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Perinatal Distribution of Appetite Regulating Hormones in the Porcine IUGR Animal Model

Thesis submitted in fulfilment of the requirements for the degree of Doctor in Veterinary Sciences (PhD) by

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List of abbreviations

AGA	Appropriate for Gestational Age
BMI	Body Mass Index
BSA	Bovine Serum Albumin
BW	Body Weight
CCK	Cholecystokinin
CRL	Crown-Rump Length
EGF	Epithelial Growth Factor
ELISA	Enzyme Linked Immuno Sorbent Assay
FFT	Free Fraction of Tryptophan
GC	Ghrelin Cell
GH	Growth Hormone
GHS receptor	Growth Hormone Secretagogue receptor (ghrelin receptor)
GOAT	Ghrelin O-Acyl Transferase
HDL	High-Density Lipoprotein Cholesterol
HPLC	High Performance Liquid Chromatography
IGF	Insulin Growth Factor
IUGR	Intrauterine Growth Restriction
IR	Immunoreactive
LNAA	Large Neutral Amino Acid
mCPP	Meta-Chlorophenylpiperazine
NEFA	Non-esterified Fatty Acid
NGS	Normal Goat Serum
NS	Not Significant
NW	Normal Weight
Ob gene	*Obesity* gene (*Leptin* gene)
Ob-Rb receptor	Long isoform of the leptin receptor
PBS	Phosphate Buffered Saline
PF	Pig Foetuses

PFA	Paraformaldehyde
PI	Ponderal Index
RIA	Radioactive Immunoassay
RT	Room Temperature
RSD	Residual Standard Deviation
SD	Standard Deviation
SGA	Small for Gestational Age
SI	Small Intestine
TBS	Tris-Buffered Saline
TPH	Tryptophan Hydroxylase
Trp	Tryptophan
UZA	Antwerp University Hospital
V_v	Volume density
WSW technique	Weigh Suckle Weigh technique
5-HIAA	5-Hydroxyindoleacetic Acid
5-HT	5-Hydroxytryptamine, serotonin
5-HTP	5-Hydroxytryptophan

Chapter 1 Introduction

1 General Introduction

Early life (pre- and postnatal) processes, such as the regulation of appetite, have an enormous impact on programming the susceptibility to chronic diseases in adult life including obesity, cardiovascular diseases and diabetes mellitus (Barker, 2004). Many hypotheses have been put forward to explain the association between low birth weight and the increased risk to develop metabolic disorders in adult life. However, the molecular mechanisms underlying this association remain unclear. Since the gastrointestinal tract is responsible for nutrient digestion and absorption it plays a crucial role in perinatal development. Prenatal growth restriction occurs naturally in piglets from hyperprolific dams. Since the porcine gastrointestinal tract shows many similarities with the human digestive tract, the small for gestational age (SGA) piglet is suggested as an ideal animal model to study intrauterine growth restriction (IUGR) (Guilloteau *et al.*, 2010).

Although the link between nutrition and the gastrointestinal system is clear, very few studies focused on the role of the gastrointestinal system in the postnatal effects of prenatal undernutrition. Therefore, this thesis focuses on the appetite regulating hormones ghrelin, leptin and serotonin (5-hydroxytryptamine, 5-HT), which are abundantly present in the digestive tract of foetal and juvenile normal and SGA piglets. Ghrelin is the only appetite-stimulating hormone derived from the stomach (Kojima *et al.,* 1999). Leptin is considered as its counterpart and induces satiety (Campfield *et al.,* 1995). The third gastrointestinal derived hormone studied in this thesis is 5-HT. Although this monoamine is primarily recognized as a neurotransmitter, it has become clear in recent years that 5-HT is also implicated in the energy balance and satiety signalling (for review see Donovan and Tecott, 2013). As such, the results of this thesis provide information on the endocrine imbalances in the gastrointestinal tract in perinatal SGA piglets. Because of the high similarities with man, these findings are relevant for both prenatal growth restricted pigs and humans.

2 IUGR

2.1 IUGR in human medicine

IUGR is common in some mammals, such as humans and pigs, and thus forms a major problem for both human health (for review see McMillen and Robinson, 2005) and animal production (for review see Wu *et al.*, 2006).

The term IUGR refers to the failure of foetuses to achieve their intrinsic growth potential as a result of impaired foetal growth due to adverse intrauterine circumstances (Wollmann, 1998). There are several methods to distinguish IUGR foetuses from normal weight (NW) foetuses. The most common criterions are 1) an estimated weight below the 10^{th} percentile for its gestational age or 2) a body weight (BW) that is at least 2 standard deviations (SD) below the mean weight for the respective gestational age (ACOG, 2013). However, IUGR is distinct from the term SGA. More specifically, SGA neonates have a constitutional low BW, whereas IUGR neonates have a low BW because one or more documented risk factors inhibited foetal growth (Table 1.1). Hence, SGA neonates are contrarily to IUGR neonates not pathologically growth restricted. The incidence of IUGR in the total human population is estimated between 3 and 7% (for review see Romo *et al.*, 2009).

Two types of IUGR can be distinguished: symmetrical and asymmetrical. Symmetrical IUGR means that weight, length and head circumference are low. This form of IUGR usually originates early in pregnancy. IUGR is characterized as asymmetrical when the head circumference is within normal limits as a consequence of brain sparing, which will be described in section 2.3 of this thesis (Crane and Kopta, 1980 ;for review see Rosenberg, 2008).

Foetal risk factors	Placental risk factors	Maternal factors
Aneuploidies: triploidy, trisomy 13, 18 and 21	Uteroplacental vascular insufficiency	Smoking, alcohol, ilicit drugs
Congenital infections: rubella, HIV, toxoplasmosis	Hematoma	Extremes of maternal age (<16 years or > 35 years)
Russell-Silver syndrome	Infarction	Vascular diseases: hypertension, pre-gestational diabetes
		Parity
		Low maternal weight gain and nutrition

Table 1.1 Foetal, placental and maternal risk factors for the development of IUGR

Foetal causes of intrauterine growth restriction include chromosome abnormalities and genetic defects whereas maternal factors include age, weight and height, parity (number of times a female has given birth), chronic diseases, infections, impairment of nutritional status and substance abuse. Placental factors contributing to prenatal growth restriction are structural abnormalities and insufficient uteroplacental perfusion (Bernstein and Divon, 1997) (Table 1.1). When placental deficiency is involved, foetal growth is normal until the growth rate exceeds the substrate provision, generally during the third trimester (for review see Rosenberg, 2008).

2.2 IUGR in pork industry

In pork industry, pregnant sows are not monitored like pregnant women. Therefore, the distinction between IUGR and SGA in piglets is different compared to what has been defined in human paediatrics (see section 2.1). Body proportionality, measured by ponderal index (PI), provides a valuable indication of mortality risk in piglets (Baxter *et al.*, 2008). Therefore, piglets with a significantly lower BW compared to their normal littermates that have a normal allometry are classified as SGA piglets whereas IUGR piglets display a disproportional allometry (Bauer *et al.*, 1998).

The major goal of animal production is to enhance its efficiency in order to meet consumers demand. However, due to the use of hyperprolific sows with a high ovulation rate, pork industry faces the highest amount of naturally occurring IUGR seen in domestic mammals (for review see Wu *et al.*, 2006; Wang *et al.*, 2008). Low birth weight has an enormous impact on neonatal survival in the pig industry, as evidenced by the higher rate of pre-weaning deaths in this BW category occurring within the first 72 h post partum (Quiniou *et al.*, 2002). Hence, the increase of low birth weight and within-litter variation in birth weight is not only an economic threat in pork industry but also has its ethical restraints on animal welfare.

The high ovulation rate in these hyperprolific sows causes intrauterine crowding (Dziuk, 1968; for review see Foxcroft *et al.*, 2006). The percentage of prenatal growth restricted piglets ranges from 7% when the litter consists of 11 piglets to 25% in case of a larger litter size (Martineau *et al*., 2009). The negative impact of intrauterine crowding on BW probably results from a lower nutrient supply during gestation. Indeed, when litter size increases, the uterine blood flow also increases but to a lower extent than the number of foetuses (Père and Etienne, 2000). Hence, foetal growth rate is reduced as a consequence of undernutrition. However, one study suggests that foetal growth rate is less sensitive to intrauterine crowding than to placental insufficiency (Vallet *et al.*, 2003). Moreover, maternal immaturity is also a risk factor for developing prenatal growth restricted piglets (for review see Wu *et al.*, 2006). Domestic mammals are often bred when they are still immature in order to maximize their production performance. Because mother and foetus both need nutrients during pregnancy, the risk for perinatal growth restricted piglets increases (for review see Wu *et al.*, 2006). Although the sow is undernourished, the piglets' milk intake is set to high priority. Hence, the sow will mobilise body tissue reserves in order to maintain milk production (Eissen *et al.,* 2000). However, others describe the concept 'maternal constraint' in sows that were food restricted. More specifically, the litters from sows that were food restricted did show a reduced foetal weight when litter size increased whereas this negative relationship between litter size and

birth weight was absent in litters from sows that were fed ad libitum (Musser *et al.,* 2004).

Adequate colostrum intake is essential for neonatal piglets in order to develop immune protection and to prevent hypoglycaemia to which they are prone (Le Dividich and Noblet, 1984). However, prenatal growth restricted piglets have an impaired vitality, hence are less capable to move to the udder and suckle colostrum (Fraser and Rushen, 1992; Tuchscherer *et al.*, 2000). Because of this reduced milk intake, prenatal growth restricted piglets suffer from impaired neonatal health and survival. Besides its negative impact on pre-weaning survival, available results indicate that low birth weight pigs have a poorer carcass and meat quality (Milligan *et al.*, 2001; for review see Foxcroft *et al.*, 2006).

Intrauterine malnutrition also may have its implications in later development since it impairs the structure and functions of many organs in the foetus. Low birth weight as a consequence of IUGR is indeed one of the major causes of perinatal morbidity (Bernstein *et al.*, 2000; for review see Wu *et al.*, 2006) and increased risk of metabolic diseases in adult life, such as obesity, impaired glucose tolerance and cardiovascular diseases (Poore and Fowden, 2002; Barker, 2004; Poore and Fowden, 2004a) in both humans and pigs.

2.3 IUGR and its long-term consequences: perinatal programming

2.3.1 The thrifty phenotype hypothesis

The association between an adverse intrauterine environment and the long-term metabolic consequences has led to the concept of 'perinatal programming'. Programming of satiety and hunger signalling occurs during the neonatal period and as such influences appetite and food intake later in life (for review see Cripps *et al.*, 2005).

The thrifty phenotype hypothesis states that the growing foetus, which is exposed to intrauterine malnutrition, uses at least 2 strategies in order to survive (Hales and Barker, 1992; for review see Brenseke *et al.,* 2013). The first strategy is assuring brain

growth by diverting nutrients to the brain at the expense of body growth and the development of other organs. Hales and Barker argued that foetal malnutrition reduces β cell mass and islet cell function (Hales and Barker, 1992). These pancreatic impairments track on into adult life, when they are associated to diabetes. The thrifty phenotype hypothesis also affects the growth of the liver. Two of its functions: regulation of cholesterol and blood clotting are permanently disturbed (Barker *et al.,* 1993). Reductions in adipose tissue and skeletal muscle mass also have been proposed (Desai *et al.,* 1996; Shepherd *et al.,* 1997). The consequences of foetal malnutrition on the gastrointestinal system will be thoroughly discussed in section 3.3.

Secondly, adult diseases originate through foetal adaptations to adverse events, such as undernutrition, which results in permanent changes in endocrine and metabolic processes (Hales and Barker, 1992). These permanent changes include increased hepatic gluconeogenesis, enhanced release of fatty acids and glucose uptake from adipose tissue. This metabolic reprogramming occurs to promote survival under poor postnatal nutrition. However, if the child/piglet is born in adequate nutritional conditions, this will conflict with the earlier reprogramming. Thus, metabolic diseases may occur in later life (Hales and Barker, 1992). The 'Barker hypothesis' proposes that organs and associated functions undergo this programming during critical periods in embryonic and foetal life, which determines the physiological and metabolic responses in adulthood (Barker, 1998). Several studies showed that these critical periods extend until the neonatal period (Rolland-Cachera *et al.*, 2004; Waterland, 2005).

2.3.2 The foetal salvage hypothesis

This hypothesis challenges the thrifty phenotype hypothesis. Whereas the latter proposes a 'brain sparing' mechanism at the expense of other organs such as the pancreas (resulting in β cell hypoplasia), the foetal salvage hypothesis suggests that the malnourished foetus develops peripheral insulin resistance to ensure that adequate amounts of glucose are delivered to organs which are more essential for

survival such as the brain (Hofman *et al.*, 1997; for review see Brenseke *et al.*, 2013). This reduced insulin sensitivity stimulates β cells to secrete more insulin to achieve normal glycaemia and would eventually lead to β cell exhaustion.

Neonatal piglets are prone to hypoglycaemia due to the absence of brown adipose tissue and low glycogen storage. Additionally, the metabolism of the newborn pig is governed completely by the concentration of circulating glucose concentrations. A fall in blood sugar concentration in the starving newborn is associated with a general functional collapse of the whole body, which is also called the 'baby pig syndrome' (Goodwin, 1957). When newborn pigs are treated with insulin, blood glucose levels decrease in such a way that it resembles the state that is seen during starvation (Goodwin, 1957). Hence, neonatal piglets do not seem to suffer from peripheral insulin resistance. It should be noted that the porcine liver only provides 15% of the available glycogen whereas in human infants the liver provides 40% of glycogen (Mellor and Cockburn, 1986). This is of interest because liver glycogen is the primary source of circulating glucose in the unfed newborn (Shelley and Neligan, 1966). Prenatal growth restriction however, has little or no effect on the quantities of glycogen in piglets (Mellor and Cockburn, 1986; De Vos *et al.*, under revision). Altogether, these results question whether the foetal salvage hypothesis explains the association between low birth weight and long-term metabolic consequences in the pig.

2.3.3 The catch up growth hypothesis

Catch up growth is the period during which juveniles compensate their delayed growth in order to obtain their genetically determined size. It may occur at any time during the growth process, but it is most commonly observed during the first 2 years of life (in humans) (Leger *et al.*, 1996). This compensatory growth regularly results in overcompensation. Consequently, the individual exceeds normal BW with often an excessive fat deposition. They thereby develop an increased risk for the occurrence of metabolic disturbances (Cianfarani *et al.*, 1999; Ong *et al.*, 2000; for review see Brenseke *et al.,* 2013).

Studies in rats have shown that early postnatal nutrition and growth indeed can program body size in later life (McCance, 1962). These results emphasize that besides the prenatal period, the early postnatal period also is involved in the programming of diseases later in life (Guilloteau *et al.*, 2009). One study showed that catch up growth also occurs in the pig and is directly associated with impaired glucose tolerance at 12 months of age (Poore and Fowden, 2002). The latter finding hence supports the catch up growth hypothesis in prenatal growth restricted piglets. Another study showed that, despite the relative higher daily weight gain in low birth weight piglets, the growth retardation persisted during the whole 70 days they examined those piglets (Morise *et al.,* 2011).

2.4 Pig as a metabolic IUGR animal model

Human epidemiological studies link the metabolic syndrome with prenatal undernutrition (Barker, 2004). These epidemiological studies provide substantial evidence for the 'foetal origins of adult disease' theory. However, because of the difficulties inherent to long-term studies and because of the ethical restrictions of performing pathogenetic studies in children, research also focused on animal models. IUGR can be experimentally induced in several species, such as rodents and sheep. The sheep is a large animal model, which facilitates surgical procedures, allowing repetitive sampling from non-anaesthetized pregnancies (for review see Barry *et al.*, 2008). These procedures include severe maternal caloric restriction and surgical procedures to induce placental insufficiency (for review see Armitage *et al.*, 2004; Barry *et al.*, 2008). However, the ruminant metabolism of the sheep, which acquires its nutrients from plant-based food by fermentation, is difficult to extrapolate to the omnivorous metabolism of humans. In rodents, guinea pigs and rabbits with an herbivorous metabolism, several approaches have been used to induce IUGR (reviewed by Haugaard and Bauer, 2001) (Table 1.2).

Model	Approach	Animal
Nutritional model	Maternal fasting	• Rodentia • Guinea pig
	Protein restriction	• Rodentia
Surgical model	Uterine Artery Ligation	• Rodentia • Guinea pig
	Electrically induced thermal placental injury	• Rabbit
Hypoxia model	Subjecting pregnant model to hypoxia	• Rodentia
Drug Induced model	• Glucocorticoids	• Rodentia
	• Inhibitors of 11 β-hydroxysteroid dehydrogenase	• Rodentia
	• Dihydroergotamine	• Guinea pig

Table 1.2 IUGR models from rodents and rabbits (reviewed by Haugaard and Bauer, 2001)

One study however, critically appraised the rodent model where the uterine artery is bilaterally ligated (Neitzke *et al.,* 2008). The surgical procedure did not lead to IUGR nor to catch up growth in the offspring in these rodent models. Moreover, no data indicating increased diabetogenic or adipogenic risk were present in the offspring of the rodent model (Neitzke *et al.,* 2008). Additionally, gastrointestinal organogenesis begins around towards the end of gestation in rodents, whereas in humans it already starts during the first trimester (Buddington, 1994). The gastrointestinal development of domestic animals, including pigs, is more similar to humans compared to rodents (for review see Sangild, 2006; Guilloteau *et al*., 2010), which will be described in more detail in part 3.2 in this chapter.

Interestingly, IUGR occurs naturally in piglets from hyperprolific dams. Moreover, the pig nearly reproduces all of the phenotypic pathological consequences of IUGR such as increased adiposity (Poore and Fowden, 2004b) and glucose intolerance (Poore and Fowden, 2002). Catch up growth in the first month of life was also directly

associated with impaired glucose tolerance when the pigs reached 1 year of age (Poore and Fowden, 2002). In the context of this thesis, the pig is also an ideal animal model since this species shows strong similarities to humans in term of changes in energy metabolism during development (reviewed by Mota-Rojas *et al.*, 2011). The process of foetal metabolis is complex as it involves the interaction of mother, placenta and foetus. Several studies in human and animal models have shown that glucose is the primary source of energy for the foetus (for review see Kalhan, 2000). However, in pigs foetal glucose levels are also influenced by the number of foetuses in the litter (Comline *et al.,* 1979). Foetal glucose metabolism is directly dependent on foetal plasma glucose concentrations. The utilization of foetal glucose is augmented by insulin produced from the foetal pancreas of which the concentrations increase as gestation proceeds. Hence, glucose utilization in insulin sensitive tissues such as skeletal muscle, liver, heart and adipose tissue, increases during gestation (for review see Mota-Rojas *et al.*, 2011).

At birth, the newborn has to maintain normoglycemia. Prenatally, glucose levels are maintained by the transplacental transfer of glucose from the mother. At birth however, there is a critical period when the newborn depends on its own hepatic glycogen stores to maintain blood glucose levels until it suckles. As already mentioned in section 2.3.2, neonatal piglets have less hepatic glycogen stores compared to human neonates, hence are more prone to hypoglycaemia.

3 Gastrointestinal system

3.1 Microscopical morphology

The porcine gastrointestinal system resembles morphologically the human digestive system (for review see Guilloteau *et al.*, 2010). The wall of the digestive tube consists of 4 layers that show a basic histological organisation. These layers are the tunica mucosa, tela submucosa, tunica muscularis and tunica serosa (Figure 1.1).

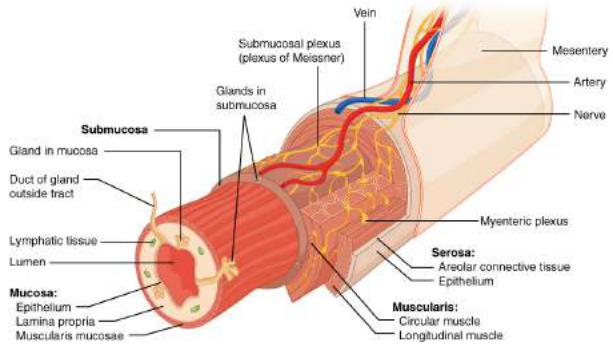

Figure 1.1 Histological organisation of the gastrointestinal tract (adapted from http://cnx.org/content/m46506/latest/?collection=col11496/latest)

Because the digestive tract has differing functions along its length, the morphology of these layers is different in the various parts of the gastrointestinal system. The tunica mucosa is the innermost layer of the gastrointestinal tract and consists of an epithelium and glands that extend into the underlying layer of loose connective tissue, the lamina propria. The gastric glands of the pars fundica, also called the oxyntic glands indeed extend the length of this tunica mucosa. Each gland consists of three regions (Figure 1.2). The junction between the gastric pit and the gastric gland is called the isthmus. The upper part of the gland is called the neck, whereas the deepest portion is called the base of the gland. In the glands of the pars fundica, different cell types can be found (Figure 1.2). The parietal cells, also called the oxyntic cells, are involved in gastric acid secretion. The mucous neck cells produce mucus in order to protect the mucosa against the corrosive nature of this gastric acid. The chief cells secrete pepsinogen, which is converted into pepsin by the acid environment of the stomach. Pepsin is an enzyme, which is involved in degrading food proteins into peptides. The enteroendocrine cells are specialized endocrine cells of the gastrointestinal tract. They produce and secrete hormones in a paracrine way, hence signal to nearby cells or in an endocrine way by secreting directly into the bloodstream. These type of cells wil be thoroughly discussed in section 3.4.

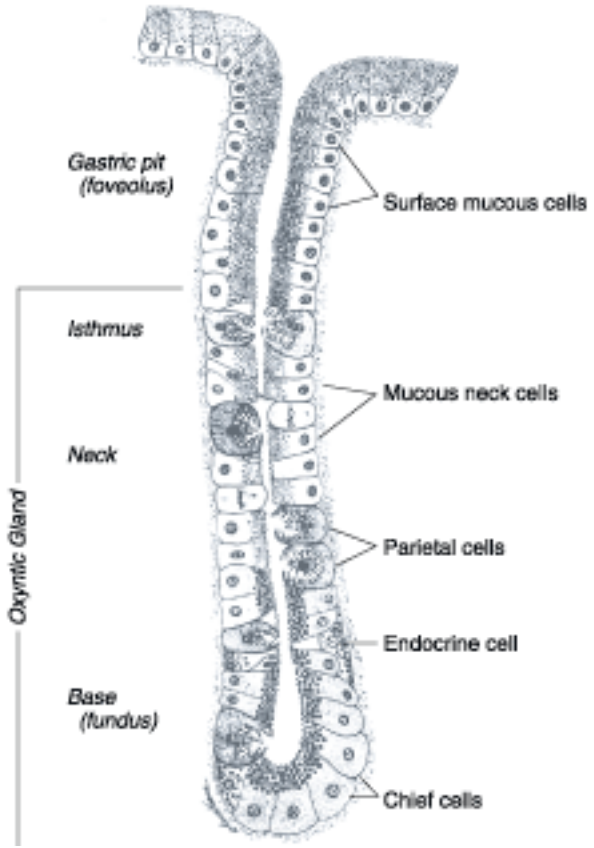

Figure 1.2 Schematic representation of an oxyntic gland
http://rezidentiat.3x.ro/eng/ulcereng.htm

The outer boundary of the tunica mucosa contains an inner circular and an outer longitudinal layer of smooth muscle and is called the tunica muscularis mucosae. The tela submucosa is located under the tunica mucosa and consists of connective tissue and a submucosal (Meissner's) nerve plexus. In large animals this submucosal nerve plexus can consist of an inner (Meissner's) and an outer submucous (Schabadasch's) plexus (Gunn, 1968; for review see Timmermans *et al.*, 2001). Underneath the tela submucosa, the tunica muscularis is formed by two layers of smooth muscle cells in the intestine: an inner circular and an outer longitudinal layer. Between these two muscle layers, the myenteric (Auerbach's) nerve plexus is situated. The stomach contains three muscle layers in the tunica muscularis: an inner oblique muscle layer, a middle circular layer and an outer longitudinal muscle layer. The tela serosa is a thin layer of loose connective tissue that surrounds the visceral organs.

The different layers of the small intestine (SI) show a developmental growth pattern. Studies in perinatal piglets have shown that the volume density (V_v) of the tunica mucosa (Van Ginneken *et al.*, 2001; Van Ginneken and Weyns, 2004) increases after birth while the V_v of the tela submucosa and tunica muscularis decrease after birth (Van Ginneken and Weyns, 2004). The two latter layers increased only after weaning (Van Ginneken *et al.*, 2001). Hence, while the growth of the tunica mucosa is related to birth related changes, the development of the tela submucosa and tunica muscularis is more linked to the changes at weaning. Moreover, the different layers of the SI also show regional differences. The tunica muscularis is thicker in the ileum, the distal part of the SI, compared to the other intestinal regions to facilitate the transfer of chyme from the SI into the large intestine (Van Ginneken and Weyns, 2004).

3.2 Perinatal development of the gastrointestinal tract: species differences

The development of the gastrointestinal tract is closely related to body growth and is affected by nutrition and feed intake. The gastrointestinal system is not fully mature at birth (Gershon and Thompson, 1973) since it has to adapt postnatally to dietary changes (Henning, 1981) and bacterial colonisation (for review see Bailey *et al.*, 2005; Perez *et al.*, 2007). In humans, the maturation process of the gastrointestinal system starts relatively early (during the first part of gestation) and progresses slowly. Rodents still have a very immature gastrointestinal system at birth whereas in domestic animals, like pigs, the timing and rate of gastrointestinal maturation are intermediate. In pigs, major maturational processes take place both prenatally and shortly after weaning (for review see Sangild, 2006) (Figure 1.3).

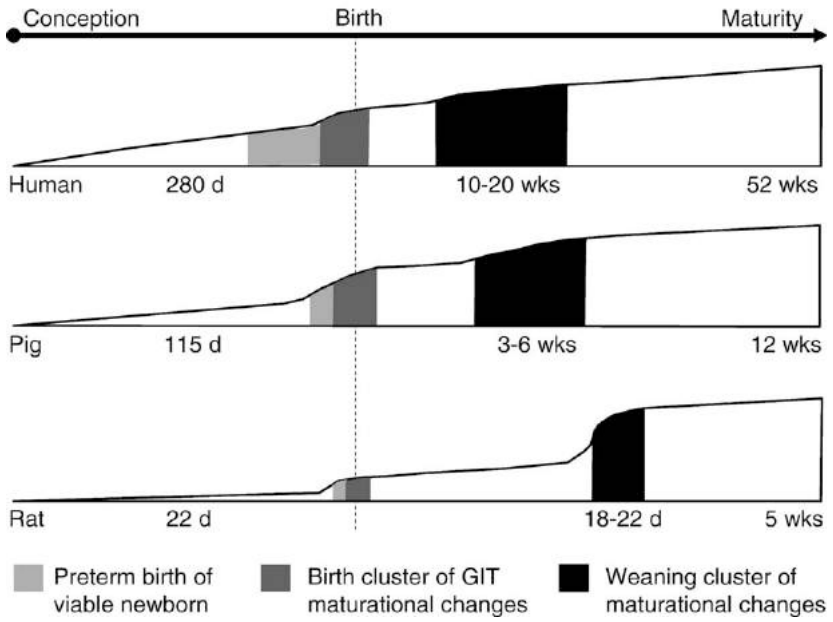

Figure 1.3 The timing of gastrointestinal maturation in three different mammalian species Reprinted from (Sangild, 2006)

As Figure 1.3 shows, gastrointestinal maturation is mainly clustered in two developmental periods: around birth and weaning. At these time points, dietary habits change and hence are accompanied by profound adaptations (for review see Sangild, 2006). As such, the perinatal development of the gastrointestinal system can be divided into three phases.

