinoaldini, J.J.M. Swarts, B.J.M. Van de Heijning (2004). Exogenous but not endogenous insulin-like growth factor I (IGF-I) postpones puberty onset in the female rat. *Submitted*

Curriculum vitae

Saeed Zeinoaldini was born on March 30 th , 1969 in Khansar (Iran). He graduated from high school in Golpayegan city (Iran) in 1988. In the same year he was accepted as a BSc student in the field of Animal Science at the Isfahan University of Technology (Isfahan, Iran) and he achieved the title of 'distinguished student' at that university. Afterwards he was accepted as MSc student in Animal Physiology at the University of Tarbiat Modarres in Tehran (Iran) and obtained his MSc in 1996. After graduating, Saeed joint the teaching staff members of the Ilam University of Iran (Ilam, Iran) and he worked there for six years. During this time he was responsible for giving courses on subjects related to Animal Physiology and Biochemistry. Also, he has worked as Head of Agriculture Faculty for two years and as Deputy of Financial and Administrative of the Ilam University for almost three years.

In October 2001, he was awarded a grant of full PhD scholarship by the Ministry of Science, Research and Technology (MSRT) of Iran to study at the Human and Animal Physiology group, Wageningen University, the Netherlands. His research work was on "*the effects of leptin on puberty onset in female rats"*. He finished his PhD project within four years with financial support of MSRT of Iran. Since February 2004, he has become a member of "Society for the Study of Reproduction (SSR)", "The American Physiological Society (APS)" and "The Endocrine Society". After his return to Iran, Saeed will begin his work as Assistant Professor in the University of Ilam (Ilam Iran).

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پس، نهایتا می توان نتیجه گرفت که هورمون ***لپتین*** یکی از عوامل اساسی آغازگر بلوغ جنسی موش های ماده است.

با استفاده ازاثر محدودیت غذایی بر تاخیر زمان وقوع بلوغ جنسی به عنوان یک مدل آزمایشی، درادامه آزمایشات، هورمون رشد به صورت مرکزی، به موش های ماده نابا لغ تزریق شد. تزریق مرکزی هورمون رشد باعث تاخیر زمان وقوع بلوغ جنسی در حیواناتی شد که بدون محدودیت غذایی بودند؛ اما، درحیوانات با محدودیت غذایی عامل تسریع بلوغ جنسی شد. بعلاوه، میزان هورمون ***لپتین در*** پلاسما در حیوانات با تزریق هورمون رشد، بدون توجّه به وضعیت تغذیه ای آنها، به صورت چشمگیری بیشتر ازگروه شاهد بوده و با ادامه دریافت هورمون رشد، سطح هورمون ***لپتین*** در طول آزمایش افزایش یافت.

در مرحله بعد، آنتی بادی هورمون رشد به صورت مرکزی، به موش های ماده نابا لغ تزریق شد؛ این کار باعث تسریع زمان وقوع بلوغ جنسی فقط در حیواناتی شد که مقدارغذای آنها محدود شده بود، ولی تاثیری در گروه شاهد نداشت. تاثیرتزریق مرکزی هورمون سوماتوستاتین، درهردو گروه، که در مطالعه بعدی انجام شده بود شبیه آنتی بادی هورمون رشد بود. نتایج این آزمایشات اخیر با نتایج تزریق مرکزی هورمون رشد که تنها باعث تسریع زمان وقوع بلوغ جنسی در حیواناتی شده بود که توام با محدودیت غذایی بودند، مطابقت دارد.

درسری پایانی آزمایشات، ابتدا به صورت مرکزی، عامل رشد شبیه انسولین شماره1، به موش های ماده نابا لغ تزریق شد؛ این عمل باعث تاخیرمعنی داردرزمان وقوع بلوغ جنسی حیواناتی شد که بدون محدودیت غذایی بودند، امّا درحیواناتی که محدودیت غذایی اعمال شده بود تاثیری نداشت. سپس، عامل رشد شبیه انسولین شماره1 با تزریق آنتی بادی آن به صورت مرکزی مهارشد؛ این کاراگرچه باعث افزایش میزان هورمون ***لپتین در*** پلاسما شده بود، امّا تاثیری برزمان وقوع بلوغ جنسی نداشت. بنابراین، بر اساس این آزمایشات به نظر می رسد که عامل رشد شبیه انسولین شماره1 درروند بلوغ جنسی اثری نداشته یا لااقل تاثیرآن مستقل از هورمون ***لپتین*** باشد.

نهایتاً از مجموعه ی این مطالعات می توان نتیجه گرفت که:

هورمون لپتین، که عمدتاً بوسیله بافت چربی درحیوانات و انسان تولید ودرخون ترشح می شود، یک عامل مهم و اساسی درتنظیم هورمونی بلوغ جنسی بوده؛ ودرواقع، این هورمون " حلقه ی مفقوده " بین وضعیت تغذیه ای حیوان ازیک طرف ومغز(هیپوتالاموس) ازطرف دیگراست. بعلاوه، ما حدس می زنیم که بین هورمون رشد و هورمون لپتین درآغازکردن بلوغ جنسی یک رابطه دوجانبه همراه با همکاری وجود دارد.