The first phase is the prenatal phase, which is characterised by minimal stimulation from the gastrointestinal lumen. Most of the structural elements are already present in the prenatal period. In the pig, the SI grows more rapidly than the body itself in the week before parturition. Its relative weight increases 70-80% over the last 3 weeks of gestation (McPherson *et al.,* 2004; for review see Sangild *et al.*, 2000).

The second phase is the neonatal stage, which is associated with milk intake. Hence, the gastrointestinal tract must cope the transition from parenteral nutrition via the placenta before birth to enteral milk consumption after birth. The previously mentioned birth cluster ensures that the gastrointestinal system grows and matures very rapidly in the weeks before birth. It has been shown that several hormonal factors influence this phase of gastrointestinal development (for review see Sangild

et al., 2000). At birth, the functional immaturity of the hormonal systems is compensated by peptides present in colostrum and milk, like insulin growth factor (IGF), epithelial growth factor (EGF) and insulin (for review see Wagner *et al.*, 2008). Moreover, these bioactive substances are known to stimulate gastrointestinal mucosal proliferation and facilitate the closure of the neonatal gastrointestinal tract (Takeda *et al.,* 2004; for review see Wagner *et al.*, 2008). The physiological significance of these milk-born factors is widely accepted and supported by the following: 1) higher concentrations of these hormones in colostrum compared to mature milk; 2) presence of specific receptors for these bioactive substances throughout the whole gastrointestinal tract and 3) relative resistance and stability of these factors for the proteolysis in the gastrointestinal tract (for review see Wagner *et al.*, 2008). Hence, maternal milk supports the postnatal development of the gut as an endocrine organ until it is adequately developed (Guilloteau *et al.*, 1992; Xu, 1996; Blattler *et al.*, 2001). The prenatal phase affects the postnatal gastrointestinal function particularly during the first postnatal days.

The third phase is the post-weaning stage during which the digestive system has to adapt to solid food (for review see Zabielski *et al.*, 2008). Since this study focuses on the suckling period, the changes inherent to the weaning stage are not discussed in this thesis.

Which maturational cluster is the most pronounced, depends on the species. In species with a relatively mature gastrointestinal tract at birth (e.g. domestic animals) the birth cluster is more pronounced, whereas the weaning cluster is more important in animals with a less developed gastrointestinal system at birth (e.g. rodents). The information for human infants is limited. However, it is likely that the human gastrointestinal system gradually matures, hence with less pronounced maturational clusters, because of the earlier development of the gastrointestinal tract compared to the gastrointestinal development in other species (Figure 1.3) (for review see Sangild, 2006).

3.3 Consequences of IUGR on gastrointestinal development

Gastrointestinal development in mammals is preprogrammed (for review see Sangild, 2006). However, this process can be enhanced or diminished depending on the intrauterine and early postnatal conditions. Gastrointestinal maturation is indispensable for optimal digestion and absorption of nutrients. In large domestic animals, such as pigs, birth takes place just after the finalisation of several maturational changes in essential organs, including the gastrointestinal tract (Bjorklund *et al.*, 1987; for review see Van der Lende *et al.*, 2001).

Compared to normal piglets and infants, IUGR individuals have smaller organs and have a dysfunctional gastrointestinal system. IUGR is associated with a proportionally greater intestinal length but thinner intestinal and gastric wall (Xu *et al.,* 1994). The intestinal surface is also reduced due to a reduced villus number size and a thinner intestine (Shanklin and Cooke, 1993; Xu *et al.*, 1994; Wang *et al.*, 2005; D'Inca *et al.*, 2010a; D'Inca *et al.*, 2010b).

An impaired gastrointestinal system may have implications on the further development and body growth of the IUGR infant since the gastrointestinal tract is the only means of acquiring nutrients after birth. Indeed, IUGR neonates are predisposed to feeding intolerance, and digestive diseases early in postnatal life (Lesage *et al.,* 2004; Bozzetti *et al.,* 2013; for review see Bozzetti *et al.*, 2013). Morphological and physiological alterations in intestinal development, such as structure atrophy and impaired nutrient absorption, can be responsible for the latter outcomes (Tillig *et al.*, 1995; Bjornvad *et al.*, 2005).

3.4 Enteroendocrine cells: development and distribution

Unlike endocrine cells in the pancreas, enteroendocrine cells are scattered as individual cells throughout the intestinal mucosa. Very little is known about how the direction of these region-specific expression of these hormones is determined. Enteroendocrine cells derive from pluripotent intestinal stem cells in the intestinal crypts. Differentiation of these enteroendocrine cells is controlled by the sequential expression of basic helix loop helix transcription factors Math1, Neurogenin 3 and

NeuroD (for review see Li *et al.,* 2011). As cells exit the stem cell compartment at the crypt base, they can become absorptive enterocytes or secretory cells (goblet cells, Paneth cells or enteroendocrine cells). Math1 specifies the secretory cell lineage (Yang *et al.,* 2001) whereas Neurogenin 3 is responsible for the enteroendocrine line specification (Jenny *et al.,* 2002). NeuroD defines a subset of the enteroendocrine cells (Naya *et al.,* 1997). Although the key functions of these transcription factors have been uncovered, there are still many unanswered questions in exactly how these factors control the multitude of endocrine cell types. The mammalian gastrointestinal tract indeed has the largest population of hormone producing cells in the body (for review see Rehfeld, 1998). More specifically, the gastrointestinal system has at least 15 different enteroendocrine cell types, which are categorized according to their morphology, location and hormone expression (for review see Hocker and Wiedenmann, 1998). The initial stimulus to release these hormones is the ingestion of food. Food provides nutrition stimulation in the gastrointestinal epithelial cells and mechanical stimulation. These signals further stimulate the release of peptides and other transmitters from the gastrointestinal mucosa where they can act locally or enter the bloodstream to circulate to distant target tissues. As such, chemical messengers from the gastrointestinal tract can affect the whole body.

During the early postnatal period, intestinal mucosal growth is not only manifested by increasing its size and weight, but also in profound tissue remodelling, i.e. the exchange of cell types leading to a modification of gastrointestinal function (Zabielski *et al.*, 2005). This epithelial remodelling process also depends on local regulators that are involved in the control of proliferation and programmed cell death, like IGF and other hormones from colostrum and milk, such as leptin and insulin (for review see Godlewski *et al.*, 2005; Zabielski *et al.*, 2005). The enteroendocrine cells of the gastrointestinal tract, together with the circulatory levels of their hormones, develop relatively early in gestation in both humans and domestic animals compared to rodents (Alumets *et al.*, 1983; Adrian *et al.*, 1995) (Table 1.3). Since this thesis focuses on ghrelin, leptin and 5-HT, we will only describe the corresponding endocrine cell types.

Ghrelin is known to be produced by the X/A-like cells in rodents (Dornonville de la Cour *et al.*, 2001) and the P/D1- cells in humans (Date *et al.*, 2000). In pigs, ghrelin is also secreted by a distinct cell type, i.e. the ghrelin cell (GC) in the gastrointestinal tract (Wierup *et al.*, 2007). In the adult porcine stomach, ghrelin is additionally detected in parietal as well as principal cells of the fundus, possibly to regulate gastric acid secretion. The immunoreactivity of ghrelin in the gastric mucosa is not different in fasted or fed pigs (Vitari *et al.*, 2010). Ghrelin in the rat and human foetal pancreas is produced by the endocrine ε cell type (Wierup *et al.*, 2002; Wierup *et al.*, 2004; Andralojc *et al.*, 2009). The origin of ε cells remains controversial. Ghrelin has also been described to be present in α cells in rats and humans (Date *et al.*, 2002) and in β cells in humans (Volante *et al.*, 2002). The abundance of ghrelin in the foetal pancreas suggests that ghrelin might regulate β-cell development.

Leptin is secreted by the chief cells of the oxyntic mucosa (Bado *et al.*, 1998) whereas 5-HT is secreted by the enterochromaffin cells in the gastrointestinal tract (Erspamer and Asero, 1952). Interestingly these enterochromaffin cells in the gastrointestinal tract are the major source of total body 5-HT (for review see Gershon, 2013).

As already mentioned, many questions remain how different enteroendocrine cells differentiate in the intestinal epithelium. Likewise, in regards to the hormones ghrelin, leptin and 5-HT the exact differentiation mechanisms are still unrevealed. Ghrelin and serotonin cells are still present in Neurogenin 3-null mice (Jenny *et al.,* 2002). However, other transcription factors than basic helix loop helix factors are also implicated during enteroendocrine cell differentiation. Instead of controlling the global differentiation of enteroendocrine cells like the basic helix loop helix factors, these factors like Nkx2.2 are likely to play a role in fine-tuning cell specification decisions within the enteroendocrine population. Interestingly, the number of ghrelin expressing cells is increased in Nkx2.2 deficient mice. Further analysis suggested that GCs are specified at the expense of other enteroendocrine cells (Desai *et al.,* 2008).

Cell type	Species	Location	Period	Reference
Ghrelin cells	Rodents	Stomach	Third trimester of gestation	(Hayashida *et al.*, 2002)
	Human	Stomach, intestine	Second trimester of gestation	(Rindi *et al.*, 2002)
	Pig	Stomach, intestine	Third trimester of gestation	Chapter 3
Leptin cells	Rodents	Stomach	Weaning period	(Oliver *et al.*, 2002)
	Human	Stomach, intestine	Second trimester of gestation	(Aparicio *et al.*, 2005)
	Pig	Stomach, intestine	Third trimester of gestation	Willemen, unpublished data
Enterochromaffin cells (5-HT)	Rodents	Stomach intestine	Third trimester of gestation (near term)	(Ekelund *et al.,* 1985; Branchek *et al.,* 1989)
	Human	Stomach, intestine	Second trimester of gestation	(Singh, 1963)
	Pig	Stomach, intestine	Second trimester of gestation	(Van Ginneken *et al.*, 2001)

Table 1.3 Gastrointestinal distribution of ghrelin, leptin and enterochromaffin cells during the prenatal period in rodents, humans and pigs

In section 2.3 several theories explaining the epidemiologically evidenced link between IUGR and the metabolic syndrome in later life were described. However, mechanisms underlying these hypotheses are still not clear.

Given the importance of the gastrointestinal system and its hormones in perinatal development, it is of interest to explore the hormonal distribution in the

gastrointestinal system and its morphological alterations that are associated with IUGR. Since satiety and hunger signalling are programmed during the perinatal period, the next sections describe ghrelin (section 4), leptin (section 5) and 5-HT (section 6), three gastrointestinal hormones that are implicated in perinatal development and appetite regulation.

4 Ghrelin

4.1 Synthesis and regulation

This study focuses on ghrelin because it is the only identified appetite-stimulating hormone derived from the gastrointestinal tract (Kojima *et al.*, 1999). Human ghrelin is synthesized from a 117 amino acid consisting pre-prohormone. Cleavage of preproghrelin results in two ghrelin molecules, i.e. a 28 amino acid form (C-terminal Arg) or a 27 amino acid form (C-terminal Pro) (Hosoda *et al.*, 2003). Porcine ghrelin is synthesized from a 118 residue consisting pre-propeptide by post-translational cleavage (for review see Dong *et al.*, 2009) (Figure 1.4). The third residue, a serine, is acylated with *n*-octanoic acid or *n*-decanoic acid. Ghrelin O-Acyl Transferase (GOAT) is the enzyme responsible for this acylation. Ghrelin is a highly conserved hormone across vertebrate species, especially in the N- terminus (for review see Kojima *et al.*, 2008). This suggests that the biological activity is determined in the N-terminus, where the octanoylated serine residue is localised.

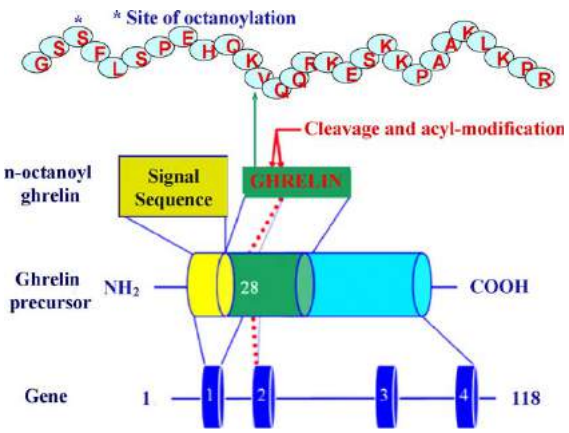

Figure 1.4 Porcine ghrelin. Reprinted from (Dong *et al.*, 2009)

Acylated ghrelin is thought to be the biologically active form because of its high binding and activating capability to the known ghrelin (growth hormone secretagogue, GHS) receptor. This receptor has 2 isoforms, GHS receptor 1a that is involved in the control of growth hormone (GH) secretion, and GHS receptor 1b, of which the function remains unknown (Gnanapavan *et al.*, 2002). Recent data suggest that des-acyl ghrelin probably also has specific biological roles, like cardioprotection (Li *et al.*, 2006). Des-acyl ghrelin is also abundantly present in both stomach and blood (Hosoda *et al.*, 2000). Acylated ghrelin is highly unstable because a fatty acid is attached to Ser3. Hence, des-acyl ghrelin might represent either a pre-form of acylated ghrelin or as its product of deacylation (Hosoda *et al.*, 2004).

Circulating ghrelin is mainly synthesized and secreted by X/A-like cells in rodents and P/D1-cells in humans, which are embedded in the oxyntic glands (Sakata *et al.*, 2002a; for review see Kotunia and Zabielski, 2006). GCs account for about 20% of the endocrine cell type population of the oxyntic glands in humans and rats (Date *et al.*, 2000). In humans and rats, the number of gastric GCs increases during postnatal development (Hayashida *et al.*, 2002; Wierup *et al.*, 2002). Patients who undergo bariatric gastrectomy exhibit a 76% decrease in plasma ghrelin levels compared with healthy control subjects (Leonetti *et al.*, 2003). Thus, although the stomach is the major source of ghrelin, other sources for circulating ghrelin clearly exist. The SI, the second major source of ghrelin, is a possible candidate (Sakata *et al.*, 2002a).

Ghrelin is present in a proportion of X/A-like endocrine cells in the mucosal villi and crypts of the small - and to a lesser extent the large intestine (Date *et al.*, 2000). Intestinal GCs can be classified into opened-and closed-type cells. Triangular, elongated-shaped opened-type cells are with their apical processes in contact with the lumen, whereas round-shaped closed-cell types are not. The stomach only has closed-type cells. In general, opened-cell types receive luminal information such as nutrients and pH whereas closed-cell types are triggered by hormones, neuronal stimulation or mechanical distension. This different distribution of opened-and closed type cells in the gastric and intestinal mucosa demonstrates that the regulatory mechanisms of ghrelin release will be different in the stomach and the

intestine (Sakata *et al.*, 2002a). Ghrelin is also present in the pancreas, pituitary gland, hypothalamus, lung, placenta (Gualillo *et al.*, 2001; Horvath *et al.*, 2001; Korbonits *et al.*, 2001; Wierup *et al.*, 2002; Santos *et al.*, 2006), mammary gland (Gronberg *et al.*, 2008), ovary (Caminos *et al.*, 2003) and testis (Gaytan *et al.*, 2004). However, the biological relevance of these different ghrelin sources is still unclear.

Total ghrelin plasma levels increase pre-prandially and decrease post-prandially (Cummings *et al.*, 2001). The mechanisms by which nutrients suppress ghrelin secretion are still not known. However, data suggests that signals from the circulatory system rather than from the gastro-intestinal based sensing system (Shiiya *et al.*, 2002) suppress ghrelin release. Ghrelin secretion only declines when nutrients leave the stomach and are adsorbed in the circulation (Williams *et al.*, 2003). Although ghrelin secretion is indeed dependent on circulating nutrients, it seems logical that also messengers, like neurotransmitters and hormones are involved. However, contradictory results in literature make it difficult to draw any conclusions about possible messengers regulating ghrelin secretion. For example, the role of insulin as hormonal messenger in the endogenous regulation of ghrelin still remains unclear. Some studies showed an inverse relationship between insulin and ghrelin (Mohlig *et al.*, 2002; Saad *et al.*, 2002; Flanagan *et al.*, 2003; Ni *et al.*, 2010). Others failed to demonstrate this relationship (Caixas *et al.*, 2002; Schaller *et al.*, 2003; de la Cour *et al.*, 2007).

4.2 The role of ghrelin in perinatal development and IUGR

The pancreas, stomach and placenta contribute to the foetal pool of ghrelin (Gualillo *et al.*, 2001; Chanoine and Wong, 2004) while later on stomach ghrelin expression postnatally increases to adult levels (Hayashida *et al.*, 2002).

Gastric GCs develop long before chief cells (Rindi *et al.*, 2002). This latter finding is of interest because leptin, the appetite-modulating opponent, is secreted by chief cells (Bado *et al.*, 1998). In rats, the number of gastric GCs increases as the stomach grows (Hayashida *et al.*, 2002). Moreover, a study in rats has shown that the distribution of

gastric GCs extend from the base to the glandular neck when the pups age (Sakata *et al.*, 2002b).

Both the pre- and postnatal period show a widespread distribution of ghrelin. This widespread distribution suggests that ghrelin exerts its biological activity by two different mechanisms of action: locally produced ghrelin may act via a paracrine effect on cells expressing ghrelin receptors and gastric ghrelin can exert its activity via an endocrine effect. A study in knock out mice demonstrates that ghrelin is not required for perinatal development (Sun *et al.*, 2003). Nevertheless, the presence of ghrelin in the placenta (Gualillo *et al.*, 2001), neonatal pancreas (Wierup *et al.*, 2002), pituitary (Kamegai *et al.*, 2001) and hypothalamus (Torsello *et al.*, 2003) suggests a perinatal role for ghrelin in the programming of energy balance.

Besides the different anatomical origin, the nutritional regulation of ghrelin also seems to differ between the pre- and postnatal periods. During the postnatal period, fasting causes a marked increase in circulating ghrelin concentrations (Hayashida *et al.*, 2002). A previous study showed that weaned piglets indeed have higher circulating ghrelin levels after feed deprivation (Salfen *et al.*, 2003). In rat foetuses, circulating ghrelin concentrations are unaffected by the maternal fasting status despite a marked decrease in foetal glucose and insulin concentrations (Chanoine and Wong, 2004). Nevertheless, acylated ghrelin concentrations increase in the foetal pancreas during maternal fasting. Hence, ghrelin might mediate the effects of maternal nutrition on the developing pancreas (Chanoine and Wong, 2004). As such, ghrelin can contribute to the programming of metabolic pathways in response to perinatal environmental signals such as nutrition (Desai *et al.*, 2005). During late gestation, ghrelin may prepare the foetus for extrauterine life by inducing adiposity (Tschop *et al.*, 2000), stimulating food intake (Wren *et al.*, 2001), maintaining glucose levels (Broglio *et al.*, 2001) and stimulating GH secretion (Sun *et al.*, 2004).

The role of ghrelin on weight gain during the perinatal period remains unclear. It is possible that ghrelin contributes to feeding initiation and positive energy balance. A modest association was found between lower umbilical cord blood ghrelin concentrations and slower weight gain in humans (James *et al.*, 2004). There is also

discrepancy in literature about the correlation between birth weight and ghrelin concentrations. Some studies indicate that SGA infants have higher ghrelin concentrations compared to appropriate for gestational age (AGA) infants (Farquhar *et al.*, 2003; Onal *et al.*, 2004). These higher ghrelin levels could result in a sustained orexigenic drive and therefore contribute to catch up growth (Chiesa *et al.*, 2008). However, others did not find this correlation (Kyriakakou *et al.*, 2008).

5 Leptin

5.1 Synthesis and regulation

Leptin, the protein product of the obesity (*ob)* gene, is a 16 kDa hormone that regulates energy homeostasis and food intake by providing afferent signals to the hypothalamus (Campfield *et al.*, 1995). It is mainly synthesized by adipose tissue but other important sources are the placenta and umbilical cord (Ashworth *et al.*, 2000; Akerman *et al.*, 2002), colostrum and breast milk (Casabiell *et al.*, 1997), and the stomach (Bado *et al.*, 1998).

In the gastrointestinal system, leptin has been described in the fundic mucosa, mainly in pepsinogen-secreting chief cells but also in the parietal cells and rarely in endocrine leptin cells of rats and humans (Cinti *et al.*, 2000; Sobhani *et al.*, 2000; Cammisotto *et al.*, 2005; for review see Cammisotto and Bendayan, 2012). The leptin secreting chief cells are mainly localized in the lower half of the gastric fundus (Cammisotto *et al.*, 2005). In 18 h fasted adult pigs, leptin was detected in the lower half of the fundic glands. In 2 h fed adult pigs, leptin was detected all along the fundic glands (Vitari *et al.*, 2010). In these pigs, leptin was expressed in endocrine leptin cells, but also in chief and parietal cells in the gastric mucosa (Vitari *et al.*, 2010). This study also found that leptin immunoreactivity was highest when the pigs were fed compared to fasted pigs. Interestingly, leptin cells are adjacent to GCs in the gastric mucosa in the lower half of the stomach in rats possibly with the aim of regulating ghrelin secretion in a paracrine way (Zhao and Sakai, 2008).

Leptin secretion of the chief cells was found to be an exocrine secretion (for review see Cammisotto and Bendayan, 2012). Endocrine and exocrine leptin cells in the

gastric mucosa are able to secrete leptin towards the blood circulation or into the gastric juice (Cammisotto *et al.*, 2005). Exocrine secreted leptin survives the hydrolytic gastric juice by forming a complex with its soluble receptor (Figure 1.5). This soluble receptor is also synthesized from the stomach and the leptin-soluble leptin receptor complex forms at the gastric chief cell secretory granules before it gets released (Cammisotto *et al.*, 2005). Previous studies also found expression of the long isoform of the leptin receptor Ob-Rb in the basolateral plasma membrane of gastric cells and therefore this suggests a paracrine/autocrine role of leptin in the stomach (Bado *et al.*, 1998; Cinti *et al.*, 2000). Next, the leptin-soluble leptin receptor complex migrates to the duodenum. Luminal leptin in the digestive tract originates from gastric chief cells or maternal milk (Smith-Kirwin *et al.*, 1998; Groschl *et al.*, 2001; Cammisotto *et al.*, 2005). Transmembrane leptin receptors at the luminal membrane of the duodenum, jejunum and ileum interact with the luminal leptin. *In vivo* and *in vitro* studies have shown that the luminal leptin-leptin receptor interaction in the gut regulates intestinal absorption and mucosa renewal, as such contributing to gut homeostasis (Lostao *et al.*, 1998; Kiely *et al.*, 2005). Thus, leptin is actively transcytosed by the enterocytes of the duodenum where it binds its soluble receptor again. This newly formed complex is secreted baso-laterally into the intestinal mucosa to reach the circulation. Circulating leptin is mainly originating from white adipose tissue, which secretes leptin through a constitutive pathway (Zhang *et al.*, 1994), but the decreased- and increased leptin concentrations before and after meal consumption originate from the digestive tract (Cammisotto *et al.*, 2005; Cammisotto *et al.*, 2006). Binding to its soluble receptor increases the stability and the half-life time of leptin in the blood (Cammisotto *et al.*, 2006). Hence, leptin reaches the hypothalamus where it regulates food intake.

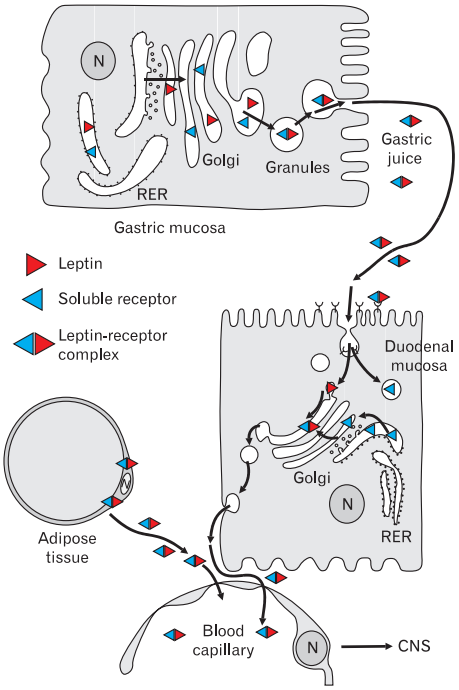

Figure 1.5 Schematic overview illustrating the secretion of leptin by the gastric chief cell and the adipocyte. Reprinted from (Cammisotto and Bendayan, 2012)

The functional Ob-Rb receptor is also detected in the human colon at the apical plasma membrane of the colonocytes (Buyse *et al.*, 2001). In the adult pig, both leptin and its receptor were observed in the basolateral membrane of enterocytes and colonocytes and in the apical membrane of these cells (Hansen *et al.*, 2008). The authors from this study concluded that leptin acts mainly on the basolateral membrane, indicating that leptin uses an endocrine pathway in both the small- and large intestine. It is known that the epithelial intestinal cell lining acts as a barrier and normally does not engage endocytosis after gut closure (Rodewald, 1970). However the transcytosis pathway of gastric leptin through enterocytes to reach the blood circulation clarified the role of luminally secreted gastric leptin (Cammisotto *et al.*, 2007). Hence, the exocrine or endocrine secretion of gastric leptin constitutes a gastroenteric axis, which coordinates the gastrointestinal role of leptin (Cammisotto *et al.*, 2005). Exocrine luminal leptin acts directly on the intestinal cells through their specific receptors on the enterocyte microvilli. There, it regulates the transport of nutrients and stimulates uptake of glucose and regulates lipid transport (Lostao *et*

al., 1998; Morton *et al.*, 1998; Buyse *et al.*, 2001). Although it is possible that luminal gastric leptin reaches the SI, it is unlikely that it reaches the colon. Hence, this suggests that gastric leptin may reach the colonic mucosa in a classical endocrine fashion via the transcytosis pathway in the SI.

Leptin from adipocytes and from the gastric mucosa is released at different times after the onset of food intake. The gastric mucosa secretes leptin within minutes after food intake whereas adipocytes need several hours for releasing leptin (Cammisotto *et al.*, 2010). These two pools of leptin have different purposes. Gastric exocrine secretion of leptin participates in the short-term regulation of food intake, including delay of gastric emptying, secretion of gastrointestinal hormones (Anini and Brubaker, 2003; Kamegai *et al.*, 2004) and absorption of nutrients by the intestinal wall. More specifically, leptin increases peptides (Buyse *et al.*, 2001) and decreases fat and carbohydrate absorption (Morton *et al.*, 1998). Adipose tissue leptin on the other hand, regulates food intake in steady state conditions.

The mechanism how leptin mediates short-term feed intake is still unclear. However, insulin is an interesting candidate since it is the major regulator of energy utilization. Moreover, insulin levels behave the same way as leptin levels: they decrease with short-term fasting (Boden *et al.*, 1996) and increase after feeding (Kolaczynski *et al.*, 1996). One human study indeed confirmed that insulinemia is a physiological mediator of leptinemia (Saad *et al.*, 1998). These data suggest that insulin can be the signal mediating the effect of caloric intake on leptin secretion. Others described that leptin decreases neuronal 5-HT synthesis to inhibit appetite in mice (Yadav *et al.*, 2009; Yadav *et al.*, 2011). Leptin also slows gastric emptying and promotes gastric distension by potentiating the effect of cholecystokinin (CCK). This induces satiety in rhesus macaques (Moran and McHugh, 1982).

5.2 The role of leptin in perinatal development and IUGR

In adults, leptin is known to primarily regulate energy balance during short and long term changes in nutritional state. The role for leptin as a nutritional signal and the concept 'appetite' in utero is still unclear. However, some findings from animal

studies have shown that leptin does not inhibit appetite during intrauterine and early postnatal life. In contrast, these studies showed that leptin promotes swallowing and hyperphagia, thus contributing to growth and serving as an adaptive response to overcome the physiological weight loss during the first postnatal days (Roberts *et al.*, 2001; El-Haddad *et al.*, 2004). Others however, demonstrated that leptin supplied by maternal milk could play a role in the short-term regulation of postnatal feeding behaviour by acting as a satiety signal (Sanchez *et al.*, 2005). This is in contrast to another study which hypothesized that leptin also might initiate enteric feeding during the postnatal period (Mostyn *et al.*, 2001).

There is accumulating evidence for the involvement of leptin in perinatal growth of mammals (Christou *et al.*, 2002). Before birth, leptin concentration may be a marker for foetal growth and maturation of tissues. The widespread distribution of leptin with its receptors in the developing foetus provides indirect evidence for this hypothesis. Moreover, the correlation of leptin levels with placental weight and with a number of foetal growth indices such as BW and length, head circumference, PI and adiposity further strengthens this finding (Hassink *et al.*, 1997; Varvarigou *et al.*, 1999; Valuniene *et al.*, 2007).

It has been previously demonstrated that leptin in foetal umbilical cord blood is likely synthesized and secreted by the foetus itself since the maternal and foetal leptin values are not correlated with each other (Schubring *et al.*, 1997). Foetal adipose tissue and other foetal tissues like the placenta and stomach are thought to be sources for circulating leptin (Hoggard *et al.*, 1997; Forhead *et al.*, 2002; Aparicio *et al.*, 2005). Hence, this widespread prenatal distribution of leptin suggests endocrine actions in the foetus, which might be important for foetal growth and development. Additionally, since leptin synthesis can be modified by parameters like insulin, thyroid hormones and oxygen availability in utero, leptin concentrations can be altered by changes in the intrauterine environment.

Prenatally, leptin is present in human amniotic fluid (Schubring *et al.*, 1997). Amniotic fluid contains growth factors and hormones, which are important mediators of gastrointestinal development (Adrian *et al.*, 1995). These mediators can be

transported across the foetal epithelium by endocytosis (Weaver *et al.*, 1990). Human foetuses swallow amniotic fluid at 10 weeks of gestation. Hence, these amniotic fluid components can mediate their effects from that moment on (Buddington, 1994). Thus besides the endocrine effects of circulating leptin, it is possible that amniotic leptin also may exert its effects in the growth and functional development of the foetal digestive organs through luminal pathways since its receptor is also expressed on the apical membranes of the SI (Barrenetxe *et al.*, 2002).

Postnatally, leptin has been found in human and porcine maternal milk (Casabiell *et al.*, 1997; Whitley *et al.*, 2009). In humans, leptin concentrations are 30 to 150 fold higher in breast milk compared to milk formula and its milk concentrations are correlated with maternal and/or infant plasma concentrations (Houseknecht *et al.*, 1997; Smith-Kirwin *et al.*, 1998). In suckling animals leptin appears to influence gastrointestinal maturation (Oliver *et al.*, 2002; Wolinski *et al.*, 2003). Leptin has been implicated in the maturation of intestinal mucosa in the early postnatal days and seems to have a protective effect against cell apoptosis and autophagia in the neonatal gut epithelium (Wolinski *et al.*, 2003; Godlewski *et al.*, 2005). Interestingly, leptin supplementation in pigs has been shown to partially reverse the IUGR phenotype by correcting growth rate and body composition (Attig *et al.*, 2008).

Leptin might exert its growth promoting potential directly on target organs in order to promote cell differentiation and organ maturation. Another possibility is that leptin acts through the stimulation of the hypothalamic-pituitary axis to promote general growth. The first mentioned possibility is supported by the fact that leptin's Ob-Rb receptor is expressed in its peripheral target organs (Lin *et al.*, 2000). At the central level, leptin has been shown to stimulate GH secretion, like its appetite stimulating opponent ghrelin (Tannenbaum *et al.*, 1998; Ramsay *et al.*, 2004). Interestingly, the somatotropic axis is disturbed in IUGR (Woodall *et al.*, 1996).

In humans, foetal and newborn weight is significantly correlated with the umbilical cord leptin concentrations. Hence, growth restricted neonates have lower leptin levels (Jaquet *et al.*, 1998; Pighetti *et al.*, 2003; Martos-Moreno *et al.*, 2009). These

results indicate that the development of adipose tissue and fat mass are the main determinants of leptin levels. In contrast to other large mammals, neonatal piglets have very little body fat and lack brown adipose tissue (Trayhurn *et al.*, 1989; Herpin *et al.*, 2002). Colostrum intake is therefore essential to provide the piglets sufficient energy. It is generally accepted that at parturition, increased milk leptin concentrations occur. This coincides with the time when neonates are best able to absorb large proteins through the gastrointestinal tract, hence can optimally absorb orally ingested leptin (Savino *et al.*, 2004; Whitley *et al.*, 2009). During the first week of life, the body fat percentage rises from 2 to 15% (Manners and McCrea, 1963).

Previous data indicate that IUGR children develop an adaptive leptin resistance with higher leptin levels beneficial for their catch up growth to increase their energy balance (Jaquet *et al.*, 1999). Another hypothesis clarifying the higher leptin concentration during catch up growth is a defect in adipose tissue function. Adipose tissue development in IUGR children is characterized by a dramatically reduced body fat mass at birth (Lapillonne *et al.*, 1997) followed by a drastic increase in weight and growth during the first year of life. Likewise, a study demonstrated a compensatory development of perirenal adipose tissue in low birth weight piglets. The proportion of perirenal adipose tissue was similar between normal and low birth weight piglets at 28 days of age whereas at day 7 the amount of perirenal adipose tissue was still lower in low birth weight piglets compared to their normal littermates (Morise et al., 2009). This postponed increase can have its effects on the sensitivity of the systems regulating leptin synthesis and secretion in adipose tissue. The increased risk of developing obesity in adult life seen in IUGR children supports this finding (Ravelli *et al.*, 1976; Barker, 2004).

6 Serotonin (5-hydroxytryptamine, 5-HT)

6.1 Synthesis and regulation

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine found in the gastrointestinal system, brain and blood platelets. Despite the fact that most research focuses on brain 5-HT, the vast majority of 5-HT does not reside in the brain of mammals, but in

the gastrointestinal system. Serotonin is also accepted as an appetite regulator, both in the central nervous system as in the periphery (for review see Donovan and Tecott, 2013). Hence, this monoamine perfectly fits in the scope of this study.

The first step in the 5-HT-synthesis from its precursor tryptophan (Trp) is mediated by tryptophan hydroxylase (TPH), which is also the rate-limiting enzyme of this synthesis. There are two isoforms of TPH: TPH1 and TPH2. While TPH1 is essential for 5-HT biosynthesis in the enterochromaffin cells, TPH2 is critical for 5-HT synthesis in neurons (Cote *et al.*, 2003, Walther *et al.*, 2003). The latter isoform is not only expressed in the brain, but also in the enteric nervous system (for review see Gershon and Tack, 2007; Neal *et al.*, 2009).

5-HT is an amine that is mainly produced in the enterochromaffin cells of the gastrointestinal tract, from which it is released in the blood circulation (Erspamer and Testini, 1959). Platelets rapidly bind and store 5-HT, hence little amounts of this amine are found in plasma (Frishman *et al.*, 1995). Brain 5-HT is produced in raphe nuclei in the brain stem and released throughout the brain. Concentrations of brain 5-HT are related to mood changes, sleep and appetite regulation (for review see Bell *et al.*, 2001; Feijo Fde *et al.*, 2011). In view of appetite, the predominant role of 5-HT signalling in the central nervous system is the suppression of food intake (reviewed by Donovan and Tecott, 2013). Although the gastrointestinal tract is the major source of 5-HT, the actual roles of 5-HT are difficult to be completely elucidated. The main difficulty of defining the gastrointestinal role of 5-HT is related to the fact that the gastrointestinal tract has two 5-HT sources: the mucosa and the enteric nervous system (Figure 1.6). Moreover, specific receptor subtypes of 5-HT are widespread and show an overlapping distribution in the gastrointestinal system, which makes the identification of the specific gastrointestinal roles of 5-HT even more complicated. In the gastrointestinal system, 5-HT is released by a range of stimuli, most potently by mucosal stroking (Linden *et al.*, 2003).

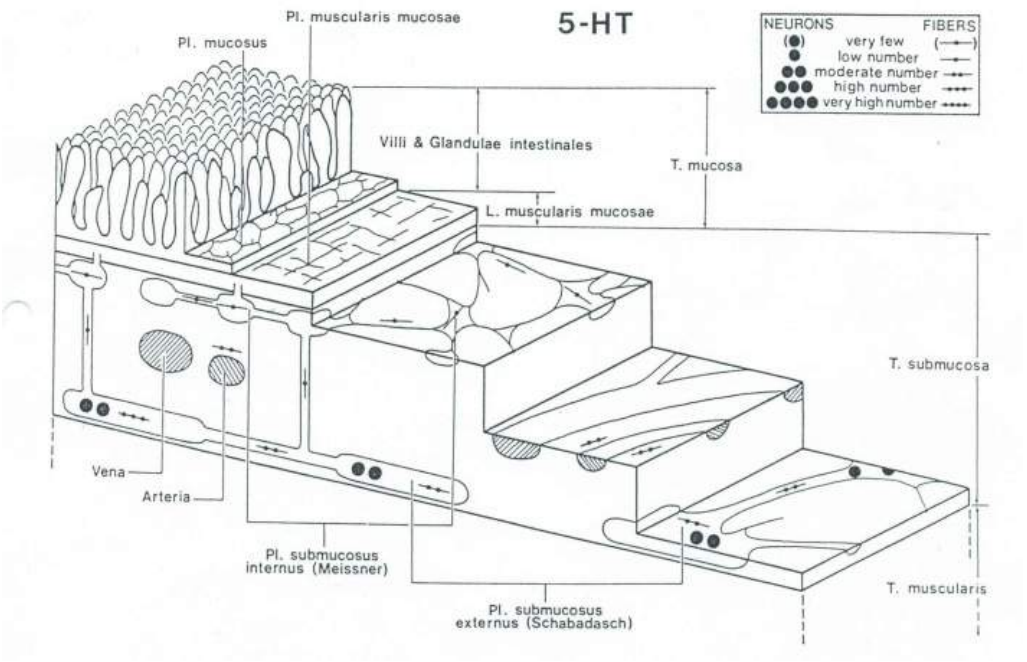

Figure 1.6 Schematic representation of the distribution of 5-HT in the enteric nervous system of the small intestine. Besides its pronounced presence in the intestinal epithelium, 5-HT is also abundantly present in the enteric nervous system. Reprinted from (Timmermans *et al.,* 1990).

5-HT controls hunger and satiety through different receptors in the central nervous system with discrete functions. The 5-HT2C receptor seems to be the most important one that regulates food intake as a satiety inductor (Lam *et al.*, 2008) whereas 5-HT1B receptors are involved in regulating meal size (Grignaschi and Samanin, 1992). Since 5-HT can not cross the blood brain barrier, it cannot impact the energy balance circuits in the central nervous system (Merrit *et al.,* 1978). However, circulating 5-HT levels that act in the periphery also have been shown to affect energy balance and appetite (Simansky, 1996). Peripheral 5-HT administration also decreases food consumption and accelerates satiety (Pollock and Rowland, 1981; Fletcher and Burton, 1986; Edwards and Stevens, 1991; Grignaschi and Samanin, 1992; Simansky *et al.*, 1992).

The precursor of 5-HT, Trp is also known to be implicated in regulating appetite. In pigs, Trp deficiency is associated with a reduction in feed intake and appetite, hence with impaired growth (Henry *et al.*, 1992; Henry *et al.*, 1996; Eder *et al.*, 2001). One

possible mechanism of Trp regulating appetite is the sensitivity of the brain to amino acid balance. The brain may serve as a kind of chemosensor that initiates depression of feed intake after such an imbalance (Le Floc'h and Seve, 2007). Interestingly, a recent porcine study (Zhang *et al.,* 2007) showed that Trp's effect on appetite regulation might be mediated through ghrelin, another molecular appetite regulating protagonist of this thesis.

Trp is a protein constituent of the normal diet (Tagliamonte *et al.*, 1973). This amino acid is the only one known to bind serum albumin in physiological conditions (McMenamy and Oncley, 1958). Hence, there are two known fractions of this amino acid: one bound to albumin and one free (McMenamy and Oncley, 1958). The total Trp pool contains 5-50% of the free fraction of Trp (FFT), depending on the physiological condition (Knott and Curzon, 1972). FFT passes through the blood brain barrier, is taken up by 5-HT neurons and hydroxylated by TPH. Then, 5-hydroxytryptophan (5-HTP) is formed, decarboxylated and subsequently 5-HT is formed (Boadle-Biber, 1993). Infusion of 5-HTP causes a reduction of food intake in weaning pigs (Zhang *et al.*, 2007). The authors from this study assume that the satiety effect of 5-HTP is caused by increased brain 5-HT levels in these pigs (Zhang *et al.*, 2007).

Several mechanisms have been proposed for the brain transfer of Trp. A specific transport system has been postulated (Yuwiler *et al.*, 1977). Besides this specific mechanism, other competition-based methods have been proposed. As such, the amount of Trp passing through the brain may depend on its binding to albumin (McMenamy and Oncley, 1958). Moreover, large neutral amino acids (LNAA) share the same carrier at the blood brain barrier and would compete with Trp for transport to the brain (Oldendorf, 1971).

6.2 The role of serotonin and its precursor tryptophan in perinatal development and IUGR

6.2.1 Tryptophan

Trp is almost entirely free in the serum of postnatal rats (Bourgoin *et al.*, 1977). This is in contrast to adult rats, in which Trp has the ability to bind to serum albumin. Three factors can account for the relative lack of albumin binding of Trp in the newborn rat: 1) a lower concentration of albumin. This corresponds to results from porcine samples, which showed that serum of newborns has very low concentrations of total proteins, including low levels of albumin (Martin *et al.*, 2005). Hence, piglet's serum undergoes a rapid metabolic maturation process with regard to its proteins, evolving from a foetal pattern to an adult one. Interestingly, a study in piglets has shown that heavier piglets have higher plasma albumin levels compared to their littermates with a lower BW (Tuchscherer *et al.*, 2000) whereas in human IUGR infants this finding is not supported (Hernandez-Rodriguez *et al.*, 2009); 2) an inhibition of binding by non-esterified fatty acids (NEFA's); this finding however has not been confirmed in a recent human study (Hernandez-Rodriguez *et al.*, 2009); 3) a decreased number of available sites for tryptophan on albumin (Bourgoin *et al.*, 1977).

One study demonstrated a diminished affinity of Trp to plasma albumin in intrauterine growth restricted rodents, which determined the higher levels of FFT seen in these rodents malnourished in utero (Hernandez-Rodriguez *et al.*, 2009). Because of these higher FFT levels, rats malnourished in utero have an accelerated brain synthesis of 5-HT (Hernandez *et al.*, 1989; Manjarrez *et al.*, 1998; Manjarrez *et al.*, 2005). It is known that 5-HT has a neurotrophic role in the foetal brain; hence an increased 5-HT metabolism can reflect permanent changes in brain neurogenesis. Besides elevated FFT levels, studies have also demonstrated an increase in the ratio of FFT to total Trp (Miller *et al.*, 1977; Manjarrez *et al.*, 1998). Altogether, these data suggest that the metabolism of 5-HT is increased in the perinatally malnourished brain. This imbalance of Trp in favour of FFT in perinatally malnourished animals and

humans suggest an elevated transport of this amino acid to the brain with a possible enhancement of 5-HT synthesis as shown in IUGR rats and infants (Manjarrez *et al.*, 1988; Hernandez *et al.*, 1989).

Increased total Trp plasma concentrations have also been reported in IUGR infants (Hernandez *et al.*, 1989; Manjarrez *et al.*, 2005; Hernandez-Rodriguez *et al.*, 2009). Interestingly, a recent study in human foetuses demonstrated that Trp can be used as biological marker for foetal growth retardation since the Trp levels of IUGR foetuses were up regulated compared to AGA foetuses (Favretto *et al.*, 2012). These results are however in contrast to a study performed in porcine IUGR foetuses, in which lower Trp umbilical plasma levels were detected (Lin *et al.*, 2012). The authors from this recent study assume that this Trp level impairment seen in IUGR piglets might be due to impaired placental transport (Avagliano *et al.*, 2012).

Tryptophan is the fourth limiting amino acid for growth in pig diets. A recent study showed an improved growth performance of pigs with increasing supplemental Trp (Shen *et al.*, 2012). Additionally, there is evidence that Trp increases food intake and growth in weaning pigs (Henry *et al.*, 1992; Ettle and Roth, 2004). Hence, when Trp supply is limited compared to the other essential amino acids, this will influence protein synthesis negatively and finally growth rate (Le Floc'h and Seve, 2007). The Trp alterations in IUGR humans and animals described earlier indeed confirm that foetal nutrient deficiency may play an important role in the pathophysiology of IUGR and emphasize the importance of further investigating the pathogenesis of IUGR.

6.2.2 Serotonin (5-HT, 5-hydroxytryptamine)

Previously it has been shown that low birth weight infants have lower 5-HT concentrations probably due to a fall in platelets (Berman *et al.*, 1965; Christensen *et al.*, 2006). In a human study, blood 5-HT levels in IUGR infants did show a negative correlation with FFT (Hernandez *et al.*, 1989). It has been shown that neonatal 5-HT levels rapidly double after birth to nearly adult levels. Enteral feeding probably causes this increased gut 5-HT release (Anderson *et al.*, 2004).

There are species-specific differences regarding the first appearance of 5-HT immunoreactive (IR) cells in the gastrointestinal tract. In rodents, these cells only appeared near term in the stomach (Ekelund *et al.*, 1985). This is in contrast to the human and porcine stomach, where enterochromaffin cells are already present midway through gestation (Stein *et al.*, 1983; Facer *et al.*, 1989; Zabel *et al.*, 1995; Van Ginneken *et al.*, 2001) (Table 1.3). Interestingly, these cells show age-dependent regional differences in the stomach of mid-gestational pig foetuses (PF) (Van Ginneken *et al.*, 2001). This species-specific difference might be explained because the gastrointestinal system of rodents is still immature at birth compared to larger mammals as already described in this chapter (section 3.2) (for review see Sangild, 2006).

6.3 The enteric nervous system

The enteric nervous system is a large network of neurons and glial cells which is located along the entire length of the gastrointestinal tract. This network has been divided in myenteric ganglia, with most of its neurons located between the longitudinal and circular muscle layers or in submucosal ganglia, where the neurons are within the submucosal connective tissue, as described in section 3.1. There are two different submucosal ganglionic neural networks in the intestinal tract: the plexus submucosus internus (Meissner), located in the innermost part of the submucosal layer and the plexus submucosus externus (Schabadasch), situated adjacent to the circulatory smooth muscle layer. The differing distributions of neuron cell types in these two plexuses support the hypothesis that these plexuses not only reflect two morphological separate neuronal networks, but also have different functions (for review see Timmermans *et al.*, 1990).

This autonomous nervous system in the gastrointestinal tract controls and regulates many gut functions, such as motility, secretion and hormone release. The ability of the enteric nervous system to mediate gastrointestinal behaviour independently of the central nervous system requires a complex network of phenotypic diverse neurons. Although 5-HT is mainly localised in the gastrointestinal enterochromaffin

cells, it is also implicated as a neurotransmitter of descending myenteric interneurons (for review see Gershon, 2009). As a neurotransmitter, 5-HT plays an important role in signalling between these myenteric interneurons and secretory responses. Consequently, 5-HT has been implicated in a range of human gastrointestinal motility disorders (for review see Spiller, 2007). Gastrointestinal motility depends more on neuronal than on mucosal 5-HT and the development of other late-born enteric neurons requires neuronal 5-HT (for review see Li *et al.*, 2011). The distribution of serotonergic IR nerve cells in the enteric nervous system is species dependent. In the guinea-pig SI, 5-HT neurons were detected only in the myenteric plexus (Furness and Costa, 1982) whereas in humans and pigs, these neurons are also located in the submucosal plexus (Griffith and Burnstock, 1983; Timmermans *et al.*, 1990).

7 The involvement of ghrelin, leptin and serotonin in the development of the metabolic syndrome

The metabolic syndrome is a combination of metabolic risk factors: abdominal obesity, elevated blood pressure, elevated fasting plasma glucose, high serum triglycerides and low high-density lipoprotein cholesterol (HDL) levels. When these disorders occur together, the risk of developing diabetes and cardiovascular diseases is higher compared to when one of these risk factors occurs alone (DeFronzo and Ferrannini, 1991). The global prevalence of this syndrome is approximately 16% in humans and is growing at an alarming rate. The European prevalence of obesity with metabolic syndrome varies between 24.6 % in an Italian cohort study to 65% in a Finnish female population cohort study and in men from 43% in the Italian cohort to 78% in the Finnish cohort (Van Vliet-Ostaptchouk *et al.,* 2010). A multitude of research has been undertaken in order to improve our knowledge. Much of this research has been done in rodent models. There are however fundamental differences in metabolism and physiology between humans and rodents. The high metabolic and physiologic similarities between humans and pigs, are already described in section 2.4. Moreover, the pig is generally considered to be the

optimum non-primate model for investigating the metabolic syndrome (for review see Spurlock and Gabler, 2008; Litten-Brown *et al.*, 2010).

Leptin, the *ob* gene product, is one of the best-known hormonal markers for obesity. Children with a rare leptin mutation, suffering from leptin deficiency, develop morbid obesity in early childhood and have important growth dysfunctions (Montague *et al.*, 1997; Fatima *et al.*, 2011). Likewise, neonates that have either low leptin levels (e.g. SGA infants) (Ben *et al.*, 2001) or high leptin levels (e.g. offspring from mothers with gestational diabetes) (Gross *et al.*, 1998; Persson *et al.*, 1999) also have a higher risk of developing obesity and diabetes mellitus type 2 compared to children with normal leptin levels at birth (Martin-Gronert and Ozanne, 2005). Another finding which substantiates the fact that leptin is indeed involved in the pathogenesis of the metabolic syndrome is the marked elevation of plasma leptin levels in obese adults and children (Gil-Campos *et al.*, 2008; Gil-Campos *et al.*, 2010). This supports a leptin resistance mechanism in obese patients. Likewise, the postprandial suppression of its appetite regulating opponent ghrelin is significantly reduced in obese adults and children (Le Roux *et al.*, 2005). Moreover, obese children show an increase in ghrelin 3 hours after meal intake similar to the fasting values whereas in NW children this increase was absent (Gil-Campos *et al.*, 2010). This finding suggests that the orexigenic effects of ghrelin return faster to baseline levels in obese children compared to NW children. This explains partially the early recovery of appetite usually observed in obese persons. A negative correlation between ghrelin levels and the incidence of diabetes type 2 and insulin resistance has also been shown (Ikezaki *et al.*, 2002; Broglio *et al.*, 2003; Poykko *et al.*, 2003; Katsuki *et al.*, 2004). However, it is still unclear if low ghrelin levels are a risk factor or are a compensatory response.

Circulating 5-HT also has been shown to affect glucose homeostasis in a complex way. Peripheral 5-HT seems to impact glucose homeostasis by two opposing mechanisms, presumably depending on dose, route or other conditions. In some studies, peripheral 5-HT administration has been found to increase circulating blood glucose levels whereas in other studies peripheral 5-HT produced hyperinsulinemia, an action resulting in a reduction of glucose levels (Ekholm *et al.,* 1971; Hajduch *et*

al., 1999; Moore *et al.,* 2005). The 5-HT induced hyperglycemia corresponds to the fact that acute hyperglycaemia is associated with increased platelet aggregability, with consequently an increased 5-HT secretion (Sakamoto *et al.*, 2000). Other studies assume that 5-HT-induced hyperglycemia is due to inhibition of glucose uptake by the liver and muscle tissue (Hajduch *et al.*, 1999; Moore *et al.,* 2005). The association between 5-HT and hyperinsulinemia presumably occurs via stimulation of pancreatic β cells by 5-HT (Ekholm *et al.,* 1971). High circulating 5-HT concentrations have been demonstrated in diabetic patients (Barradas *et al.*, 1988). Moreover, urinary 5-hydroxyindoleacetic acid (5-HIAA) concentrations, a derivative end product of 5-HT, were higher in diabetic patients compared to normal subjects (Takahashi *et al.*, 2002). They also showed a positive correlation with these 5-HIAA concentrations and plasma glucose levels. A link between 5-HT and diabetes is also supported by the reduced FFT and FFT/total Trp ratio seen in diabetic children (Herrera *et al.*, 2003). Interestingly, a recent study has shown increased 5-HIAA levels in subjects with metabolic syndrome (Fukui *et al.*, 2012). Another study however did find reduced circulating levels of 5-HT in obese patients. Interestingly, this study showed a negative correlation between circulating 5-HT levels and body mass index (BMI) (Hodge *et al.*, 2012).

To conclude, ghrelin, leptin and 5-HT levels are altered in subjects with the metabolic syndrome, in which endocrine appetite regulation is disturbed. Interestingly, ghrelin, leptin and 5-HT interact during appetite regulation. The antagonistic relationship between ghrelin and leptin has been illustrated in literature as the 'ghrelin-leptin tango' (Cummings and Foster, 2003). However, this relationship between ghrelin and leptin remains controversial since several studies failed to show a direct negative correlation (Ikezaki *et al.*, 2002; Soriano-Guillen *et al.*, 2004) (Figure 1.7). Nonetheless, studies agree that ghrelin and leptin secretion is regulated oppositely. Indeed, while the secretion of ghrelin is promoted by hypoglycaemia (Shiiya *et al.*, 2002), leptin secretion is induced by hyperglycaemia (Fruhbeck and Salvador, 2000). Ghrelin and insulin concentrations are inversely correlated (Saad *et al.*, 2002) whereas insulin induces leptin secretion (Saad *et al.*, 1998) (Figure 1.7).