خلاصه

عوامل متعددی در تنظیم زمان وقوع بلوغ جنسی درمحورهیپوتالاموس- هیپوفیز- گناد دخالت دارند. " ***لپتین***" وضعیت تغذیه ی حیوان و وجود مقدارکافی ازچربی ذخیره شده (به عنوان منبع بلند مدت انرژی بدن) را به مراکز مغزی گزارش کرده، و بدین وسیله فرایند تولید مثل (بلوغ جنسی) در حیوان آغاز می شود. علاوه بر این، محورهورمون رشد- عامل رشد شبیه انسولین شماره1 نیز تصور می شود که در تنظیم زمان وقوع بلوغ جنسی دخیل باشد.

هدف اصلی این تز بررسی این نظریه اخیر است که ***لپتین*** ممکن است مستقیماً یا غیرمستقیم ازطریق محور هورمون رشد- عامل رشد شبیه انسولین شماره1 ، عامل آغازکننده بلوغ جنسی باشد.

ابتدا، یک سری آزمایش های پایه و تشریحی انجام شد. زمان وقوع بلوغ جنسی با تشخیص اولین بازشدگی- واژن تعیین شد. نتایج این آزمایشات یک همبستگی قوی مثبت بین مقدارچربی بدن و هورمون ***لپتین*** پلاسما نشان داد ؛ ضمناً میزان ***لپتین*** پلاسما درطول زمان قبل ازبلوغ تدریجاً درحا ل افزایش بود. سپس، ما اثرات محدودیت غذایی را بر زمان وقوع بلوغ جنسی، مقدارچربی، سطح ***لپتین*** پلاسما و دمای بدن مورد بررسی قراردادیم. محدودیت غذایی اعمال شده در طول این مطالعات معادل 70 درصد انرژی بوده (30% کمتر ازگروه شاهد) ولی سایراجزاء غذا (شامل میزان پروتیین، موادمعدنی و ویتامین ها) مانند گروه شاهد بوده است. ِاعمال محدودیت غذایی زمان وقوع بلوغ جنسی رابه وضوح به تاخیرانداخت. درطول آزمایش، مقدارچربی، هورمون***لپتین*** پلاسما، و دمای بدن در حیواناتی که غذایشان محدود شده بود به طورچشمگیری کمتراز گروه شاهد بود.

درسری بعدی آزمایشات، با استفاده از نتایج مطالعات پایه قبلی و اثر محدودیت غذایی بر تاخیر زمان وقوع بلوغ جنسی به عنوان یک مدل آزمایشی استفاده شد، وبه منظور بررسی اینکه آیا ***لپتین*** عامل آغازگر بلوغ جنسی هست یا نه، ***لپتین*** ابتدا به صورت مرکزی (ازطریق بطن سوم مغز) و سپس ازطریق زیرپوستی (احشایی) به وسیله پمپ های کوچک اسمزی که مقداراندکی ازهورمون را به طور مداوم و ثابت انتقال می دهد، به موش های ماده نابا لغ تزریق شد؛ و درسری بعدی، به صورت مرکزی، آنتی بادی هورمون ***لپتین*** به وسیله این پمپ ها، تزریق گردید.

تزریق مرکزی ***لپتین*** نه تنها تاخیر بلوغ ناشی از محدودیت غذایی را جبران کرد بلکه زمان وقوع بلوغ جنسی را نیز درحیوانات بدون محدودیت غذایی، جلو انداخت. همچنین، تزریق زیرپوستی این هورمون، تاخیر بلوغ ناشی از محدودیت غذایی را شبیه تزریق مرکزی آن جبران نمود. بنابراین، ما در این سری از آزمایشات، تاثیر مثبت هورمون ***لپتین را*** چه به صورت مرکزی و چه زیرپوستی بر زمان وقوع بلوغ جنسی موش های ماده نابا لغ، به وضوح نشان دادیم. بالعکس، تزریق مرکزی آنتی بادی هورمون ***لپتین،*** زمان وقوع بلوغ جنسی را به تاخیر انداخت.

تنظیم هورمونی بلوغ جنسی درموش های ماده:

آیا لپتین یک حلقه مفقوده است؟

سعید زین الدینی

روز هجران و شب فرقت یار آخر شد
زدم این فال و گذشت اختر و کار آخر شد

آن همه ناز و تنعم که خزان می فرمود
عاقبت در قدم باد بهار آخر شد

شکر ایزد که به اقبال گله گوشهٔ گل
نخوت باد دی و شوکت خار آخر شد

صبح امید که شد معتکف پردهٔ غیب
کو برون آی که کار شب تار آخر شد

بعد ازاین نیز به آفاق دهم از دل خویش
که به خورشید رسیدیم و غبار آخر شد

آن پریشانی شب های دراز و غم دل
همه در سایهٔ گیسوی نگار آخر شد

با درم نیست ز بدعهدی ایام شکوه
قصهٔ غصه که در دولت یار آخر شد

ساقیا لطف نمودی قدحت پر می باد
که به تدبیر تو تشویش خمار آخر شد

در شمار ار چه نیاورد کسی حافظ را
شکر کان محنت بیرون ز شمار آخر شد

تقدیم به:

پدر و مادر مهربانم؛

همسر دلسوز و فداکارم؛

و گل های زندگی ام زهرا و امیرحسین

به نام خداوند جان و خرد

کزین برتر اندیشه برنگذرد