In vivo animal experiments showed that leptin can induce its satiety effect by modulating brain 5-HT synthesis (Oury and Karsenty, 2011). A functional relationship between leptin and 5-HT was demonstrated by the observed increased circulating leptin levels after treatment with 5-HTP, a precursor of 5-HT (Yamada *et al.,* 2000, 2006). Interestingly, one of these studies demonstrated that hyperleptinemia only was induced when 5-HTP was peripherally, hence not centrally injected in mice (Yamada *et al.,* 2000). Evidence exists that serotonergic signalling is also involved in ghrelin release. Fenfluramine and meta-Chlorophenylpiperazine (mCPP), both 5-HT releasing agents, decreased plasma ghrelin levels (Nonogaki *et al.,* 2006). Additionally, ghrelin secretion is enhanced by Trp supplementation in weanling pigs, which increased weight gain (Zhang *et al.*, 2007).

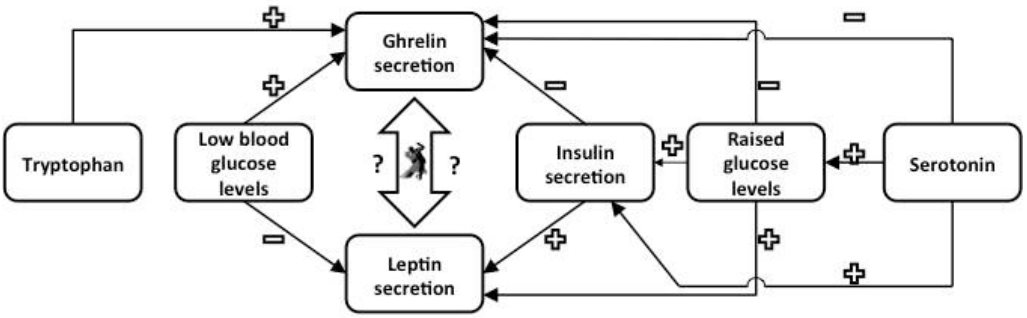

Figure 1.7 Schematic representation of the ghrelin-leptin tango, the correlation between serotonin, ghrelin and leptin and the involvement of ghrelin, leptin and serotonin in glucose homeostasis

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Chapter 2 Aims

The high perinatal mortality seen in IUGR humans and pigs can be attributed to an impaired development of the gastrointestinal system (Shanklin and Cooke, 1993; Xu *et al.,* 1994). Moreover, prenatal growth restricted foetuses alter their metabolic and endocrine pathways in order to survive. These developmental endocrine changes permanently affect the physiology and metabolism in these newborns, thereby predisposing them to endocrine and metabolic disorders later in life (Hales and Barker, 1992; Barker, 1998). Because IUGR occurs naturally in pigs and the porcine gastrointestinal system resembles the human digestive tract (for review see Guilloteau *et al.*, 2010), the prenatal growth restricted SGA pig is a good animal model to study the consequences of IUGR linked to the gastrointestinal system.

Since adequate feed intake is important to compensate prenatal growth restriction and affect the risk for metabolic diseases in later life, this thesis focuses on the role of three gastrointestinal derived appetite regulating hormones in piglets: ghrelin, leptin and 5-HT. We have chosen these particular hormones because ghrelin and leptin levels are correlated with anthropometric measurements such as ponderal index and birth weight (Chiesa *et al.,* 2008) whereas 5-HT metabolism is impaired in IUGR children (Hernandez-Rodriguez *et al.,* 1989; Hernandez-Rodriguez *et al.,* 2009). Hence, we aim to link the presence of these essential neuro- and endocrine components of the gastrointestinal system and appetite regulation to the BW and age of our animal models and to their metabolic profile (glucose and insulin levels). As final objective, we want to determine whether IUGR has an effect on the morphology of the porcine gastrointestinal system with possible consequences later in life. Overall, these results will provide a better insight in how endocrine signals go astray when gut and body development in piglets is hindered in utero. This could aid in explaining metabolic and growth impairments that occur later in life. To this purpose the following objectives were put forward:

Chapter 3: Since the endocrine and metabolic pathways are disturbed in SGA piglets we hypothesize that these alterations can be reflected in an adapted

- gastrointestinal distribution of endocrine GCs
- serum concentration of ghrelin

Furthermore, we hypothesize that the immature gastrointestinal system of SGA piglets is reflected in a changed gastric (pars fundica) morphology.

Chapter 4: Since the endocrine and metabolic pathways are disturbed in SGA piglets we hypothesize that these alterations can be reflected in an adapted

- intestinal distribution of enterochromaffin cells
- serum concentrations of 5-HT, together with its precursor Trp

Furthermore, we hypothesize that the immature gastrointestinal system of SGA piglets is reflected in a changed intestinal morphology.

Chapter 5: Interestingly, the molecular protagonists of this study, ghrelin, leptin and 5-HT are known to be involved in energy homeostasis. This homeostasis is a complex balance between food intake and energy expenditure and includes the regulation of nutrient levels, in particular glucose. Hence, this section will correlate the serum concentrations of ghrelin, leptin and 5-HT and relate these to glucose and insulin levels in order to detect the developmental adaptations in endocrine appetite regulation of the SGA piglet.

These data will be a valuable asset in the follow up of the naturally occurring IUGR seen in pigs, hence will help to optimize animal production. Moreover, since the pig has proven to be a valuable animal model for humans, this information will improve our understanding of the pathological consequences of IUGR in both humans and piglets.

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Chapter 3 Ghrelin in the gastrointestinal tract and blood circulation of perinatal low and normal weight piglets

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1 Abstract

Ghrelin, the 'hunger' hormone, is an endogenous GH secretagogue that exerts a wide range of physiological functions. Its perinatal presence suggests that ghrelin might be involved in growth and metabolism processes during intrauterine and postnatal life. IUGR neonates have altered endocrine and metabolic pathways due to malnutrition during foetal development. These changes might include an altered gastrointestinal presence of GCs. Since ghrelin is mainly secreted by the stomach this altered presence might be reflected in its serum concentrations. SGA pigs appear to be a natural occurring model for IUGR children. Therefore, the first aim of this study was to investigate the presence of gastrointestinal GCs expressing active ghrelin in NW foetal and postnatal piglets compared to their SGA littermates using immunohistochemistry in combination with stereological methods. Secondly, total ghrelin serum concentrations of these piglets were analysed with a porcine radioactive immunoassay (RIA). In addition, the growth of the gastric pars fundica in NW and SGA piglets was analysed stereologically. Corresponding to humans and rats, it was shown that opened and closed-type IR GCs are distributed along the entire gastrointestinal tract of perinatal NW and SGA piglets. However, in contrast to the rat's stomach, the porcine GCs do not disperse from the glandular base to the glandular neck during perinatal development. Furthermore, stereological analysis demonstrated that NW neonates have a higher amount of gastric cells expressing active ghrelin compared to SGA piglets, which could result in higher milk consumption during the neonatal period. This finding is however not reflected in total serum ghrelin levels, which showed no difference between normal and SGA piglets. Moreover, the stereological V_vs of the fundic layers demonstrate a similar growth pattern in SGA and NW piglets.

2 Implications

In humans and pigs, IUGR leads to higher perinatal mortality. To adapt, IUGR foetuses alter their metabolic and endocrine pathways. Ghrelin, the 'hunger' hormone, is mainly expressed in the gastrointestinal tract and forms a possible link between nutrition and development. This study compared the ghrelin expression in the gastrointestinal tract and blood circulation of SGA piglets with normal littermates. Together with the results of the growth of the gastric pars fundica, these findings may improve our understanding of the impact of IUGR on endocrine appetite control and gastric development in perinatal piglets.

3 Introduction

The high morbidity associated with IUGR in humans and animals can be attributed to an impaired development of various organs, such as those of the gastrointestinal system (D'Inca *et al.,* 2010b). Consequently, IUGR neonates are prone to food intolerance, decreased fat absorption and digestive diseases during early postnatal life (Xu *et al.,* 1994; Lee *et al.,* 2001). Additionally, the developmental changes caused by poor foetal growth permanently affect the physiology and metabolism of the offspring, thereby predisposing these individuals to endocrine and metabolic disorders in adult life (Hales and Barker, 1992; Barker, 1998).

Ghrelin, the 28 amino acid GH-releasing appetite regulator, was first discovered in the rat and human stomach (Kojima *et al.,* 1999). As an endocrine hormone, it is also present in the circulatory system and high ghrelin levels can also be detected in colostrum (Aydin *et al.,* 2006). Ghrelin IR epithelial cells have been observed from the stomach to the colon in perinatal rodents (Sakata *et al.,* 2002a), humans (Rindi *et al.,* 2002) and postnatal cattle, sheep, pigs and horses (Hayashida *et al.,* 2001; Vitari *et al.,* 2012). In the gastric mucosa of rodents, GCs get distributed from the glandular base to the glandular neck when rat pups grow older (Sakata *et al.,* 2002b). Its perinatal presence and important physiological and endocrine functions indicate that ghrelin might play a role in gastrointestinal development and possibly also in its IUGR-associated adaptations (Wang *et al.,* 2005). Only a few studies describe the role of

ghrelin in the development of the gastrointestinal system of newborn and suckling animals (Kotunia and Zabielski, 2006). This absence of knowledge, together with the important role of feed intake regulating postnatal growth contributed to the focus on the perinatal period in this study. Since the gastrointestinal system develops differently in rodents and humans (Sangild, 2006), it is highly relevant to examine the distribution of GC in the perinatal gastrointestinal tract of the pig as a closer-to-human model. Indeed, not only is the naturally occurring SGA pig thought to be a suitable model for IUGR (Cooper, 1975), the gut of the pig is more comparable with the human gastrointestinal tract than that of rodents with regard to development, physiology and morphology (Sangild, 2006). The porcine model represents most of the symptoms associated with the metabolic syndrome in adult life seen in IUGR children, such as increased adiposity (Poore and Fowden, 2004) and glucose intolerance (Poore and Fowden, 2002). Additionally, IUGR alters gastrointestinal morphology in postnatal piglets (Xu *et al.,* 1994; Wang *et al.,* 2005; D'Inca *et al.,* 2010b). However, it is not known whether these alterations persist until weaning.

Since the metabolic and endocrine processes are disturbed in IUGR, normal feeding behaviour is crucial for both IUGR children and SGA piglets to achieve a normal postnatal growth rate. Therefore, we first investigated whether the distribution of the orexigenic GCs is altered both qualitatively and quantitatively in the gastrointestinal system of the porcine IUGR animal model during perinatal development. Next, we determined whether the amount of the gastrointestinal GCs expressing active ghrelin was related to the serum concentration of total ghrelin. We hypothesize that the immature gastrointestinal system of SGA piglets is reflected by an altered gastric morphology, thereby contributing to lower growth rates.

4 Materials and methods

4.1 Animals and experimental design

Perinatal piglets with body BWs ranging within 0.5 standard SD of the mean litter BW were considered as NW piglets, whereas piglets with BW lower than 1.5 SD of the

mean litter BW were defined as SGA piglets. PF from the third trimester of gestation were obtained from a local slaughterhouse. The age of the PF was estimated by measuring the crown-rump length (CRL) (Evans and Sack, 1973). Postnatal pigs from different days (d) of age (0 d, 3 d, 10 d and 28 d) were collected at a commercial farm from multiparous sows (Finnish Yorkshire x Belgian Landrace) and transferred within 30 minutes to the laboratory of Applied Veterinary Morphology. In general, piglets on commercial Belgian farms are weaned at the age of 4 weeks. In this study, piglets were not weaned *sensu stricto,* but immediately removed from the sow. All piglets were euthanized by severing the common carotid arteries under deep barbiturate anaesthesia (sodium pentobarbital, 200 mg/kg, Kela Laboratoria, Hoogstraten, Belgium) immediately upon arrival. Age and gender-matched pairs consisting of foetal and postnatal NW and SGA piglets were selected. This resulted in 5 pairs of piglets per age group. The sample collection was organised as such that the paired NW and SGA piglets were processed simultaneously. This study was approved by the Ethical Committee on Animal Experimentation from the University of Antwerp.

4.2 Sample collection

Blood from postnatal piglets was collected during exsanguination. Serum specimens were allowed to clot for 20 min at room temperature (RT) and were subsequently centrifuged at 4°C at 1,500 x g. The gastrointestinal tract was immediately removed after euthanasia and processed on ice. The empty weight of the stomach was recorded and only the pars fundica was retained for further sampling. The length of the SI was measured and divided into 3 equal-length segments corresponding to the proximal, middle and distal SI. The colon was divided into a proximal and distal part. After rinsing in 0.01 M phosphate buffered saline (PBS) (pH 7.4), samples were fixated for 2 h in 4% (w/v) paraformaldehyde (PFA) in distilled water at RT. The fixative was subsequently washed out overnight with PBS. From each sample a full thickness biopsy was taken (8 mm biopsy punch, Miltex, Plainsboro, New Jersey, USA) and processed to paraffin blocks of which 4 µm vertical sections were made. The gastric pars fundica was stereologically analysed using systematic randomly retained (i.c.

every 5th section after trimming the tissue block in a random position) sections that were processed for immunohistochemistry.

4.3 Immunohistochemistry

After rehydration the sections were incubated in Tris-EDTA (pH 9) (Dako, Glostrup, Denmark) and heated in a microwave oven (15 min, 90 W) to retrieve antigenicity. Sections were allowed to cool down for 15 min (RT). After rinsing 3 times for 5 min with 0.05 M Tris-buffered saline (TBS) (pH 7.4), endogenous peroxidase activity was depleted by incubating the sections in 3% (v/v) H_2O_2 in methanol (10 min; RT). Non-specific staining was blocked with normal goat serum (NGS) (1:5, Dako, Glostrup, Denmark), diluted in TBS enriched with 0.3% Triton X-100 (v/v) and 1% (w/v) Bovine Serum Albumin (BSA) for 30 min at RT. Subsequently, sections were incubated for 2 h at RT with purified polyclonal rabbit IgG against a human peptide from the N-terminus of acyl ghrelin, diluted with the same buffer as NGS (1:300, Alpha diagnostic International, San Antonio, USA). Following 3 TBS wash steps for 5 min, the sections were incubated for 1 h at RT with anti-rabbit Envision® (Dako, Glostrup, Denmark). After 2 wash steps for 5 min with TBS and 1 wash step for 5 min with distilled water, positive reactions were revealed by incubating the sections with the chromogen 3,3'-diaminobenzidine (Dako, Glostrup, Denmark). The sections were counterstained with Carazzi's hematoxylin (Klinipath, Olen, Belgium), dehydrated and mounted with glycerol.

4.3.1 Qualitative analysis

Immunostained sections from the small (proximal, middle and distal parts) and large intestine (proximal and distal parts) were qualitatively analysed and scored for the presence (+) or absence (-) of closed- and opened-type GCs with an Olympus BX41 microscope (Olympus Belgium, Aartselaar, Belgium). The data are presented as percentage of positive intestinal samples.

4.3.2 Quantitative analysis- Stereology

For the quantitative analysis, an Olympus BX50 microscope connected to a computer running the software program Cast 2 (Olympus Belgium, Aartselaar, Belgium) was used. One single investigator performed the analysis blinded to the age or BW of the pigs from which the samples were collected.

The V_vs of the tunica mucosa, tela submucosa, tunica muscularis and ghrelin IR cells were estimated by using a point grid at magnification 400x. The different V_vs were calculated using the following stereological equation:

$$V_V (Y, \text{reference volume}) = [\Sigma P (Y) / \Sigma P (\text{reference volume})]$$

Σ P (Y) refers to the number of points hitting the region of interest and *Σ P (reference volume)* refers to the number of points hitting the reference volume. The entire gastric wall was used as the reference volume of the tunica mucosa, tela submucosa and tunica muscularis. The reference volume of the ghrelin IR epithelial cells was the tunica mucosa. In order to determine the distribution of the ghrelin IR cells, the reference volume of the tunica mucosa associated with the fundic glands was divided into three equal parts: base, middle and neck of the glands. In addition to the V_v related to the entire tunica mucosa, the V_vs of the ghrelin IR cells were also determined in these three different parts of the fundic glands.

The optimal density of the stereological grid (number of points), the number of sections, and the number of fields were estimated as described previously (Gundersen and Jensen, 1987) and resulted in analysing approximately 30 fields of vision in at least 15 systematic random sections of each tissue block.

The following equation was used in order to estimate the weight of the different fundic layers and GC:

$$W (est) = V_v (Y) \times W (\text{weight reference volume})$$

W (est) represents the estimated weight of the region of interest, $V_v (Y)$ is the V_v of the region of interest and *W (weight reference volume)* is the estimated weight of the reference volume. The weight of the pars fundica was used as reference weight for the fundic layers. The latter weight was determined by dividing the weight of the empty

whole stomach by four (Frappier, 2006). Next, the weight of the GC was estimated by multiplying the weight of the pars fundica tunica mucosa by the V_v of the GC.

4.4 Serological analysis

Total serum ghrelin levels were measured with a porcine ghrelin RIA kit (Phoenix Pharmaceuticals, Belmont, California, USA). The protocol was performed on a MULTIGAMMA 1261 gammacounter and analysed with the software program MultiCalc 1224 (Perkin Elmer, Zaventem, Belgium). Since the active form of ghrelin is highly unstable in blood, the predominant form in serum is unacylated ghrelin. Therefore, this study focused on total (active and unacylated) ghrelin serum concentrations. Some observations had values lower than the detection limit of the kit. Treating these observations as missing values would cause bias since it would selectively remove observations with low values. Thus, the values below the detection limit were set equal to the detection limit itself (100 pg/ml).

4.5 Statistical analysis

The effects of BW and age on the volume, weight and distribution in the fundic glands (base, middle, neck) of the GCs; weight and volume of the fundic layers together with the serum ghrelin concentrations were studied by fitting linear mixed models with BW and age as predictors. Age was entered as a categorical variable in most analyses, unless a clear linear trend was found upon visual inspection. In case the distribution of the residuals after regression was non-normal, regressions were performed on the logarithm of the outcome. To take into account for the relatedness between observations within the same litter, a random intercept term for litter was added to the model. Adding a random slope term for weight did not lead to a significant improvement in the model fit for any of the variables tested. To fit the regression model for the fixed effects, a stepwise backward model building strategy was applied; starting with the model that includes age, BW and their interaction, whereby the interaction term was first tested for significance. In case the interaction term was not significant, this term was removed from the model and a model including only the main effects for age and BW was fitted. Significance of the fixed effect terms in the

equation was tested using the F-test with a Kenward-Roger correction for the numbers of degrees of freedom. In case one of the factors (age or region) was significant, a posthoc test was performed with a Tukey correction for multiple testing. A *P*-value below 0.05 was considered significant.

All statistical calculations were performed in the software package R version 2.13.1 (www.r-project.org). Mixed models were fit using the lme4 package. The F-test with Kenward-Roger correction was performed using the package pbkrtest, and the posthoc test with Tukey correction was carried out as implemented in the multcomp package. Graphs were generated using the lattice package or Excel.

5 Results

5.1 Distribution of ghrelin cells along the gastrointestinal tract

In general, ghrelin IR cells were distributed throughout the gastrointestinal tract both pre- and postnatally in NW and SGA piglets. Ghrelin endocrine cells were abundant in the fundic mucosa, but were less numerous in the intestinal mucosa. Moreover, the presence of GCs diminished from the small to the large intestine in both NW and SGA piglets (Figure 3.1).

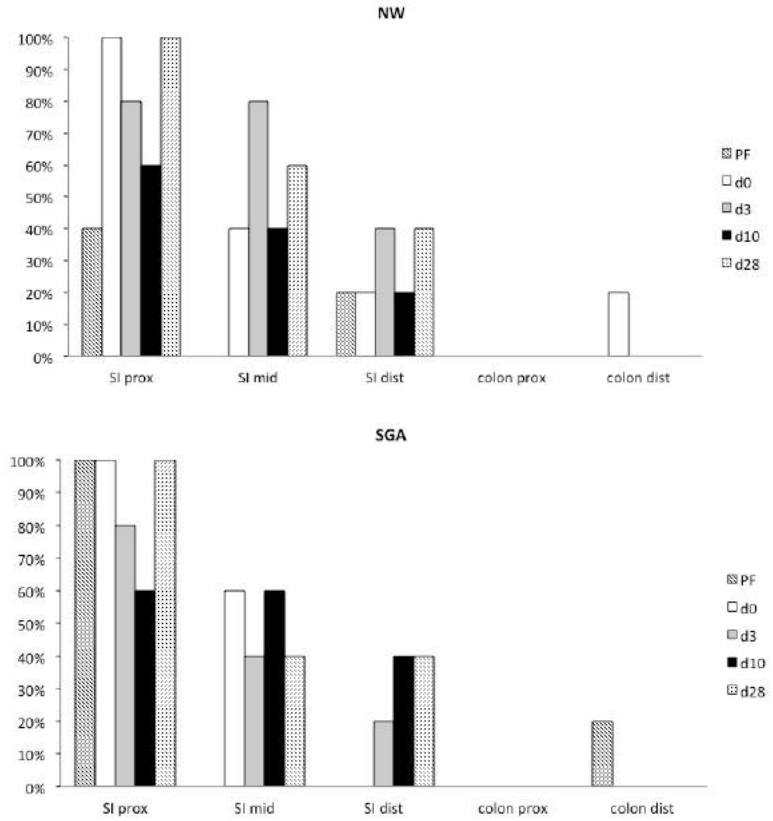

Figure 3.1 Graph showing the perinatal intestinal distribution of GCs in NW and SGA pigs according to age. The GCs population diminishes from the SI to the colon in both NW and SGA piglets. The data are presented as percentages of positive intestinal samples.

Ghrelin endocrine cells were scattered in the epithelia of the intestinal crypts and villi. Both opened and closed-type cells were observed in the porcine intestine (Figure 3.2), whereas in the porcine gastric fundic glands only closed-type GCs were present.

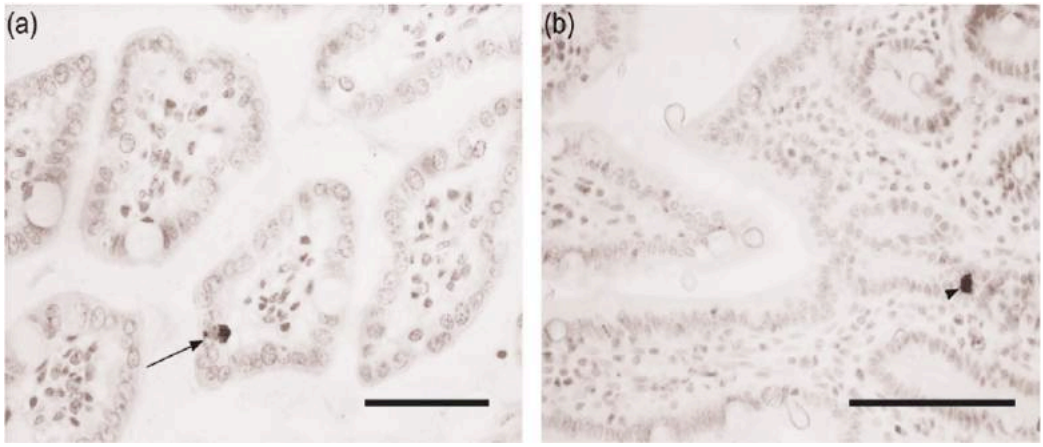

Figure 3.2 Triangular shaped opened-type GC (arrow) in the proximal part of the SI of a SGA foetal piglet (a), scale bar= 50 µm. Closed-type GC (arrowhead) in the middle part of the SI of a d28 SGA piglet (b), scale bar= 100 µm

The V_v of GCs in the three different regions (base, middle and neck) of the fundic glands were significantly different from each other (P < 1E-10) with the highest V_v in the base, followed by the middle and the top regions. However, the differences in distribution did not differ significantly across the different age and BW groups (P > 0.05 for interaction between region X BW and region X age) (Figure 3.3).

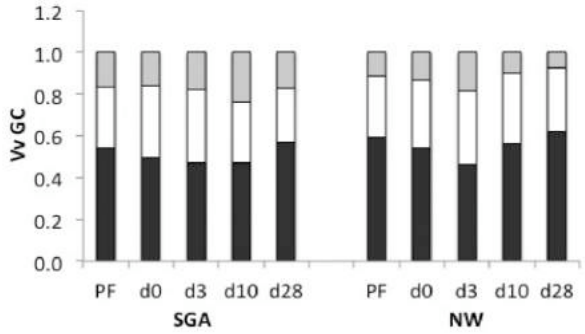

Figure 3.3 V_v of GCs in the base (black bars), middle (white bars) and neck part (grey bars) of the fundic glands in normal and SGA piglets until weaning.

5.2 Ghrelin in the perinatal gastric mucosa and circulation of SGA and normal piglets: stereological and serological analyses

The V_v of ghrelin IR epithelial cells in the perinatal gastric mucosa was similar between the different SGA age groups (P = 0.47). Remarkably, a significant interaction was found between age (categorical) and BW (P = 0.006), implying that age-related differences in the V_v of ghrelin IR cells were not uniform across the SGA and NW piglets. A separate analysis showed that in NW piglets the V_v of ghrelin IR epithelial cells of the neonates was significantly higher compared to the other NW age groups (P = 0.0019) (Figure 3.4 and Figure 3.5).

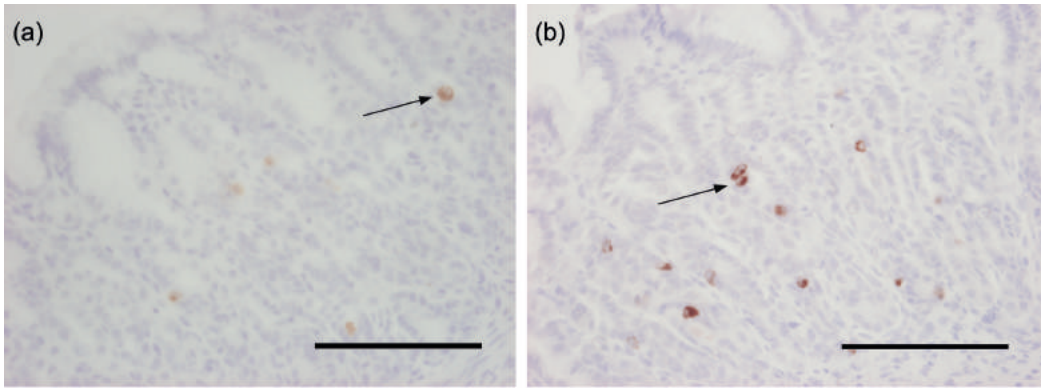

Figure 3.4 Round-shaped closed type ghrelin epithelial cells in the pars fundica of a d0 SGA (a) and NW (b) piglet. Scale bars= 100 µm.

The estimated weight of ghrelin IR epithelial cells increased exponentially with age in both NW and SGA piglets. Therefore, age was entered as an ordinal variable into the linear regression model. The increase in weight was highly significant (P = 0.007), but the rate of increase was not significantly different between normal and SGA piglets (P = 0.52 for the interaction between weight and age). However, piglets from the NW group had on average a significantly higher weight of GCs compared to their SGA littermates (P = 0.03) (Figure 3.5).

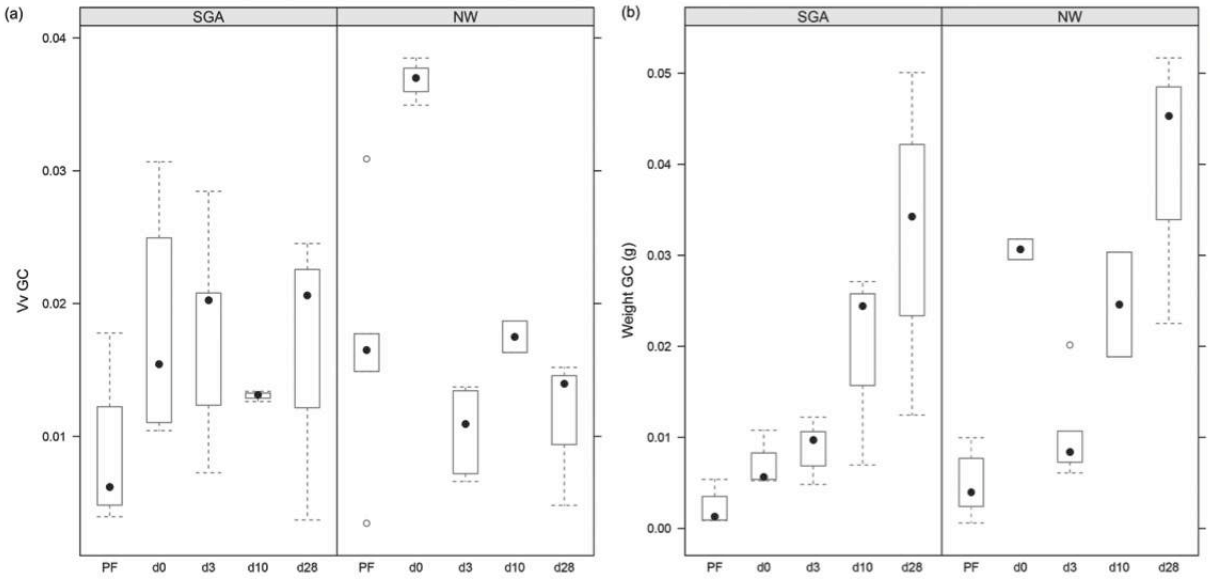

Figure 3.5 V_v (a) and weight (b) of fundic ghrelin epithelial cells in relation to age shown in boxplots, with the data grouped into two panels according to BW.

In contrast to the stereological analysis of GCs expressing active ghrelin, total ghrelin serum concentrations did not show an age (P = 0.96) or a BW (P = 0.41) effect (Figure 3.6).

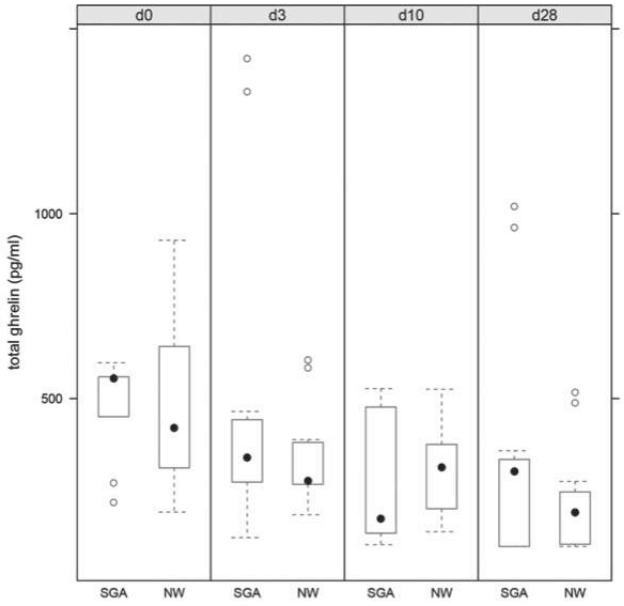

Figure 3.6 Boxplots showing the serum concentrations of total ghrelin according to BW, with the data grouped into 4 panels according to age.

5.3 Volume densities and weights of the pars fundica and its layers: tunica mucosa, tela submucosa and tunica muscularis

The relative weights of the pars fundica (g/kg BW) did not show an age effect, but a significant difference between NW and SGA piglets was found. The SGA piglets had on average higher relative fundic weights compared to their normal littermates (Table 3.1). Although the V_v of the tunica mucosa remained more or less constant during development, the weight of the tunica mucosa significantly increased during postnatal development, with the most pronounced increase from day 10 onwards. The log(weight) increased linearly with age. On average, NW piglets had higher tunica mucosa weights compared to their SGA littermates. However, the increase in tunica mucosa weight during development was not different between normal and low weight piglets (Table 3.1).

No significant effect of BW was seen in the V_v of the tunica muscularis, but a difference according to age was found (P = 0.02). Age groups d10 and d28 differed significantly from the foetal and the d3 age groups (Figure 3.7). The weight of the tunica muscularis mimicked the changes seen in the tunica mucosa: an increase postnatally in both SGA and NW piglets. This age effect was highly significant with the log of the weight increasing linearly with age. Like the weight of the tunica mucosa, the most pronounced increase was found from d10 onwards. Moreover, a significant effect of BW was observed, with the NW piglets showing significantly higher values compared to their SGA littermates. However, the age related differences in the tunica muscularis weight were not significantly different between NW and SGA piglets (Table 3.1).

A significant difference in V_v of the tunica submucosa was present between age categories (P = 9E-5), with foetal pigs having significantly higher V_vs compared to all other age categories (Figure 3.7).

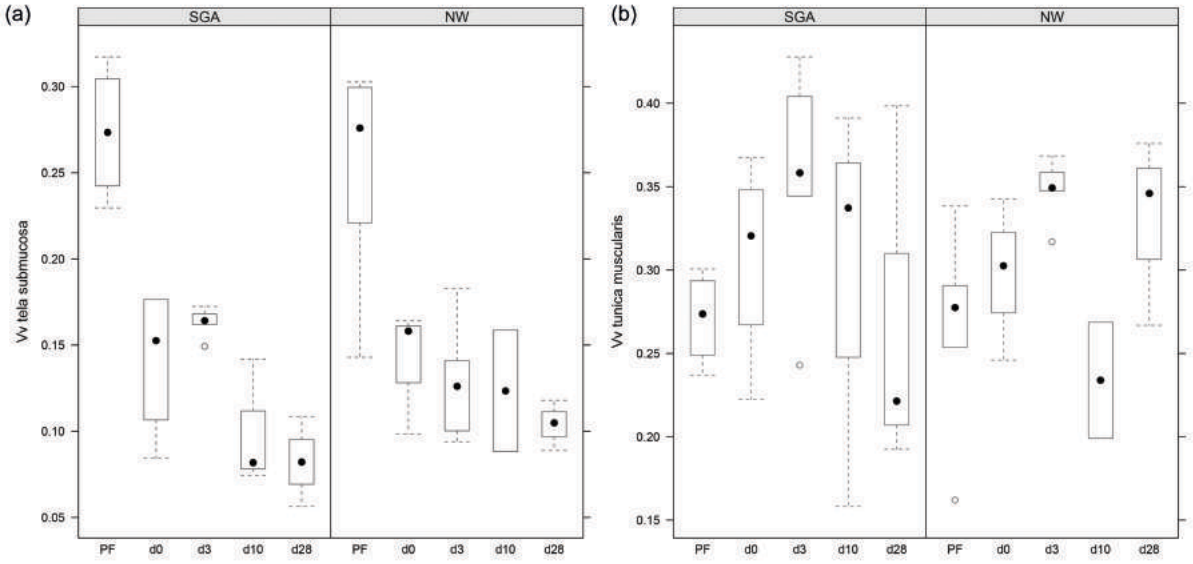

Figure 3.7 Boxplots showing the V_vs of the tela submucosa (a) and tunica muscularis (b) according to age, with the data grouped into two panels according to BW.

No BW related differences were observed and the differences between age groups were not different between NW and SGA littermates. Similar to the other layers of the fundic gastric wall, the weight of the tela submucosa increased during postnatal development in both SGA and NW piglets, with the d10 and d28 piglets being significantly different from the other age groups. The mean weight of the tela submucosa was higher in NW piglets compared to SGA littermates. Similar to the other fundic layers, the increase in tela submucosa weight during development did not differ between normal and low BW littermates (Table 3.1).

		Age					RSD	*P*-Value[1]	
		PF	d0	d3	d10	d28		Age	Weight
Body Weight (kg)	NW	0.75	1.91	1.59	3.63	8.01	0.17	<1E-16	8.7E-6
	SGA	0.53	0.79	0.93	2.53	5.41			
Weight pars fundica (g)/ body weight (kg)	NW	1.30	1.36	1.66	1.63	1.45	0.18	NS	0.0007
	SGA	1.64	1.72	1.72	1.63	1.65			
Weight tunica mucosa (g)	NW	0.27	0.84	1.00	1.37	3.79	0.30	<1E-16	0.0017
	SGA	0.24	0.41	0.55	1.40	2.35			
Weight tela submucosa (g)	NW	0.23	0.42	0.36	0.72	1.21	0.16	6.9E-5	NS
	SGA	0.23	0.20	0.26	0.49	0.71			
Weight tunica muscularis (g)	NW	0.48	1.34	1.51	3.80	6.52	0.30	<1E-16	8.8E-5
	SGA	0.40	0.74	0.75	2.88	5.69			

Table 3.1 Effect of age on the weight of the gastric pars fundica and its distinct layers (estimated by V_v) in NW and SGA piglets. NS= not significant; RSD= residual standard deviation.

6 Discussion

In this study, SGA piglets have on average higher relative fundus weights compared to their NW littermates. This observation corresponds with previously published data (D'Inca *et al.,* 2010a). The weights of the different fundic layers are in general higher in NW animals compared to their SGA littermates. This accords with a previous study, which demonstrated similar differences in the thickness of the gastric layers between normal and growth restricted piglets (Xu *et al.,* 1994). However, the developmental growth pattern, i.e. the age related increase in mucosal weight, did not differ between normal and low weight piglets. The mucosal weights were estimated from tissues that had been fixated in PFA and afterwards embedded in paraffin. These procedures can induce tissue type dependent shrinkage and compression. Therefore, the weights of

[1] No significant interaction was found between age and BW for all variables, implying that the increase in weight according to age is not different between NW and SGA piglets

the fundic layers determined by this stereological approach probably underestimate the real *in vivo* weights. However, the scope of this study was to compare these quantities among the different piglet groups from which the tissues had been similarly prepared. Since it was expected the shrinkage and compression to be the same in all groups, we concluded that the various layers of the porcine gastric pars fundica do not show BW dependent morphological diversification during development. These results provide valuable information about the developmental changes in the gastric morphology, which will assist in the interpretation of the maturation of gastric functioning in both normal and SGA piglets.

This study shows that GCs populate the entire gastrointestinal tract of the pig during the perinatal period. As in humans, most of the ghrelin-expressing cells are located in the piglet's stomach (Rindi *et al.,* 2002). Similar as in foetal and adult man and adult pig, the density of GCs gradually decreases from the SI to the colon (Rindi *et al.,* 2002; Wierup *et al.,* 2007). Similar to rats, GCs of the porcine intestinal tract exist as two cell types, i.e. round shaped closed-type cells and triangular shaped cells that are open into the lumen. Opened-type cells react to luminal information such as pH and nutrients, whereas closed-type cells are stimulated by hormones, neuronal stimulation or mechanical distension (Sakata *et al.,* 2002a; Vitari *et al.,* 2012).

Although closed-type GCs are present along the entire length of the fundic glands, the majority remains located at the glandular base, even when the pigs reach the weaning age. These results contrast with a study in rodents, in which GCs spread from the base to the neck of the gastric glands with increasing age (Sakata *et al.,* 2002b). Nonetheless, a recent study did observe this spreading in the oxyntic mucosa in older pigs (from 28 days to 7 months of age) (Vitari *et al.,* 2012). Altogether, this pinpoints species dependent timing of gastrointestinal maturation. In most rodents, the maturational changes are rather late and quick and occur mostly around weaning. In contrast, in humans, gastrointestinal maturation occurs rather early and progresses relatively slow. In large domestic animals such as pigs, gastrointestinal maturation timing is intermediate and takes place both pre- and postnatally shortly after weaning (Sangild, 2006). It is possible that the quick maturation process in rats highlights the

difference in GC distribution during the weaning period and hence this altered distribution is only visible in pigs after weaning.

During 'neonursing', colostrum is continuously available for a period of 11 hours after the start of farrowing (Lewis and Hurnik, 1986). The higher weight of epithelial ghrelin IR cells that we found in NW d0 piglets can possibly be attributed to this phase of nursing. Specifically, this larger number of GCs might implicate higher ghrelin secretion, which stimulates milk intake. However, this hypothesis is not supported by our serological analysis. This discrepancy might be explained by the fact that the immunohistochemical analysis determined the amount of GCs expressing the acylated, active form of ghrelin, whereas the serological analysis measured both active and unacylated ghrelin levels. Unacylated ghrelin is, in contrast to its acylated form, not able to bind the ghrelin receptor and because of this, was initially considered as being physiologically inactive. Today, accumulating evidence indicates that unacylated ghrelin is also involved in metabolic processes via a separate signaling system (Toshinai *et al.*, 2006). The present study demonstrated comparable serum ghrelin levels in both SGA and normal piglets at all time points studied. This accords with human data, which failed to show any difference between IUGR and normal infants (Kyriakakou *et al.*, 2009). Others however, demonstrated higher ghrelin levels in SGA neonates (Farquhar *et al*., 2003). Hence, the specific role of ghrelin in perinatal growth remains unclear. Further studies determining both circulating active and unacylated ghrelin levels are necessary in order to define the specific roles of these two ghrelin forms in IUGR.

The lower weight of GCs expressing active ghrelin in SGA piglets can further complicate sufficient milk consumption. This accords with previous studies, which emphasize that birth weight is an important factor regulating milk intake (Milligan *et al.,* 2001; Devillers *et al.,* 2007). On the other hand, appropriate milk intake may contribute to the maturation process of the gastrointestinal system and may thereby influence the amount of GCs. Because of their low birth weight, SGA piglets are not able to compete with larger siblings for colostrum (Hendrix *et al.,* 1978). Although most piglets establish to own a particular teat, many SGA piglets presumably fail,

resulting in lower and insufficient intake of high quality colostrum (Depassille *et al.,* 1988). However, the present study has shown that the growth pattern of the fundic layers is not disturbed in perinatal SGA piglets. Therefore it can be assumed that the developing stomach of SGA piglets has the same structural morphology as in NW piglets and hence possesses the same necessary components for mature functioning.

Weaning is considered one of the most stressful periods that has a negative impact on feed intake and BW control, hence influencing ghrelin secretion (Salfen *et al.*, 2004). Our results did not show an effect at 28 days of age (age of weaning) on the amount of gastric GCs nor total ghrelin serum concentrations. Hence, in our study the impact of weaning on ghrelin homeostasis in the gastrointestinal system is not evaluated. However, research of Du and colleagues indicates that changes only appear 10 days after weaning (Du *et al.,* 2007). Interestingly, exogenous ghrelin induces weight gain in weaning piglets (Salfen *et al.*, 2004). Therefore, further research is needed on GC development and ghrelin secretion in piglets within the weaning period.

One limitation might be that ghrelin levels are not measured after a fasting period. To circumvent this, each NW-SGA pair has been collected and processed simultaneously. Moreover, the statistical analysis takes the relatedness of observations within litters, hence within NW-SGA pairs, into account.

Another issue of this study might be that the samples were collected from an uncontrolled environment, more specifically a commercial farm. Since significant differences in the gastric distribution of ghrelin endocrine cells can be observed in this uncontrolled environment, the sample collection in the commercial farm does not confound or complicate the interpretation of our results. Moreover, these results might provide insight in both human and (domestic) piglet's physiology. Interestingly, a recent study has shown that enteral administration of ghrelin in neonatal piglets influenced both intestinal growth and intestinal epithelial cell turnover (Slupecka *et al.*, 2012). Since IUGR induces intestinal growth impairment (D'Inca *et al.*, 2010b), the knowledge of an altered gastric GC distribution in SGA piglets may be used for the preparation of milk formulas for neonates suffering from an insufficient development of the gastrointestinal system.

7 Conclusions

To our knowledge, this is the first study investigating gastrointestinal and circulating ghrelin in intrauterine growth restricted piglets by comparing the serum levels of total ghrelin and number of GCs expressing active ghrelin in the gastrointestinal tract in perinatal SGA piglets and normal littermates. Accordingly, it was demonstrated that NW newborns have a higher number of gastric GCs compared to their SGA littermates. These results emphasize the importance of further research to circumvent the vicious circle of insufficient perinatal nutrition and gastrointestinal development in the pathology of IUGR, thereby having a permanent effect on the physiology and growth of both IUGR infants and SGA piglets.

8 Acknowledgements

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Chapter 4 Enteric and serological distribution of serotonin and its precursor tryptophan in perinatal low and normal weight piglets

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Perinatal growth restriction is not related to higher intestinal distribution and increased serum levels of 5-hydroxytryptamine in piglets

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Enteric and serological distribution of serotonin and its precursor tryptophan in perinatal low and normal weight piglets

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1 Abstract

Perinatal mortality is high among SGA piglets and continues to be an economic burden and threat to animal welfare. Since the physiological role of 5-HT in perinatal development and gastrointestinal function in the pig remains unknown, the aim of this study was to assess the enteric distribution of 5-HT cells and to determine 5-HT together with its precursor tryptophan in serum of perinatal normal and SGA piglets. To this purpose, proximal and distal parts of the SI were processed for immunohistochemistry to assess the presence of 5-HT endocrine cells. Serum 5-HT was measured with enzyme linked immuno sorbent assay (ELISA), while its precursor, i.e. FFT together with albumin-bound tryptophan and total tryptophan were analysed with high performance liquid chromatography (HPLC) in postnatal piglets. In addition, the morphological growth patterns of the different intestinal tissue layers of both normal and SGA piglets were stereologically analysed. The stereological V_v of 5-HT enteroendocrine cells showed a significant interaction effect between age and region. Indeed, the amount of 5-HT cells in both the proximal and distal part of the SI tended to decrease according to age, with the lowest values detected at day 3 postpartum. No differences could be observed related to BW. Interestingly, the serum concentration of 5-HT was higher in normal piglets compared to SGA piglets. Moreover, the ratio FFT to total tryptophan was significantly affected by age and BW. Normal piglets had on average a lower FFT/total tryptophan ratio compared to SGA piglets. An approximate linear decrease was observed with increasing age. Finally, the immaturity of the intestinal system of SGA piglets was not reflected in altered V_vs of the different intestinal layers. To conclude, although no BW effect could be detected in the distribution of enteric 5-HT cells, serum 5-HT and the ratio of FFT to total tryptophan ratio showed significant differences between normal piglets and their SGA littermates.

2 Implications

The use of hyperprolific sows in pork industry increases the prevalence of prenatal growth restricted piglets, characterised by reduced survival rates. 5-HT is prominently present in the gastrointestinal system and regulates feeding behaviour and BW. This study investigated the enteric distribution of 5-HT cells and the concentration of this hormone along with its precursor tryptophan in serum of perinatal SGA and normal littermates. These results - combined with the morphological analysis of the SI - will give insight into the endocrine programming and morphological adaptations of the SI of SGA piglets.

3 Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter that regulates feeding behaviour and BW (Lam *et al.*, 2010). Two independent 5-HT systems exist: one is present in the brain and one in the periphery. The central nervous system only synthesizes 2% of the total amount of 5-HT whereas 95% of 5-HT is derived from the gastrointestinal tract (Erspamer, 1953; Twarog and Page, 1953). In the latter, 5-HT is mainly present in enterochromaffin cells whereas only a small amount is located in the enteric nervous system (Erspamer, 1954). In research, most attention has been focussed on the role of brain 5-HT although enteric 5-HT also plays a key role as a growth factor, hormone and as a neurotransmitter (Gershon, 2013).

Litters from hyperprolific sows often display a natural form of IUGR. Prenatal growth restriction results in SGA pigs characterized by high perinatal mortality and morbidity (Quiniou *et al.*, 2002). Due to intrauterine malnutrition, SGA piglets develop altered endocrine pathways, as known for the IGF system, in order to survive (Fowden *et al.*, 2005; De Vos *et al.*, 2013). Since 5-HT enhances GH secretion, which in turn stimulates IGF production (Musumeci *et al.*, 2013), we hypothesize that this altered endocrine balance is reflected in an altered intestinal distribution of enterochromaffin cells. In the blood circulation, FFT is an important marker for encephalic 5-HT synthesis, while most 5-HT in blood is derived from the

gastrointestinal tract (Erspamer and Testini, 1959; Tagliamonte *et al.*, 1973; Manjarrez *et al.*, 1998). Intriguingly, infants and rats suffering from IUGR show elevated plasma FFT levels compared to normal weight infants and littermates (Hernandez *et al.*, 1989). Moreover, IUGR impairs gastrointestinal morphology in neonatal pigs (Wang *et al.*, 2005; D'Inca *et al.*, 2010a). Since 5-HT promotes mucosal growth (Gershon, 2013), we hypothesize that the possible altered 5-HT levels in SGA piglets affect the intestinal morphology in these piglets.

To conclude, the aims of this study were to investigate whether the perinatal intestinal distribution of 5-HT cells is altered in SGA pigs compared to their normal littermates. Moreover, 5-HT synthesis from both the periphery and the brain were compared in normal and SGA pigs during postnatal development by analysing serum 5-HT and FFT levels. Finally, we determined the impact of IUGR on the morphological growth pattern of the different intestinal layers.

4 Material and methods

4.1 Animals and experimental design

Piglets with BWs ranging within 0.5 SD of the mean litter BW were considered as NW piglets, whereas piglets with BW lower than 1.5 SD of the mean litter BW were defined as SGA piglets as described previously (D'Inca *et al.*, 2010b; Willemen *et al.*, 2012). Mean BW of the different age and body weight groups are shown in Table 4.1. Across all age groups, NW piglets had a significantly higher BW, but in some groups the differences were larger than others (Table 4.1). PF (90-115d of gestation) were obtained from a local slaughterhouse. Their ages were estimated by measuring the CRL (Evans and Sack, 1973). Postnatal pigs from different days of age (d0, d3, d10 and d28) were collected at a local farm from multiparous sows (Finnish Yorkshire x Belgian Landrace) and transferred within 30 minutes to the laboratory of Applied Veterinary Morphology. Euthanasia of these piglets was carried out by severing the carotid arteries under deep barbiturate anaesthesia (sodium pentobarbital, 200 mg/kg, Kela Laboratoria, Hoogstraten, Belgium) immediately upon arrival. Age- and gender-matched pairs consisting of a NW and SGA piglet were selected. This resulted

in five pairs of piglets per age group. The sample collection was organised as such that the paired NW and SGA piglets were processed simultaneously.

This study was approved by the Ethical Committee on Animal Experimentation from the University of Antwerp.

		Age					RSD	*P*-value Age x Weight
		PF 90-115d	d0	d3	d10	d28		
Body Weight (kg)	NW	0.75	1.78	1.58	3.77	8.21	0.01	<0.0001[1]
	SGA	0.53	0.84	0.93	2.40	5.31		
P-value[2]		0.003	0.007	0.024	0.003	0.001		

Table 4.1 Mean body weight of perinatal NW and SGA piglets

4.2 Sample collection

Blood was collected from postnatal pigs by severing the carotid arteries after lethal barbiturate anaesthesia. After an incubation period of 20 min at RT, the blood samples were centrifuged at 4°C at 1,500 g for 10 min. After euthanasia, the gastrointestinal tract was immediately removed and kept on ice. Samples from the proximal and distal parts of the SI were taken as described previously (Willemen *et al.*, 2013). After rinsing in PBS (0.01 M, pH 7.4), these samples were fixated for 2 h in 4% PFA at RT. The fixative was washed out with PBS overnight. A full thickness biopsy was taken from each sample (8 mm, Miltex, Plainsboro, New Jersey, USA). These were subsequently routinely processed to paraffin blocks. From each sample, vertical sections with a thickness of 4 µm were taken at systematically random positions (i.e. every 5th section) and processed for immunohistochemistry and stereological analysis.

[1] A significant interaction between age and weight was found, which means that the BW differences between the SGA and NW animals are not the same across the different age categories.

[2] *P*-values upon splitting the dataset according to age and testing for a difference in mean BW between NW and SGA in each separate age group.

4.3 Immunohistochemistry

After rehydrating the sections, they were rinsed three times with TBS (0.05 M, pH 7.4). Subsequently, endogenous peroxidase activity was depleted by incubation with 3% H_2O_2 in TBS for 10 min at RT. Non-specific staining was blocked by incubating for 1 h at RT with 20% normal swine serum diluted in TBS enriched with 0.3% Triton X-100 and 1% BSA. Paraffin tissue sections were then incubated overnight (4°C) with a polyclonal rabbit anti-5-HT antibody (1/1000; Chemicon, Millipore, Billerica, MA). Sections were rinsed and subsequently incubated with a biotinylated swine anti-rabbit antibody, diluted with the same buffer as normal swine serum (1/600, 2h RT; Dako, Glostrup, Denmark). After a next rinsing step with TBS, the sections were immediately incubated with streptavidin-conjugated horseradish peroxidase (1/600, 2 h RT; Dako, Glostrup, Denmark). After two wash steps for 5 min with TBS and one wash step for 5 min with distilled water, IR cells were visualised by incubating the sections with the chromogen 3,3'-diaminobenzidine (Dako, Glostrup, Denmark). The sections were counterstained with Carazzi's haematoxylin (Klinipath, Olen, Belgium), dehydrated and mounted with glycerol.

4.4 Stereological Analysis

An Olympus BX50 microscope connected to a computer running the software program Cast 2 (Olympus, Copenhagen, Denmark) was used for the stereological analysis. One single investigator, blinded to the origin of the samples, performed the analysis.

From both the proximal and distal part of the SI, the V_vs of the tunica mucosa, tela submucosa and tunica muscularis (V_v intestinal layer, reference volume: entire SI wall) was estimated by using a point grid at magnification 200x. The following equation was used to calculate V_v (intestinal layer, SI wall*):*

$$V_v \text{ (intestinal layer, SI wall)} = [\Sigma P \text{ (intestinal layer)} / \Sigma P \text{ (SI wall)}]$$

where Σ P (intestinal layer) refers to the number of points coinciding with the specific layer and Σ P (SI wall) refers to the number of points coinciding with the entire wall of the SI.

In both the proximal and distal part of the SI, the V_v of the epithelial 5-HT IR cells was estimated. The stereological equation used to calculate the V_V *(5-HT IR cells, epithelial layer*) was defined as:

$$V_V(\text{5-HT IR cells, epithelial layer}) = [\Sigma P(\text{5-HT IR cells}) / \Sigma P(\text{epithelial layer})]$$

Σ P (5-HT IR cells) refers to the number of points hitting the IR 5-HT epithelial cells and Σ P (epithelial layer) refers to the number of points hitting the epithelial layer of the tunica mucosa.

The optimal density of the stereological grid (number of points), the number of sections, and the number of fields were estimated as described previously and resulted in analysing approximately 30 fields of vision in at least 15 systematic random sections of each tissue block (Gundersen and Jensen, 1987).

4.5 Serological analysis

Serum 5-HT levels were measured by a multispecies ELISA according to the manufacturer's protocol (Enzo Life Sciences, Lorrach, Germany) as described previously (Willemen *et al.*, 2012).

For the analysis of total Trp, the proteins present in 30 µl serum were precipitated with 60 µl perchloric acid (0.4 M). Afterwards, the samples were centrifuged for 5 min at 12,00 g at RT and the supernatant was used for HPLC analysis. FFT was recovered by first ultrafiltrating the serum samples using an Amicon Ultra 0.5 ml 50K centrifugal filter (Millipore, Overijse, Belgium) and was further prepared analogously to the total Trp procedure before analysis. The difference between total and free Trp was considered to be the fraction bound to albumin as described previously (Manjarrez *et al.*, 2005).

Serum Trp concentrations were measured by isocratic reversed-phase liquid chromatography using a C18 4 µm Nova-Pak (Waters S.A.S., Saint Quentin, France) and detected with a 2487 dual absorbance UV detector (Waters S.A.S, Saint Quentin, France) at 273 nm. The mobile phase consisted of 90% MilliQ, 10% acetonitrile and phosphoric acid (pH 2.7) at a flow rate of 1ml/min. This protocol is based on a recently published study (Sultana *et al.*, 2012). Following Trp dilutions were used in

order to create a standard curve: 7, 5, 3, 1, 0.5 and 0.25 µg/ml. The concentrations are determined by the molar extinction coefficient of 5600/(M.cm) of 1M Trp at 280 nm.

4.6 Statistical analysis

The effects of weight, age and intestinal region on the different outcome parameters were studied by fitting linear mixed models. To account for the relatedness between observations within the same litter and within the same individual, random intercept terms for litter and individual, nested within litter, were added to the model. Adding random slope terms for weight and region did not lead to a significant improvement in the model fit for any of the variables tested. To fit the optimal regression model for the fixed effects, a stepwise backward model building strategy was applied, starting from a model that included main effect terms for weight, region and age (as a categorical variable), as well as their 2-way interactions.

Significance of the fixed effect terms in the equation was tested using the F-test with a Kenward-Roger correction for the numbers of degrees of freedom. In case one of the factors (age category or region) was significant, a posthoc test was performed with a Tukey correction for multiple testing. A *P*-value below 0.05 was considered significant.

All statistical calculations were performed in the software package R version 2.13.1. Mixed models were fit using the lme4 package. The F-test with Kenward-Roger correction was performed using the package pbkrtest, and the posthoc test with Tukey correction was carried out as implemented in the multcomp package. Graphs were generated using the lattice package.

5 Results

5.1 Enterochromaffin cells in the SI: stereological analysis

In general, 5-HT cells were distributed along the entire SI both pre- and postnatally in NW and SGA piglets. The endocrine cells were located in the intestinal epithelium, covering crypts and villi (Figure 4.1).

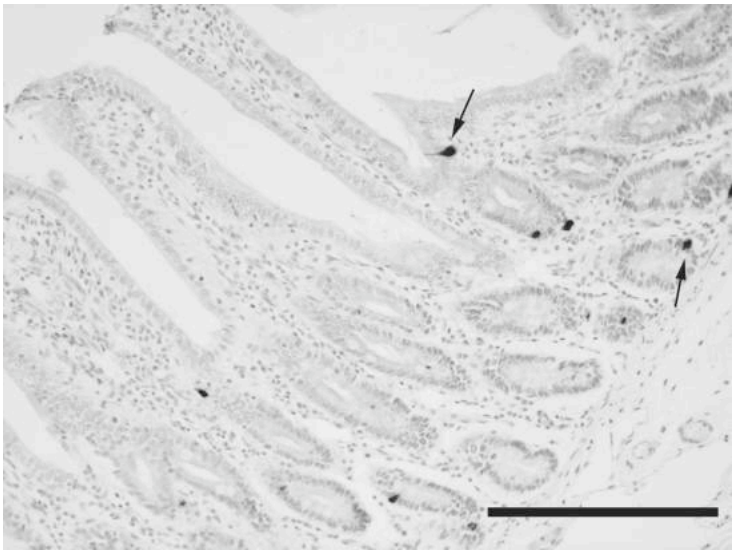

Figure 4.1 5-HT IR cells (arrows) scattered in the epithelia of crypts and villi in the distal part of the SI of a NW d28 piglet, scale bar= 200 µm.

The V_v of the intestinal enterochromaffin cells showed a significant interaction between age and intestinal region (*P* = 0.0001) (Figure 4.2). At the age of 3 days, the 5-HT cell V_v in the SI was consistently the lowest, but more pronounced differences between the age groups were observed in the distal SI. In the proximal region, the overall effect of age on enterochromaffin cell density was significant *(P* = 0.008). Posthoc testing using a Tukey correction for multiple testing, showed a significantly lower 5-HT cell V_v at d3 compared to PF 90-115d (*P* = 0.001, mean difference = 0.013) and a significantly higher enterochromaffin cell V_v at d10 compared to d3 (*P* < 0.001, mean difference = 0.015). None of the other pairwise comparisons was significant at the 0.05 level. In the distal region, the overall effect of age on 5-HT cell V_v was significant (*P* = 0.0002). Posthoc testing using a Tukey correction showed that the enterochromaffin cell V_v value at PF 90-115d was significantly higher compared to all postnatal values (Figure 4.2). Mean differences and P- values for each pair of comparison which were significant at the 0.05 level are given in Table 4.2. The postnatal measurements showed no differences (Figure 4.2).

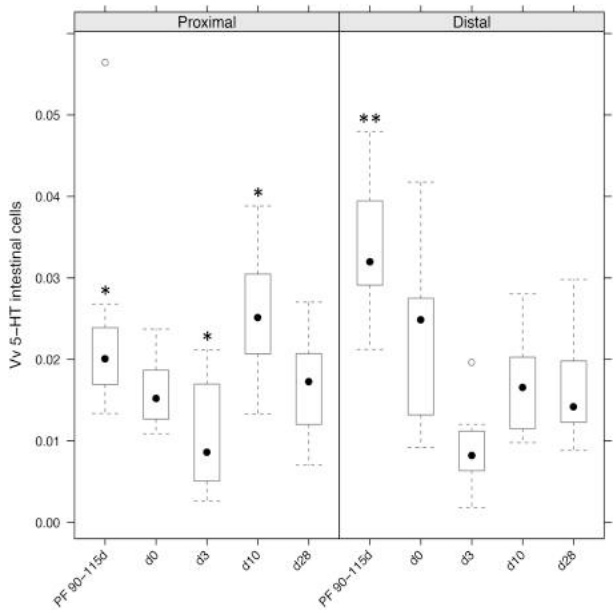

Figure 4.2 V_v of intestinal 5-HT epithelial cells in relation to age. The V_v is a dimensionless unit since it relates two volumes with the same unit (μm^3/ μm^3). The data are grouped into two panels according to the small intestinal region where the endocrine cells are located. The sample size consists of five age- and gender- matched pairs of a NW and SGA piglet per age group. The 5-HT cell V_v was the lowest at d3 in both the proximal and distal part of the SI. In the proximal part of the SI, the V_v was significantly lower compared to the foetal and d10 age groups (*, $P \leq 0.001$). In the distal part of the SI, the foetal age group had the highest V_v compared to all other age groups (**, $P = 0.0002$).

Age groups	Mean difference V_v 5-HT intestinal cells	P- value
PF 90-115d vs. d0	0.011	0.02
PF 90-115d vs. d3	0.025	<0.001
PF 90-115d vs. d10	0.017	<0.001
PF 90-115d vs. d28	0.017	<0.001

Table 4.2 Mean differences of 5-HT volume densities (V_V) from the distal part of the SI and the significant *P*- values from posthoc analysis for pairwise comparisons between prenatal piglets (PF 90-115d) and the different postnatal age groups (d0, d3, d10 and d28).

5.2 Serotonin and its precursor tryptophan: serological analysis

On average, NW piglets had more serum 5-HT compared to the SGA littermates (P = 0.008; Table 4.3). Likewise, total Trp serum concentrations of NW piglets were higher compared to SGA piglets (P = 0.001; Table 4.3). The total Trp concentration also showed an age-dependent effect (P = 0.0001; Table 4.3). More specifically, posthoc testing failed to show a difference between d0 and d3, but significant differences between all other age groups were observed. A marginal difference between d0 and d28 (P = 0.05) was observed.

When looking at FFT, post hoc analysis showed that piglets at d28 had significantly lower FFT levels compared to piglets from all three other age groups (Table 4.3). For the albumin-bound fraction of Trp, statistical analysis was performed on log-transformed values since the outcome was strongly non-normal. NW piglets had on average a greater albumin-bound Trp fraction compared to SGA piglets (Table 4.3). The effect related to age for this fraction of Trp showed a difference between early (d0 vs. d3) and late (d10 vs. d28) age, with the later stages having a significantly higher concentration compared to the early stages (Table 4.3).

The outcome of the FFT/ total Trp ratio was also strongly non-normal. Hence, statistical analyses were performed on log-transformed values. Significant main effects of age and weight were observed. The ratio FFT to total Trp was lower in NW piglets compared to SGA piglets (Table 4.3). As for the effect of age, a slightly linear decrease in outcome was observed with increasing age. Newborns (d0) did not have significantly different FFT/total Trp ratios compared to d3 piglets, but all other pairwise comparisons showed significant differences (Table 4.3).

		Age				RSD	*P*-value	
		d0	d3	d10	d28		Age	Weight
5-HT (ng/ml)	NW	906.8	1086.3	1535.1	1089.6	168.5	NS	0.008
	SGA	726.3	996.7	1120.7	934.7			
Total Trp (µg/ml)	NW	5.98	5.39	11.77	8.32	0.49	0.0001	0.001
	SGA	4.34	4.18	9.30	6.58			
FFT (µg/ml)	NW	3.07	2.99	3.09	1.27	0.15	0.001	NS
	SGA	3.05	2.35	3.13	1.28			
Albumin-bound Trp (µg/ml)	NW	2.90	2.39	8.69	7.05	0.28	<0.0001	0.003
	SGA	1.29	1.83	6.17	5.30			
FFT/total Trp	NW	0.52	0.49	0.27	0.16	0.21	0.028	<0.0001
	SGA	0.70	0.57	0.30	0.20			

Table 4.3 Effect of age on serum 5-HT concentrations, its precursor FFT, albumin-bound Trp and total Trp in NW and SGA piglets

5.3 Intestinal morphology

The V_vs of the different intestinal layers (tunica mucosa, tela submucosa and tunica muscularis) showed a similar morphological growth pattern in normal and SGA piglets (Figure 4.3) ($P > 0.05$).

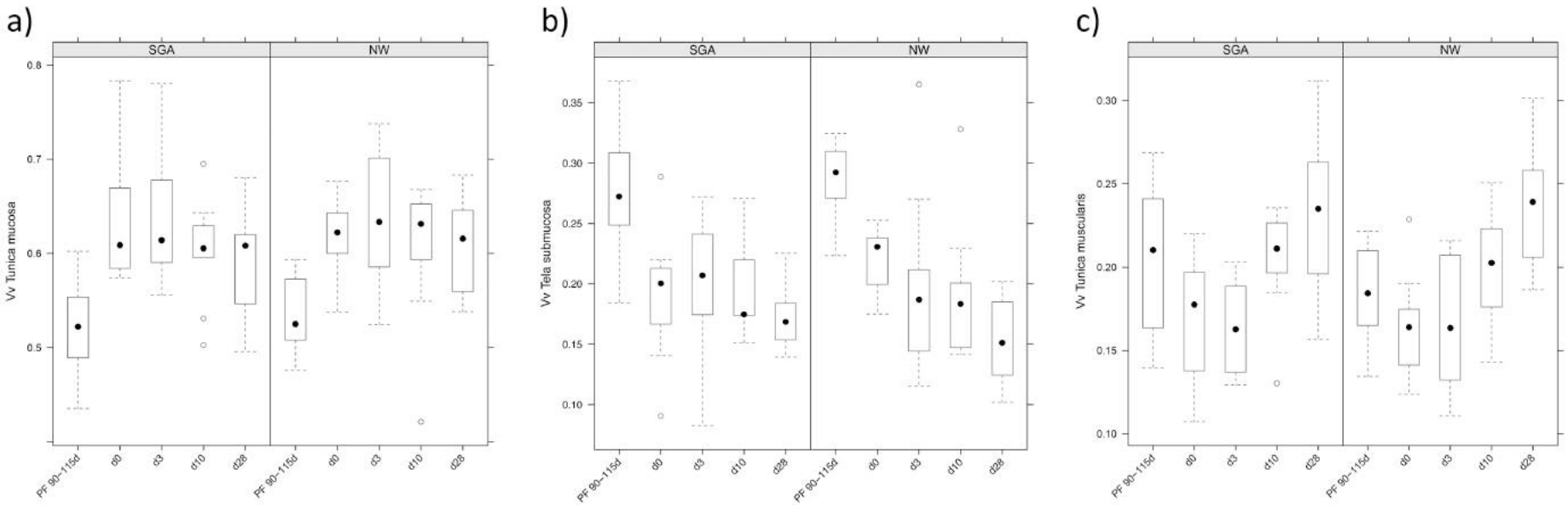

Figure 4.3 V_v of the intestinal tunica mucosa (a), tela submucosa (b) and tunica muscularis (c) in relation to age. The V_v is a dimensionless unit since it relates two volumes with the same unit (μm^3/ μm^3). The data are grouped into two panels according to BW. The sample size consists of five age- and gender- matched pairs of a NW and SGA piglet per age group. There were no significant differences in V_v of the three intestinal layers in NW and SGA piglets.

The V_v of the tunica mucosa showed a significant interaction between age and region (*P* = 0.012). A separate analysis in proximal and distal samples showed that during prenatal development (PF 90-115d) the proximal samples had a significantly reduced V_v of the mucosal layer compared to the postnatal samples (*P* = 0.0004). In the distal samples there were no significant differences between prenatal and postnatal samples (*P* = 0.10) (Figure 4.4).

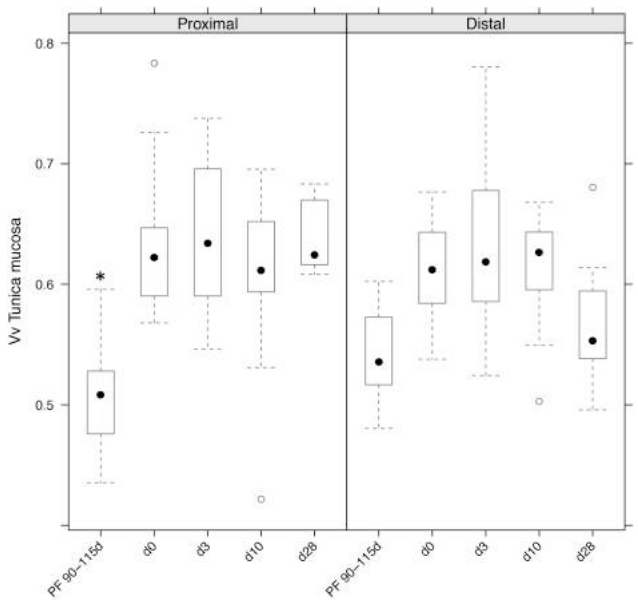

Figure 4.4 V_v of the intestinal tunica mucosa in relation to age. The V_v is a dimensionless unit since it relates two volumes with the same unit ($\mu m^3/ \mu m^3$). The data are grouped into two panels according to the small intestinal region. The sample size consists of five age- and gender- matched pairs of a NW and SGA piglet per age group. The prenatal samples of the proximal SI had a significantly reduced V_v of the mucosal layer compared to the postnatal samples (*, *P* = 0.0004).

The V_v of the tela submucosa showed a main effect of age (*P* = 0.002). Posthoc testing showed that in contrast to the tunica mucosa, the V_vs of the foetal piglets were higher compared to those in postnatal piglets (Figure 4.5). Mean differences and *P*- values for each pair of comparison which were significant at the 0.05 level are given in Table 4.4.

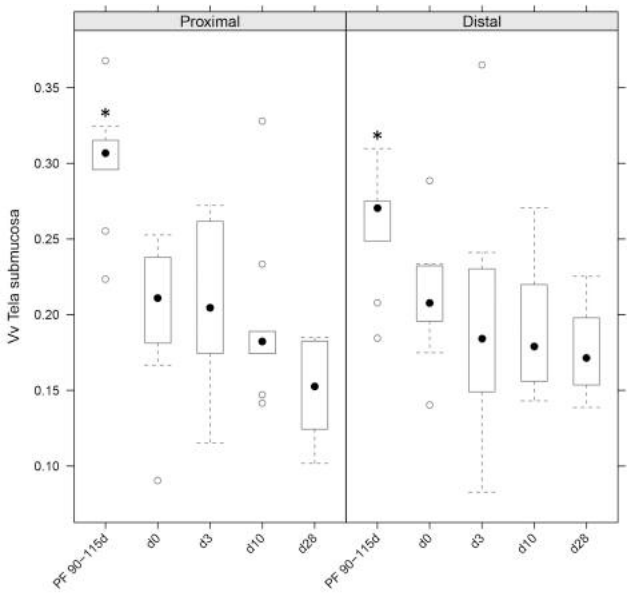

Figure 4.5 V_v of the intestinal tela submucosa in relation to age. The V_v is a dimensionless unit since it relates two volumes with the same unit (μm^3/ μm^3). The data are grouped into two panels according to the small intestinal region. The sample size consists of five age- and gender- matched pairs of a NW and SGA piglet per age group. The V_v of the intestinal tela submucosa of the foetal piglets was higher compared to the tela submucosa Vv in postnatal piglets. This finding is reflected in both the proximal and distal part of the SI (*, *P* = 0.002).

Age groups	Mean difference V_v tela submucosa	*P*-value
PF 90-115d vs. d0	0.073	0.003
PF 90-115d vs. d3	0.079	0.001
PF 90-115d vs. d10	0.087	<0.001
PF 90-115d vs. d28	0.116	<0.001

Table 4.4 Mean differences of intestinal tela submucosa volume densities (V_v) and the significant *P*- values from posthoc analysis for pairwise comparisons between prenatal piglets (PF 90-115d) and the different postnatal age groups (d0, d3, d10 and d28).

Significant effects of region (*P* = 0.01) and age (*P* = 0.001) were observed for the V_v tunica muscularis. On average, the proximal region showed lower values of V_v tunica muscularis compared to the distal region. On average, the V_v tunica muscularis is 0.017 higher in the distal region compared to the proximal region (*P* = 0.01).

Posthoc testing for age showed significant differences between PF 90-115d and d28 ($P = 0.004$), d0 and d28 ($P < 0.001$), d3 and d10 ($P = 0.03$) and between d3 and d28 ($P < 0.001$).

6 Discussion

The morphological results from this study demonstrated both region- and age-related differences in the SI. More specifically, the mucosa showed a postnatal increase in V_v, which was most pronounced in the proximal region. This is in accordance with previous data (Van Ginneken *et al.*, 2002; Van Ginneken and Weyns, 2004). Likewise, a postnatal decrease of the V_v tela submucosa (Van Ginneken and Weyns, 2004) and a drop of the V_v tunica muscularis after birth have been described (Van Ginneken *et al.*, 2002). Moreover, these authors also described a thicker tunica muscularis in the distal part of the SI (Van Ginneken *et al.*, 2002). The thicker muscle layer in this intestinal region serves to pump the small intestinal chyme into the colon. Hence, our findings of the developing intestine in normal piglets correspond to previously published data. However, the developmental growth pattern, i.e. the age-related changes of the various elements of the intestinal wall did not alter in SGA piglets. As such, this similarity between NW and SGA piglets corresponds to earlier observations on the growth pattern of the pars fundica of the stomach (Willemen *et al.*, 2013).

The present study showed that there were no significant differences in intestinal enterochromaffin cell densities between NW and SGA piglets. In contrast to earlier reports regarding the prevalence of 5-HT in the stomach of the postnatal piglet (Van Ginneken *et al.*, 2001), the V_v of the 5-HT cells in both the proximal and distal part of the SI tended to decrease with age, with the lowest values detected at 3 days postpartum. Similar results have also been demonstrated in an immunohistochemical study of the intestinal tract of the water buffalo (Lucini *et al.*, 1999). As previously suggested, the decreasing V_v of the enterochromaffin cells might be caused by an increase in mucosal tissue volume per surface area after birth (Van Ginneken *et al.*, 2002). However, this does not exclude the possibility that the higher density of 5-HT

cells in the foetal small intestinal mucosa contributes to a higher bioavailability of 5-HT. In this way, it could play a role in the development of the foetal gastrointestinal system as a growth factor and neurotransmitter (Fiorica-Howells *et al.*, 2000; Gershon and Tack, 2007; Gershon, 2013). Ristine and colleague have suggested an important role for 5-HT in the suckling ritual, based on their experiments in newborn rats (Ristine and Spear, 1984) which might explain the high neonatal (d0) density of 5-HT enterochromaffin cells seen in our study. Another possible explanation for the higher intestinal 5-HT cell density in foetal piglets is that this important peripheral 5-HT source can compensate for deficiencies in encephalic 5-HT production, since the prenatal blood brain barrier is immature (Trowbridge *et al.*, 2011).

NW piglets have higher 5-HT serum concentrations compared to their SGA littermates. This is in accordance with previous data (Berman *et al.*, 1965). These lower concentrations of 5-HT in SGA piglets might be attributed to a fall in the number of platelets, as already described in low birth weight infants (Christensen *et al.*, 2006). Another study in foetal piglets also described lower foetal Trp concentrations in IUGR (Lin *et al.*, 2012) and suggested this might be due to impaired amino acid transport through the placenta (Avagliano *et al.*, 2012). Although the previous proposition correlates with our data describing lower total tryptophan levels in SGA piglets, we cannot rule out that this latter finding is due to a lower feed intake in these piglets (Devillers *et al.,* 2007).

Other studies described an elevation of FFT and its ratio to total Trp (FFT/total Trp) in IUGR children (Manjarrez *et al.*, 1998; Hernandez-Rodriguez *et al.*, 2009). Interestingly, our results also described a higher FFT/total Trp ratio in SGA piglets. This altered ratio can be explained by the significantly higher total Trp concentrations caused by an elevated albumin-bound fraction detected in NW piglets. The binding capacity of L-Trp to plasma albumin has shown to be lower in infants with IUGR compared to normal controls (Hernandez-Rodriguez *et al.*, 2009). This might account for the lower albumin-bound fraction detected in our SGA samples.

7 Conclusions

The results from this study clearly demonstrate that 5-HT concentrations together with its precursor Trp are altered in the circulation of SGA piglets. This finding however, is not reflected in a different distribution of enteroendocrine 5-HT cells in the SI of these SGA piglets. Hence, further research is necessary to find the source of the altered circulating 5-HT concentration in SGA piglets. Moreover, the adaptation of circulating Trp in SGA piglets suggests that, like in IUGR humans and rats, the central serotonergic system may also be disturbed in the SGA piglet. Since the level of Trp clearly has an impact on neuronal 5-HT synthesis (Henry *et al.*, 1992; Shen *et al.*, 2012), the knowledge of lower tryptophan levels in SGA piglets might encourage further research concerning dietary tryptophan supplementation in these prenatal growth restricted piglets.

8 Acknowledgements

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Chapter 5 The impact of prenatal growth restriction on serum levels of appetite regulators and glucose metabolism in piglets

Under revision, Animal

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1 Abstract

Developmental alterations caused by poor prenatal growth permanently affect the physiology and metabolism of the newborn, thereby predisposing these individuals to endocrine and metabolic disorders. Ghrelin, leptin and 5-HT are appetite regulators involved in perinatal development and glucose metabolism. The aims of this study were to correlate the serum levels of ghrelin, leptin and 5-HT and to relate these to the glucose and insulin levels and the PI (BW (kg)/ CRL (m^3)) of postnatal SGA and NW piglets of different ages (d0, d3, d10 and d28 after birth). Additionally, weight and age related differences of the various above-mentioned serum parameters were determined. This study demonstrates that mainly the PI is associated with the serum levels of the appetite regulators. More specifically, ghrelin concentration is inversely correlated, whereas leptin and 5-HT levels are positively correlated with PI. In addition, NW piglets have higher leptin and 5-HT concentrations compared to their SGA littermates. Leptin levels are also influenced by age since d10 piglets show the highest serum concentrations of all age-examined groups. The correlations between the different concentrations of appetite regulators as well as their correlations with insulin and glucose levels and the PI of the piglets correspond to human data. Therefore, this study gives insight into both porcine and human developmental adaptations related to endocrine appetite regulation in case of prenatal growth restriction. Hence, this study provides additional arguments for using the SGA piglet to study the prenatal deposition of adult metabolic diseases.

2 Implications

Because of the high perinatal mortality in both humans and pigs, IUGR has an ethical and economical impact. Moreover, the IUGR associated endocrine alterations predispose these individuals to metabolic diseases in adulthood. This study compared the levels of the appetite regulators ghrelin, leptin and 5-HT in postnatal normal and SGA piglets. Furthermore, these levels were correlated with the glucose and insulin levels of these piglets together with their PI as a measure for leanness. These findings aim to render additional insight into the developmental adaptations of endocrine appetite control in SGA piglets.

3 Introduction

The thrifty phenotype hypothesis proposes that the association between poor foetal growth and the subsequent development of metabolic disorders in adult life results from prenatal growth restriction, which causes permanent changes in glucose metabolism (Hales and Barker, 1992). A relationship between glucose tolerance and birth weight has indeed been shown in human adults (Fowden *et al.*, 2005). Interestingly, it has been demonstrated that juvenile and adult pigs show an altered glucose tolerance and endocrine functioning in relation to low birth weight, illustrating that the pig could be an interesting model to study this mechanism (Poore and Fowden, 2002).

Hormones such as ghrelin, leptin and 5-HT are involved in appetite regulation. Ghrelin is a hunger-stimulating hormone that was first detected in the rat and human stomach (Kojima *et al.*, 1999). Its appetite-modulating opponent is the 'satiety hormone' leptin that is mainly produced by adipose tissue. The stomach is a non-adipose source of leptin (Bado *et al.*, 1998). 5-HT is a monoamine, which is mainly secreted by enterochromaffin cells of the gastrointestinal system. In research most attention has been paid on 5-HT secreted by the central nervous system, whereas the gastrointestinal system is the major source of this monoamine. Nevertheless, it has been shown that gastrointestinal 5-HT is also implicated in appetite regulation (Simansky, 1996).

Recent research in both animals and humans suggests that ghrelin (Verhulst and Depoortere, 2012), leptin (Fruhbeck and Salvador, 2000) and 5-HT (Watanabe *et al.*, 2011) are implicated in glucose metabolism. Indeed, an inverse relationship between the circulating concentrations of ghrelin and insulin has been shown (Saad *et al.*, 2002). In addition, insulin induces leptin production (Saad *et al.*, 1998) whereas 5-HT induces hyperglycemia and hyperinsulinemia (Watanabe *et al.*, 2011).

Ghrelin, leptin and 5-HT interact during appetite regulation. For instance, the antagonistic relationship between leptin and ghrelin is called 'the ghrelin-leptin tango' (Cummings and Foster, 2003). *In vivo* animal experiments have shown that leptin can induce its satiety effect by decreasing the synthesis or release of brain 5-HT through a leptin-5-HT axis (Oury and Karsenty, 2011). Moreover, Trp, the precursor of 5-HT, enhances ghrelin secretion, which is associated with increased weight gain in weanling pigs (Zhang *et al.*, 2007).

The description of the above-mentioned appetite regulators and energy homeostasis may provide insight into the metabolic alterations that takes place in prenatal growth restricted piglets during the perinatal period. Therefore, the aims of this study were to examine (1) whether the circulating levels of appetite regulators ghrelin, leptin and 5-HT are altered in SGA piglets compared to in their NW littermates and/or are affected by their leanness (PI), (2) whether these appetite regulators are involved in the perinatal porcine glucose metabolism by correlating their levels with glucose and insulin concentrations and, (3) whether ghrelin, leptin, 5-HT, glucose and insulin concentrations are interrelated.

4 Material and methods

4.1 Animals

Piglets with a BW lower than 1.5 SD of the mean litter BW were considered as SGA piglets, whereas piglets ranging within 0.5 SD of the mean litter BW were considered as NW piglets as described previously (Willemen *et al.*, 2013). Five age- and gender-matched pairs, consisting of a NW and SGA piglet were selected in litters from multiparous sows (Finnish Yorkshire x Belgian Landrace) housed at a local farm. The piglets were transferred within 30 minutes to the laboratory of Applied Veterinary Morphology either at d0, d3, d10 or d28 after birth and subsequently euthanized by severing the common carotid arteries under deep barbiturate anaesthesia upon arrival (sodium pentobarbital, 200 mg/kg, Kela Laboratoria, Hoogstraten, Belgium). The serum collection was organized as such that the paired NW and SGA piglets were processed simultaneously. BW and CRL were recorded. PI was calculated according to the following formula:

$$PI = BW\ (kg)/\ (CRL\ (m))^3$$

Multiple samples from the piglets in this study, including gastrointestinal tissue samples, were collected and analysed in order to answer multiple research questions within the same piglets (Willemen *et al.*, 2012; Willemen *et al.*, 2013). Therefore, repeated measurements on the same animals, hence a longitudinal study design was not possible. Another disadvantage might be that serum levels are not measured after a fasting period. To circumvent this, each NW-SGA pair had been collected and processed simultaneously. Moreover, the statistical analysis takes the relatedness of observations within litters, hence within NW-SGA pairs, into account. This study was approved by the Ethical Committee on Animal Experimentation from the University of Antwerp.

4.2 Biochemical assays

Blood glucose levels were determined with a Lifescan OneTouch Ultra glucometer® (Johnson & Johnson, Beerse, Belgium) during exsanguination. Serum samples were allowed to clot for 20 min at RT and were subsequently centrifuged at 4°C at 1,500 X g for 10 min. Insulin serum levels were analysed with a porcine ELISA (Mercodia, Uppsala, Sweden). Serum 5-HT concentrations were analysed using a multispecies 5-HT ELISA (Enzo Life Sciences, Lorrach, Germany). The serum concentrations determined by ELISA were measured with a Sunrise reader and analysed with the software program XFluor4 (TECAN, Tecan Benelux BVBA, Mechelen, Belgium). All procedures were performed according to the manufacturer's protocol.

Serum leptin levels were analysed using a multi-species leptin RIA (Millipore, St. Charles, Missouri, USA). Total serum ghrelin levels were measured with a porcine-specific ghrelin RIA kit (Phoenix Pharmaceuticals, Belmont, California, USA). The RIA samples were analysed on a MULTIGAMMA 1261 gamma counter and data were processed with the MultiCalc 1224 software (Perkin Elmer, Zaventem, Belgium).

For the immunoassays, samples were assayed in duplicate or triplicate whereas for glucose one measurement per animal was performed.

4.3 Data analysis

The values below detection limit were set equal to the detection limit itself; i.e. 100 pg/ml for ghrelin and 0.002 µg/l for insulin in order not to cause bias by removing values below the detection limit.

The effects of age, BW and PI were studied using linear mixed models. To take into account the relatedness between observations within the same litter and within the same individual, random effects for litter and individual (nested within litter) were added. For glucose, where only one measurement per individual had been taken, only a random intercept for litter was entered. As fixed effects, age (as a categorical variable), BW, their interaction and PI were entered. Significance of the fixed-effect terms was tested by a likelihood ratio test with a Kenward-Roger correction for the number of degrees of freedom. Posthoc testing for the effect of age was carried out

using a Tukey correction for multiple testing. The observed regression coefficients shown in Table 5.3 should be interpreted as the estimated average increase (+) or decrease (-) in the concerned serum parameter when the variable PI increases by 1 unit. For all models, the variance explained by the covariates was calculated by extracting the multiple R^2 from the final regression model.

The correlations between serum parameters ghrelin, leptin, 5-HT, glucose and insulin were calculated by Spearman correlations on untransformed values. Pairwise plots are shown with the Spearman correlation values.

All analyses were carried out in the software package R (www.r-project.org), using packages lmer, pbkrtest and multcomp.

5 Results and discussion

5.1 Influences of age, body weight and ponderal index (PI) on serum levels of appetite regulators and glucose metabolism

The morphometric measurements of the postnatal SGA and NW piglets as well as the serum concentrations of leptin, ghrelin, 5-HT, glucose and insulin are shown in Table 5.1 and Table 5.2 respectively.

Morphometric measurements	Birth weight	Age				RSD	*P*-values	
		d0	d3	d10	d28		Age	Body Weight
BW (kg)	NW	1.78	1.58	3.77	8.21	0.28	0.0001[1]	
	SGA	0.84	0.93	2.40	5.31			
CRL (m)	NW	0.31	0.31	0.37	0.51	0.02	6.4E-11	1E-7
	SGA	0.26	0.27	0.32	0.44			
PI (kg/m^3)	NW	57.49	56.83	73.06	63.93	11.29	0.01	NS
	SGA	50.62	56.05	71.03	62.85			

Table 5.1 Effect of age on the morphometric measurements from SGA and NW piglets.

The latter table shows that NW and SGA piglets share similar PI's. This indicates that these SGA piglets have a proportionally small length and weight, which means that their growth is restricted in a symmetrically way. It is striking that the PI shows an increase at d10, which is followed by a decrease at d28. The underlying mechanism is the following. The piglet is born with little fat. During the first week of life, the piglet grows very rapidly and fat is stored. The volumes of adipose tissues increase that fast that at the end of the first week, the piglet may have up to 20 times as much fat compared to its birth (Manners and McCrea, 1963). The PI decrease at d28 corresponds with previous data (Corson *et al.*, 2008).

[1] There is a significant interaction between age and birth weight, hence the differences between NW and SGA are not uniform across the different age groups. Splitting the dataset according to age showed that across all age groups, the NW piglets had a significantly higher BW, but in some age groups the differences were larger than in others. *P*-values upon splitting the dataset according to age and testing for a difference in mean BW between NW and SGA in each separate age group: d0 *P*= 0.004; d3 *P*= 0.02; d10 *P*= 0.002; d28 *P*= 0.001

Serum parameter	Body weight	Age				RSD	*P*-values	
		d0	d3	d10	d28		Age	Body Weight
Ghrelin (pg/ml)	NW	491.64	343.18	303.64	224.55	36.81	NS	NS
	SGA	480.00	502.18	293.73	352.91			
Leptin (ng/ml)	NW	1.53	1.30	1.95	1.55	0.15	0.01	0.03
	SGA	1.20	1.26	1.69	1.34			
5-HT (ng/ml)	NW	906.8	1086.3	1535.1	1089.6	168.5	NS	0.005
	SGA	726.3	996.7	1120.7	934.7			
Insulin (ng/l)	NW	12.70	21.50	21.50	49.20	0.15	NS	NS
	SGA	11.50	12.00	41.50	16.70			
Glucose (mmol/l)	NW	4.10	6.28	9.46	9.00	0.16	NS	NS
	SGA	3.88	5.33	8.30	8.56			

Table 5.2 Effect of age on the serum concentrations of ghrelin, leptin, 5-HT, insulin and glucose from SGA and NW piglets

Linear mixed model analysis of serum levels consistently showed a significant effect of PI on all the serum concentrations of the different hormones (Figure 5.1 and Table 5.3).

Dependent variables	Independent variables				
	Ghrelin (pg/ml)	Leptin (ng/ml)	Serotonin (ng/ml)	Insulin (µg/l)	Glucose (mmol/l)
Ponderal Index (kg/m^3)	-9.65	+0.0069	+8.78	+0.00028	+0.096
R^2	0.09	0.39	0.13	0.01	0.09

Table 5.3 Regression coefficients showing the average increase (+) or decrease (-) of the concerned serum parameter when ponderal index increases by one unit. The variance of these regressions is demonstrated by the R^2 from the final regression model.

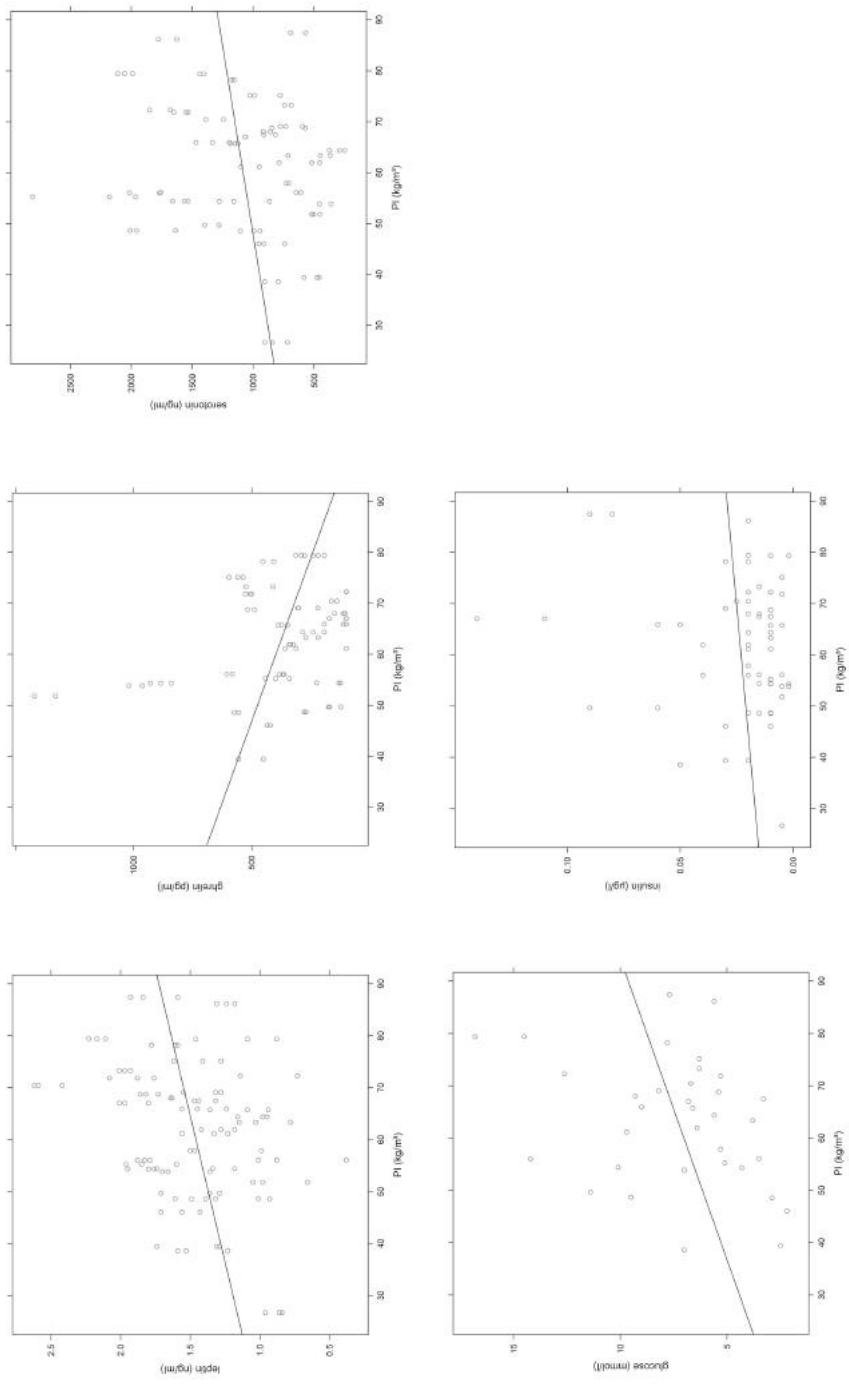

Figure 5.1 Scatterplots showing the influence of ponderal index (PI) on leptin, ghrelin, serotonin, insulin and glucose.

Ghrelin serum levels were negatively correlated with PI ($P < 2E-16$), which also has been demonstrated in humans (Soriano-Guillen *et al.*, 2004). No significant correlation with age or birth weight was observed. Analogous to observations in humans (Chiesa *et al.*, 2008), serum levels of leptin were positively correlated with PI ($P = 0.005$). In contrast to ghrelin, both age and birth weight were significantly related with serum levels of leptin, and these effects were not changed by in- or excluding PI as a covariate into the model. When correcting for PI and age, NW piglets had on average 0.21 ng/ml higher leptin levels compared to SGA piglets ($P = 0.03$), which also corresponds to human data (Bozzola *et al.*, 2010). When focussing on the association between leptin serum levels and age, the highest levels were observed at d10 ($P = 0.01$, Tukey correction for multiple testing) (Figure 5.2).

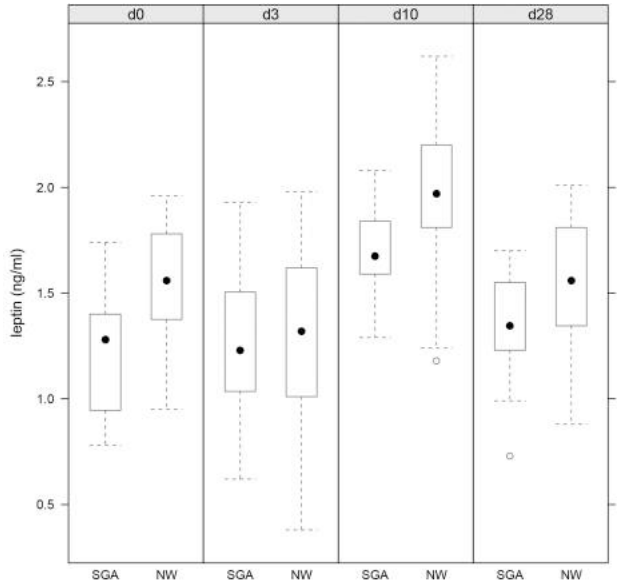

Figure 5.2 Boxplots showing the leptin serum concentrations according to birth weight. The data are grouped into four panels according to age.

Because of the small volumes of adipose tissue in neonatal piglets, colostrum and milk ingestion are essential to provide the piglets with sufficient amounts of energy. As already mentioned, the body fat percentage of piglets rises from 2% to 15% during the first week of life (Manners and McCrea, 1963), which is reflected by the significant rise of leptin serum levels on d10. Since sow milk yield peaks at 10 days

(Harrell *et al.*, 1993), the limited supply of milk at d28 can cause the decline of the leptin serum concentrations.

Glucose and insulin levels were similar in SGA and NW pigs, which is in agreement with previous observations in humans (Kyriakakou *et al.*, 2009, Bozzola *et al.*, 2010). Poore and Fowden (2002) only found an association between low birth weight and glucose intolerance in 1-year-old pigs. Like leptin, glucose (*P* = 9E-6) and insulin (*P* = 1.2E-11) are positively associated with PI (Table 5.3). In this regard, Setia *et al.* (2006) demonstrated that insulin levels are more closely related to PI than birth weight.

The positive correlation between 5-HT and PI does not correspond to previous data in adult women, which showed a negative correlation between 5-HT and BMI (Modder *et al.*, 2010). This apparent contradiction might be explained by the immature 5-HT synthesis in juvenile animals (Berman *et al.*, 1965) compared to adult women. The levels of 5-HT in NW piglets were on average 310.95 ng/ml higher compared to those in their SGA littermates (*P* = 0.005, regression coefficient adjusted for PI) (Figure 5.3).

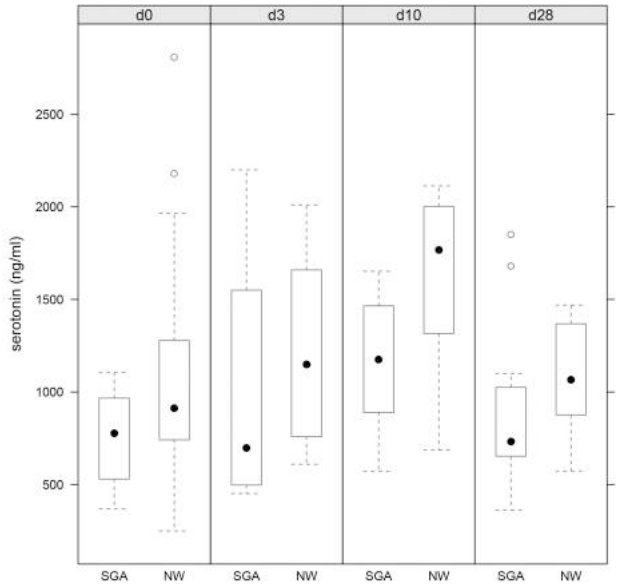

Figure 5.3 Boxplots showing the 5-HT serum concentrations according to BW. The data are grouped into four panels according to age

This is in accordance with previous data (Berman *et al.*, 1965; Willemen *et al.,* 2014). The lower concentration of 5-HT in SGA is probably related to a decrease in the number of circulating platelets (Christensen *et al.*, 2006).

5.2 Correlations between serum concentrations

Calculation of the Spearman ρ values showed that ghrelin and leptin levels are respectively negatively and positively correlated with 5-HT, glucose and insulin levels (Figure 5.4).

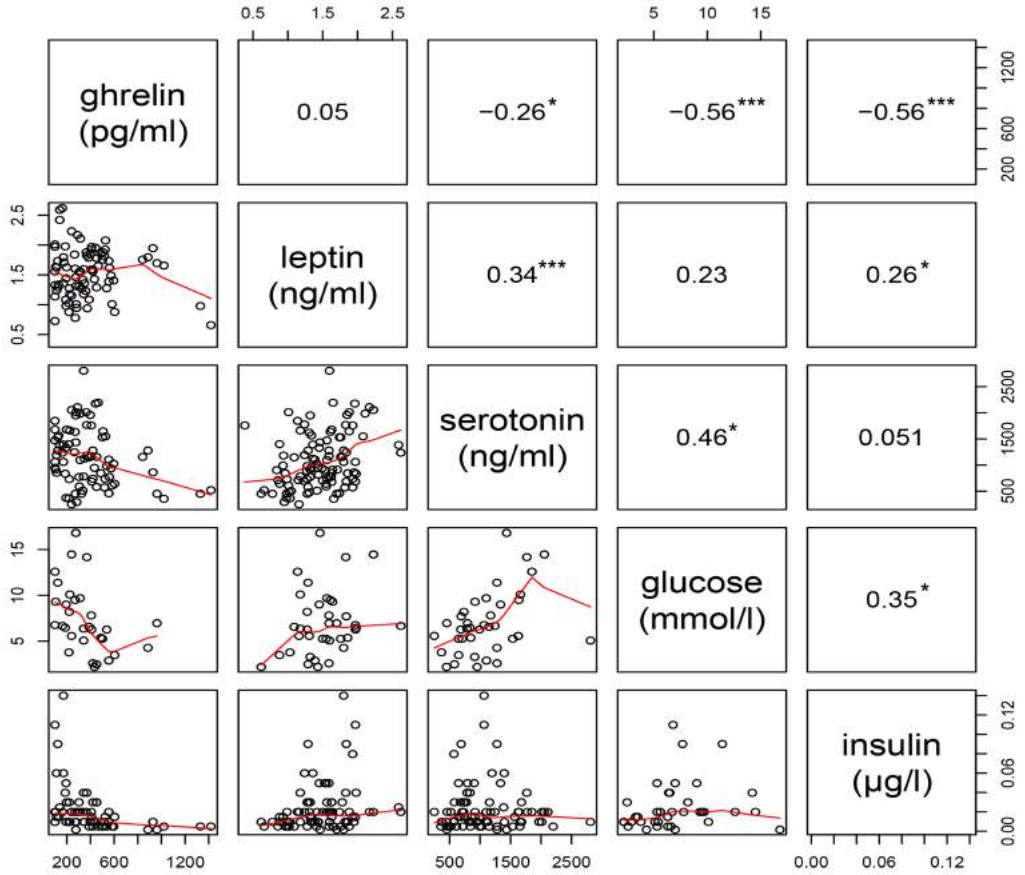

Figure 5.4 Pairwise scatterplots showing the correlations between the different serum parameters, together with their Spearman values. Each off-diagonal cell corresponds to a scatterplot of two of the variables and has the following format: the vertical axis of the plot is the variable named in diagonal element falling in the same row as the plot whereas the horizontal axis is the variable named in diagonal element falling in the same column as the plot. The Spearman values seen in this matrix have the following format: one of the variables of the pairwise correlation falls in the same row whereas the other variable falls in the same column as their respective Spearman value. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P \leq 0.001$

Surprisingly, no strong correlation between leptin and ghrelin was present. This finding has also been reported in children (Soriano-Guillen *et al.*, 2004). Notwithstanding the absence of a direct ghrelin-leptin correlation, these two 'presumed opponents' have opposite correlations with glucose, insulin and 5-HT. This emphasizes the difference in biological effects and regulation of secretion between ghrelin and leptin. While the secretion of ghrelin is promoted by hypoglycaemia, leptin secretion is induced by hyperglycaemia (Fruhbeck and Salvador, 2000; Shiiya *et al.*, 2002).

The negative association between total ghrelin and insulin levels can be attributed to the fact that postprandial insulin reduces ghrelin levels (Gil-Campos *et al.*, 2006). Indeed, there is considerable evidence that insulin and not leptin communicates the status of body fat stores to the system in order to regulate ghrelin levels (Saad *et al.*, 2002). However, other studies indicate that ghrelin, leptin and insulin interact and that leptin regulates ghrelin levels and BW (Williams and Mobarhan, 2003). The positive correlation between serum insulin and leptin levels corresponds to the insulin-induced increase in leptin expression (Ramsay and White, 2000).

The negative correlation of circulating total ghrelin levels and the positive correlation of leptin levels with 5-HT correspond to clinical data, demonstrating that ghrelin levels are decreased and leptin levels are increased after cisplatin chemotherapy (Matsumura *et al.*, 2013). Cisplatin-based chemotherapy induces gastrointestinal disorders such as nausea, vomiting and appetite loss. These disorders involve increased secretion of gastrointestinal 5-HT, which activates neural reflexes associated with intestinal secretion and motility (Cubeddu *et al.*, 1992). The positive correlation between blood glucose and serum 5-HT levels correspond to the results from a recent study which demonstrated a positive correlation between 5-HIAA with fasting plasma glucose levels (Fukui *et al.*, 2012).

6 Conclusion

To our knowledge, this is the first study describing the correlation of ghrelin, leptin and 5-HT serum levels in postnatal piglets. Moreover, this study investigated the association of these appetite regulators with low BW and glucose metabolism. Although the samples of this experiment were taken from an uncontrolled environment, i.e. a local farm, the correlations between the different serum parameters correspond to human data. The results of this study might provide insight into the physiology of both domestic piglets and humans. In addition, this study also shows that the pig is a useful model to investigate the impact of prenatal growth restriction on appetite and energy homeostasis in humans.

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Chapter 6 Discussion

1 Brief overview of the study results

Chapter 3 Ghrelin in the gastrointestinal tract and blood circulation of perinatal low and normal weight piglets

- ✓ GCs did not spread from the base to the neck of the gastric glands with increasing age
- ✓ NW newborns (d0) had a higher amount of gastric GCs compared to their SGA littermates
- ✓ Total ghrelin serum concentrations did not show an age or a BW effect
- ✓ The morphology of the gastric pars fundica of SGA piglets was not significantly different from NW piglets

Chapter 4 Enteric and serological distribution of serotonin and its precursor tryptophan in perinatal low and normal weight piglets

- ✓ The V_v of the 5-HT cells in the proximal and distal part of the SI tended to decrease with age
- ✓ NW piglets had higher 5-HT serum concentrations compared to their SGA littermates
- ✓ SGA piglets had a higher FFT/total tryptophan ratio
- ✓ The morphology of the SI of SGA piglets was not significantly different from NW piglets

Chapter 5 The impact of prenatal growth restriction on serum levels of appetite regulators and glucose metabolism in piglets

- ✓ Mainly the PI was associated with serum ghrelin, leptin and 5-HT levels
- ✓ NW piglets had higher leptin and 5-HT serum levels compared to their SGA littermates
- ✓ The correlations between the different serum parameters corresponded to human data

2 Ghrelin

This study showed that ghrelin IR cells are located in the entire gastrointestinal tract of perinatal piglets. Most of the GCs were distributed in the piglet's stomach. As in rodents and humans, the intestinal tract has opened- and closed- cell types whereas the stomach only has closed-type GCs (Sakata *et al.*, 2002a; Grönberg *et al.,* 2008). However, in contrast to rodents (Sakata *et al.*, 2002b), the fundic GCs did not spread from the base to the neck of the glands with increasing age. These data demonstrate the species-specific gastrointestinal maturation process. Rodents still have a very immature gastrointestinal system at birth and have a pronounced maturation cluster during the weaning period. This in contrast to humans and pigs, where the maturation process starts earlier and is more gradual. Thus, the quick 'catch up' maturation process in rats during the weaning period probably highlights the difference in GC distribution during the weaning period whereas this dispersed distribution is only visible in pigs after weaning (Vitari *et al.*, 2012). Since the gradual maturation process of the gastrointestinal system of the pig is more comparable to the human gastrointestinal development (for review see Sangild, 2006), these results emphasize that the piglet is a good perinatal animal model to study the development of the digestive tract.

A possible effect of the prenatal growth restriction on the appetite regulators was shown in our stereological analysis from the gastric GCs in the perinatal SGA and NW piglets. At birth (d0), growth restricted piglets had a lower amount of cells expressing active ghrelin compared to their normal littermates. The low amount of GCs in neonatal SGA piglets might discourage their milk intake compared to their normal littermates. Therefore, we performed a weigh suckle weigh (WSW) technique in other piglets but in a similar setting in the same farm to verify BW related differences in milk consumption. The results did not show a significant BW effect (P = 0.22), which is in contrast to other studies (Milligan *et al.*, 2001; Devillers *et al.*, 2007). However, the assessment of milk intake was only carried out on d0, d6 and d13 and did not focus on the neonursing period, i.e. 11 hours after farrowing (Lewis and Hurnik, 1986). This specific nursing phase may be of interest in this study since the

diminished amount of GCs was specifically present in SGA piglets immediately after birth.

Strangely, the differing amount of GCs in the SGA piglets versus NW piglets was not reflected in our serological analysis, which quantified total ghrelin levels. To validate our stereological analysis, it would be interesting to determine active ghrelin levels in the serum of normal and SGA piglets. Such analysis is complicated since active ghrelin is an extremely unstable molecule in serum. Ideally, all samples should be processed as quickly as possible. Furthermore, during blood collection, protease inhibitor treatment together with acidification of the samples is necessary.

Although some studies found IR GCs in pancreatic tissue from human adults (Andralojc *et al.*, 2009; Raghay *et al.*, 2013), the presence of these cells in the adult pancreas remains controversial. The highest ghrelin expression was found prenatally and neonatally in humans (Wierup *et al.*, 2002). Pancreatic GCs were also detected in the rat (Wierup *et al.*, 2004). Nevertheless, we failed to show IR GCs in pancreatic tissues from the piglets of both BW categories and the different ages. Until now there are no other studies that detected these specific cells in the porcine pancreas.

3 Serotonin

This study demonstrated that the V_v of enterochromaffin cells in both the proximal and distal SI tends to decrease with age. In rodents, 5-HT cells only appear near term in the gastrointestinal tract (Ekelund *et al.*, 1985; Branchek and Gershon, 1989) whereas these cells already appear in the porcine and human gastrointestinal tract in the second trimester of gestation (Stein *et al.*, 1983; Facer *et al.*, 1989; Van Ginneken *et al.*, 2001; Willemen *et al.,* unpublished data). These data as for ghrelin again signify species-specific differences in gastrointestinal maturation.

Intriguingly, these results correspond to our stereological data from the enteric plexuses. More specifically, in the myenteric, outer- and inner submucosal plexuses from both the proximal and distal SI, PF showed significantly more 5-HT immunoreactivity compared to the postnatal age groups ($P < 0.05$, unpublished results). Furthermore, this immunoreactivity was most pronounced in the distal part

of the SI (P = 0.04). Enteric 5-HT neurons are among the first early-born neurons that might influence the differentiation of other enteric neurons (Li *et al.*, 2011). Altogether, our results demonstrate that gastrointestinal 5-HT could be of importance in prenatal development.

Our serological analyses of 5-HT together with its precursor also show comparable results with human data. As in human IUGR infants, FFT/total Trp ratios are increased in SGA piglets (Manjarrez *et al.*, 1998). Trp is not just an essential amino acid, but has recently also been classified as one of the 'functional amino acids' (Wu, 2010). Functional amino acids are amino acids that regulate key metabolic processes in order to promote survival, growth and development of animals and humans (Wu, 2010). Trp deficiency in piglets is associated with a reduction in appetite and feed intake (Eder *et al.*, 2001). Until now, experimental data on the optimum dietary requirements for neonatal pigs are missing. However, results indicate that the milk yield of sows is not sufficient to provide adequate amounts of amino acids for supporting growth and development of piglets (Boyd *et al.,* 1995). Our results, showing lower total Trp levels in SGA piglets, can encourage further research concerning dietary tryptophan supplementation in these prenatal growth restricted piglets. A recent study demonstrated that Trp supplementation resulted in an increased cerebral 5-HT production and improved growth performance of 6 week old pigs (Shen *et al.*, 2012). However, this is in contrast to a study in weanling pigs, in which Trp supplementation had no effect on daily weight gain (Koopmans *et al.*, 2006). Hence, further research is necessary to investigate how amino acid supplementation might be used to treat and/or prevent IUGR in both humans and pigs.

4 Influence of growth restriction on endocrine appetite regulation and glucose homeostasis

Our study showed an altered serum profile of the appetite regulating protagonists in the prenatal growth restricted piglets. Although the demonstrated correlations do not necessarily imply a causal relationship or interaction between these molecules,

these results are comparable to the serum profile described in studies performed in IUGR children (Berman *et al.*, 1965; Soriano-Guillen *et al.*, 2004; Chiesa *et al.*, 2008; Kyriakakou *et al.*, 2009; Bozzola *et al.*, 2010) (Figure 6.1).

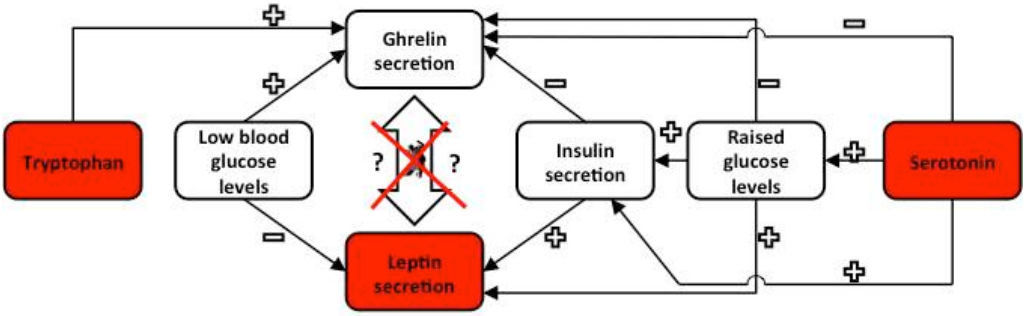

Figure 6.1 Schematic representation of the absent direct ghrelin-leptin tango, the correlation between serotonin, ghrelin and leptin and the involvement of ghrelin, leptin and serotonin in glucose homeostasis in our animal models. The levels of serotonin, its precursor tryptophan and leptin in serum of SGA piglets were all reduced compared to the serum of their NW littermates (represented in red in this figure). All the positive and negative correlations between the different molecules corresponding to what has been described in human literature are also represented in the figure.

These intriguing results perfectly fit in the scope of this thesis. By determining and correlating these different serum parameters this thesis elucidated the endocrine appetite regulation from juvenile SGA and NW piglets in order to assess whether perinatal programming disturbs the molecular mechanisms behind food intake.

However, there is a study related demerit that we would like to emphasize.

Glucose intolerance has already been described in the porcine IUGR animal model (Poore and Fowden, 2002a). However, using the domestic pig as a diabetic animal model should be nuanced. Domestic pigs are selectively bred for their ability to efficiently accumulate and store energy for later consumption by humans. Because of this selection, pigs are protected against the 'diabetic environment', i.e. an environment that favours inactivity and energy abundance (Gerstein and Waltman, 2006). Although they are protected against the toxic effects of this 'diabetogenic environment', pigs are still frequently used as diabetic animal models because of their human phenotypic similarities including their metabolism and omnivorous habits (Poore and Fowden, 2002a and 2002b and 2004a and 2004b; Bellinger *et al.*, 2006). Since IUGR occurs naturally in the domestic pig, we and other researchers

have used this model to investigate the mechanisms of metabolic programming (Hoet and Hanson, 1999; Poore and Fowden, 2002a; Litten-Brown *et al.*, 2010).

Notwithstanding there are suggestions that endocrine appetite regulation is similar in children as in adults, this finding has never been confirmed. Increased levels of ghrelin in the first days of life have been suggested to act as an 'anabolic drive' to promote feed intake (Bellone *et al.*, 2006). This corresponds to our stereological analysis, showing an increased amount of GCs in the gastric pars fundica in neonatal piglets. This 'anabolic drive' also might be stimulated by the decreased leptin and insulin levels seen in neonates (Mami *et al.*, 2005). At birth, leptin levels in piglets are also very low. In contrast to other mammals, the adipose tissue in neonatal pigs only contributes to 1% of the entire BW (Manners and McCrea, 1963), which may result in the low leptin levels. However, after 24 hours leptin levels in sow-reared piglets reach and maintain until d7 adult leptin levels (Wolinski *et al.*, 2013). Our results also show steady leptin levels until d10 when these levels are significantly increased.

5 SGA pig as IUGR animal model in our experimental set up: its strengths and drawbacks

Animal models are needed in order to unravel the pathogenesis of the disease, in this case IUGR. Moreover, because long-term studies in children are difficult to perform, it is interesting to determine the long-term consequences of IUGR using animal models in which the outcomes of the disease shows as many features as possible of the human pathological reactions. Hence, it is important to choose the animal model thoughtfully. Therefore, we evaluate below our SGA as IUGR animal model by assembling its strengths and weaknesses that we have observed during our study.

First of all, sows and piglets from a local farm, which are intended for pork industry, are not monitored like pregnant women and human infants. Therefore, the categorisation between SGA and IUGR in pigs and humans is different. In humans, the distinction between SGA and IUGR has been made according to the intrinsic growth potential of the foetus. The gestation related optimal weight (GROW)

computer generated program is often used to delineate the gestation related optimal weight for each baby, by adjusting its characteristics such as maternal height, weight or by excluding pathological factors such as smoking and diabetes (Gardosi, 2004). SGA children are constitutionally small. Hence their low birth weight is not due to the risk factors for IUGR which were listed up in section 2 of the first chapter. In pigs, the distinction between IUGR and SGA has been made according to the PI of the pig because the PI provides a valuable indication of mortality risk in piglets (Baxter *et al.,* 2008). Piglets with a significantly lower BW compared to their normal littermates that have a normal allometry are classified as SGA piglets whereas IUGR piglets display a disproportional allometry (Bauer *et al.*, 1998). Secondly, the cause of developing IUGR between piglets and humans is different. The pig is a multifoetal domestic animal. Hence, in contrast to humans, who also show IUGR pregnancies in singleton pregnancies, IUGR occurs in pigs as a consequence of intrauterine crowding (Martineau *et al.,* 2009). However, in contrast to other animal models (for review see Haugaard and Bauer 2001; Barry *et al.,* 2008), IUGR develops naturally in pigs. Moreover, as already discussed in detail in section 3.2 of Chapter 1, the gastrointestinal maturation process of the pig is comparable to the human gastrointestinal development (for review see Sangild, 2006). In addition, the pig nearly reproduces all of the phenotypic pathological consequences of IUGR such as increased adiposity (Poore and Fowden, 2004b) and glucose intolerance (Poore and Fowden, 2002a). Catch up growth in the first month of life was also directly associated with impaired glucose tolerance when the pigs reached 1 year of age (Poore and Fowden, 2002a). Therefore the SGA piglet can be considered as an isomorphic animal model for IUGR since it shares most of the symptoms seen in human IUGR.

When we consider our experimental set up: every SGA-NW age- and gender matched couple was selected from the same litter. Since the SGA and NW couple have the same genetic background, the statistical analysis takes the relatedness of the litter into account. Unfortunately, this statement cannot be assured when we compare the foetal with the postnatal age groups. More specifically, although the sample

collection, i.e. the age- and gender matched NW- and SGA couple per litter, is the same in the foetal age group, this age group was collected from a local slaughterhouse. It was not possible to determine the identity, hence the breed, of the sow during sample collection. Hence, it is possible that the PFs are obtained from different breeds compared to the postnatal piglets. Finally, the piglets from this study were sampled after a non-fasting period. Since the aim of our thesis is to link the endocrine gastrointestinal derived appetite regulators with the BW of our piglets, the non-fasting period might complicate the interpretation of our data because it is generally known that milk intake is influenced by BW (Devillers *et al.*, 2007). Albeit we did see BW related differences in the serum profile, it is possible that we missed or misinterpreted certain results. However, it should be mentioned that there are discrepancies between studies that did take a fasting period into account. In epidemiological human studies it is problematic to implement fasting blood tests because participants are not willing to fast before attending the study. As a result, some participants may not admit to have eaten before the test, increasing measurement variability and introducing bias. To avoid this issue, a recent study demonstrated that measures of insulin resistance in fasting and semi-fasting (4 hours) blood from human healthy adults were comparable (Hancox and Landhuis, 2011). Moreover, it is difficult to define the optimal fasting period in pigs. Although pigs and humans tend to ingest periodic meals, they both have a different gastrointestinal transit. In pigs, food emptying in the stomach is bimodal. About 30-40% of the contents is emptied in the first 15 minutes, followed by a sustained emptying one hour later (Pond and Houpt, 1978). Gastric emptying appears to be incomplete in the pig. Consequently, there may be food present in the stomach after 24 hours. Hence, it is very difficult to talk about a 'fasting state' in pigs when in fact there is still food in their stomach.

Altogether, our IUGR animal model has some strengths, but also some drawbacks we have to take into account. The available evidence suggests that IUGR negatively affects preweaning survival, feed utilization efficiency, body composition and meat

quality (for review see Wu *et al.,* 2006). Thus, our data also affects animal agriculture since our piglets were obtained from the average Belgian farm.

6 Future Perspectives

With this research project we investigated the perinatal distribution of ghrelin, 5-HT and leptin in the gastrointestinal tract and blood circulation of normal and SGA piglets. These results together with the data from the gastrointestinal morphology are indispensible to improve our insight on the development and maturation of the porcine gastrointestinal tract in both normal and growth restricted conditions. This knowledge will help increasing survival and improve the postnatal development of SGA piglets and help to validate the SGA piglet as an animal model to study IUGR. However, more research is needed to further unravel the mechanisms responsible for IUGR and its long-term complications.

6.1 Long term complications from IUGR: follow up studies

For the pig industry the weaning period is commercially more interesting compared to the suckling period. Thus, most studies focus on the weaning process. Indeed, feed intake during the suckling period is mainly controlled by the sow and by litter size whereas after weaning, feed intake can be controlled by solid feed management.

Follow up studies of these piglets after the weaning period may give insight into the catch up growth hypothesis. Poore and Fowden (2002a) did observe at 12 months of age that the weight of low BW piglets was no longer different compared to their NW littermates. Moreover, later on they observed that this catch up growth process had an effect on nutritional programming (Poore and Fowden, 2004a). Thus, determining the distribution of appetite regulating hormones in these older pigs is one of the future prospects that should definitively be kept in mind. This thesis already established some of the molecular mechanisms behind metabolic imprinting in SGA piglets by determining the perinatal distribution of the gastrointestinal derived appetite regulators ghrelin, leptin and 5-HT in both NW and SGA piglets.

6.2 Maternal milk, a protection against obesity?

Nutrition is an important issue during perinatal development. In humans, prolonged breastfeeding is associated with a lower risk of obesity compared to formula feeding (Savino *et al.*, 2009). The potential benefits of breastfeeding may be due to slower growth in breast fed children compared to those who are formula fed (Singhal and Lanigan, 2007), the finding that maternal milk is a source of various hormones, including ghrelin and leptin, which are involved in feeding behaviour (Casabiell *et al.*, 1997; Aydin *et al.*, 2006; Wolinski *et al.*, 2006) and that maternal milk contains bioactive nutrients that are not present in formula milk (Hamosh, 2001). In humans, breast fed babies can control the amount of milk they consume by themselves; hence they learn to regulate their own energy intake (Taveras *et al.*, 2004). In pigs however, within litter competition and thus birth weight is an important factor regulating milk intake since SGA piglets are most of the time defeated during sibling competitions (Milligan *et al.*, 2001; Devillers *et al.*, 2007).

Interestingly, one study demonstrated that the production of leptin in maternal milk might be physiologically regulated by the needs of the infant (Dundar *et al.*, 2005). This study showed that SGA infants grew more rapidly compared to AGA infants and that the human milk leptin levels were significantly lower in the SGA group (Dundar *et al.*, 2005). Moreover, it has been shown that leptin concentration in human breast milk decreases whereas ghrelin concentration increases with time during lactation (Ilcol *et al.*, 2006; Ilcol and Hizli, 2007). A recent study has shown a direct relationship between maternal milk ghrelin levels and BMI together with the weight of the infants at birth (Cesur *et al.,* 2012). Moreover, they also found a positive correlation between ghrelin milk concentrations and weight gain of the infant (Cesur *et al.,* 2012). Trp also shows a decreasing trend according to time during lactation in sow's colostrum and milk (Csapo *et al.*, 1996).

Since maternal milk is also the main source of these appetite-regulating hormones in neonatal piglets, it would be worthwhile to assess these hormonal concentrations in sow milk during different stages of lactation. Interestingly, a recent study determined leptin and ghrelin levels in sow colostrum and milk during the first week of lactation

(Wolinski *et al.*, 2013). This study suggests that sows have a different ghrelin pattern compared to human and rodent females (Wolinski *et al.*, 2013). In sow milk, ghrelin levels remained stable during the first week of lactation (Wolinski *et al.*, 2013). This is in contrast to human breast milk, where the concentration of ghrelin is lower in the first three days after delivery and then increases significantly for 4-14 days and remains stable during 180 days of lactation (Ilcol and Hizli, 2007). However, the stable ghrelin levels in sow milk correspond to the stable ghrelin levels measured in our postnatal piglets. Both human and sow colostrum and milk showed a similar decrease in leptin concentration with time during lactation (Ilcol *et al.*, 2006; Wolinski *et al.*, 2013). This unique pattern of leptin and ghrelin synthesis in the sow mammary gland is also an interesting topic to further investigate in view of postnatal development.

7 Conclusion

Several human epidemiological studies confirmed the association between low birth weight and metabolic diseases in adult life. However, epidemiological studies performed in children show conflicting data (for review see Geremia and Cianfarani, 2004). Nevertheless, early recognition of these metabolic disturbances has an enormous impact in clinical practice, since this information may establish appropriate hormone-, diet- or lifestyle-based strategies to prevent long-term metabolic consequences of IUGR. By comparing the gastrointestinal distribution and serological concentrations of appetite regulators in perinatal SGA piglets with what has been described in human studies concerning IUGR, this study contributes to the use of the SGA piglet as a metabolic IUGR animal model in which both short- and long-term consequences of IUGR can be studied. Moreover, because of the numerous morphological and physiological similarities between the human and porcine gastrointestinal system, the SGA piglet is ideally placed to study nutritional interventions. For porcine industry, the knowledge of these metabolic alterations may restore gastrointestinal development and endocrine balances in IUGR piglets by emphasizing the importance of feeding colostrum.

8 References

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Summary

Intrauterine growth restriction (IUGR) is a major cause of perinatal mortality in both humans and domestic animals, especially in pigs. This high perinatal mortality has both an ethical and economical impact in pork industry. The consequences of IUGR are often studied in animal models in which prenatal growth restriction is induced by maternal undernutrition or by bilateral uterine ligation. In pigs however, and particularly in hyperprolific sows, IUGR occurs naturally (8-25% of the piglets in a nest) when litter size exceeds the average of 11 piglets. In these large litters, there is a high prevalence of stillbirths and low viability low birth weight pigs. Since IUGR occurs naturally in the pig, this in contrast to the rodent which is often used as IUGR animal model, and the fact that the porcine gastrointestinal system resembles the human digestive system (microscopically, physiologically and its maturation process), we are convinced that the pig is a good animal model in order to study the consequences of IUGR linked to the gastrointestinal system.

Chapter 1 gives an overview of what has been cited in literature about IUGR and its long-term consequences in both humans and pigs. The high perinatal mortality in IUGR can partially be explained by an underdeveloped gastrointestinal system. Moreover, the developmental changes in the different organ systems caused by IUGR permanently affect the physiology and metabolism of the neonate. These permanent changes predispose them to endocrine and metabolic diseases in adulthood. Since adequate feed intake is important to compensate the prenatal growth restriction postnatally and the risk of developing metabolic diseases increases, this thesis focuses on the role of appetite regulating hormones. The role of ghrelin, leptin, serotonin and its precursor tryptophan in perinatal development and to metabolic parameters in the suckling period are discussed. Ghrelin is the only identified hunger-stimulating hormone derived from the gastrointestinal tract. Ghrelin concentrations in cord blood are negatively correlated with anthropometric measurements such as ponderal index (kg/m^3) and body weight. These results suggest that ghrelin with its orexigenic effects plays a physiological role in foetal adaptation to intrauterine malnutrition. The gastrointestinal system is besides of ghrelin, also the major source

of serotonin. Tryptophan is the precursor of serotonin and an essential amino acid, and as such needs to be supplied by food intake. IUGR children have both lower serotonin and tryptophan levels. Finally, as the third protagonist, leptin levels will be analysed. Leptin levels are, in contrast to its appetite-regulating opponent ghrelin, positively correlated with anthropometric measurements. Thus, IUGR newborns have significantly lower leptin serum levels compared to neonates with a normal body weight.

Hence, this thesis focuses on the distribution of three important appetite-regulating hormones in the suckling period of the pig (**Chapter 2**). We aim to link the presence of these essential neuro- and endocrine components of the gastrointestinal system and appetite regulation to the body weight and age of our animal models and to their metabolic profile (glucose and insulin levels). As final objective we want to determine whether IUGR, as a natural phenomenon, has an effect on the functional morphology of the gastrointestinal system with possible consequences later in life.

Different samples (serum, tissue samples from the stomach, small- and large intestine) were taken from porcine foeti (90-115d of gestation, determined by measuring crown-rump length) and from piglets at 0, 3, 10 and 28 days after birth. Within these age groups, piglet couples (from the same litter, gender-matched) were selected according to body weight (small for gestational age, SGA piglets: mean litter body weight- 1.5 SD; normal weight, NW piglets: mean litter body weight ± 0.5SD). In pigs, a distinction between IUGR and SGA is made according to the ponderal index since this factor gives a good indication about the mortality risk from these piglets. IUGR piglets have, in contrast to SGA piglets, besides low body weights also low ponderal indexes.

The presence of ghrelin- and serotonin containing cells was demonstrated by immunohistochemical analysis and their amount was determined by stereological methods. The serological levels of ghrelin and leptin were determined by RIA, whereas serotonin and insulin levels were analyzed by ELISA respectively.

Tryptophan serum levels were measured with HPLC whereas glucose levels were determined by a glucometer.

Chapter 3 aimed to investigate whether SGA piglets have more ghrelin cells (GCs) in the gastrointestinal tract or have higher ghrelin serum levels during the suckling period as a consequence of foetal undernutrition.

As in humans and rodents, cells expressing active ghrelin were distributed along the entire gastrointestinal system of both normal and SGA piglets. However, unlike in rodents, the GCs did not spread from the base to the neck of the pars fundica glands in the stomach when the piglets reached weaning age. Intriguingly, normal littermates had a higher amount of gastric GCs expressing active ghrelin compared to their SGA littermates at birth. This accords with previous studies, which emphasize that birth weight is an important factor regulating milk intake. This finding is however not reflected in its total (active and not active ghrelin) serum concentrations, which showed no body weight related differences. Further research will need to provide an answer whether the higher density of GCs in the gastric pars fundica of the NW (d0) piglets is also demonstrated in their serum concentrations of active ghrelin.

Chapter 4 determined the distribution of serotonin in both the proximal and distal part of the small intestine according to age and body weight of our piglets. Moreover, serotonin together with its precursor free fraction tryptophan (FFT), total tryptophan (Trp), and albumin–bound Trp were analyzed in the serum of normal and SGA piglets.

In both the proximal and distal part of the small intestine, the V_v of serotonin cells tended to decrease according to age. This finding can be explained by a postnatal increase of the tunica mucosa. In contrast to what we have described for GCs in the gastric pars fundica, the enterochromaffin cells did not show body weight related differences. However, the serum levels did show a body weight related difference. More specifically, SGA piglets had lower serotonin and total Trp levels compared to their normal littermates. Moreover, SGA piglets had higher FFT/total Trp ratios

compared to normal piglets. These findings correspond to human data and suggest that both the enteric and central serotonin system are disturbed in SGA piglets since Trp levels have an impact on neuronal serotonin synthesis. Hence, as described in human literature the serotonin metabolism is also an abnormal trait in SGA piglets, which might have its implications in further development of both the gastrointestinal tract and central nervous system.

In **Chapter 5,** the serum concentrations of ghrelin and serotonin, together with leptin, were related in function of age, BW and ponderal index. In the analysis, a disturbed glucose homeostasis as a consequence of low birth weight was also taken into account. Ghrelin, serotonin and leptin are chosen as triumvirate because they interact during appetite regulation and are known to be implicated in glucose homeostasis.

Ghrelin and leptin levels were respectively negatively and positively correlated with serotonin, glucose and insulin levels. Surprisingly, the negative correlation between ghrelin and leptin- in literature cited as the 'ghrelin-leptin tango'- was absent. However, these presumed opponents did have opposite correlations with serotonin, glucose and insulin. These latter findings emphasize the difference in biological effects between ghrelin and leptin. Indeed, while the secretion of ghrelin is promoted by hypoglycaemia, leptin secretion is induced by hyperglycaemia.

This study also showed that it was mainly the ponderal index that affects the serum parameters. More specifically, ghrelin levels were negatively associated- whereas leptin and serotonin were positively associated with ponderal index. Moreover, leptin and serotonin serum levels were lower in SGA piglets compared to their NW littermates. Leptin levels were also influenced by age since the d10 piglets showed the highest leptin serum concentrations compared to the other age groups. To conclude, the serological data from our triumvirate corresponds to what has been described in human literature.

All the results are recapitulated in **Chapter 6** and compared with data from literature in order to define the strengths and drawbacks from our SGA piglet as IUGR animal model. Sows and piglets from a local farm, which are intended for pork industry, are not monitored like pregnant women and human infants. Moreover, prenatal growth restriction occurs in pigs as a consequence of intrauterine crowding. In humans, IUGR and SGA also occur in singleton pregnancies. Nonetheless, IUGR occurs naturally in pigs. This in contrast to other animal models where IUGR needs to be induced experimentally. Prenatally growth restricted piglets also show the pathological consequences which are described in IUGR infants long term such as glucose intolerance, increased adiposity and catch up growth. Hence we can conclude that the SGA pig is an isomorphic animal model since it shares the same symptoms as human IUGR.

The results of this study might provide insight into the physiology of both domestic piglets and humans. In addition, this study also shows that the pig is a useful animal model to investigate the impact of prenatal growth restriction on appetite and energy homeostasis.

Samenvatting

Intra-uteriene groeivertraging (intrauterine growth restriction, IUGR) is één van de belangrijkste oorzaken van perinatale mortaliteit en dit bij zowel de mens als een aantal gedomesticeerde diersoorten, waaronder het varken. Deze hoge perinatale mortaliteit heeft voor de varkenssector naast een ethische ook een economische impact. De gevolgen van IUGR worden vaak bestudeerd in proefdiermodellen waar de groeivertraging wordt geïnduceerd door de maternale voederopname te beperken of door het reduceren van de uteriene bloedtoevoer via ligatie.

Bij het varken en in het bijzonder bij 'hyperprolifererende' zeugenlijnen zien we vaak het optreden van een natuurlijke vorm van IUGR (8-25% van de biggen in een nest) wanneer de nestgrootte het gemiddelde aantal van 11 biggen overschrijdt. In deze grote worpen ziet men een hogere prevalentie aan doodgeboorten en aan zwakke, weinig levensvatbare biggen met een laag geboortegewicht. Het feit dat IUGR onder een natuurlijke vorm voorkomt bij het varken –in tegenstelling tot de knaagdiermodellen die frequent worden gebruikt – en het sterk gelijkende spijsverteringsstelsel (microscopisch, fysiologisch, ontwikkeling) van het varken met het humane maag-darmstelsel, maken het varken een goed proefdiermodel om de processen ten gevolge van IUGR met een link naar het spijsverteringsstelsel te bestuderen.

Hoofdstuk 1 geeft een overzicht van wat reeds in de literatuur beschreven is over IUGR en de gevolgen op lange termijn bij zowel de mens als het varken. De hoge perinatale mortaliteit die wordt gezien bij IUGR, kan ten dele gekoppeld worden aan een onderontwikkeld gastro-intestinaal systeem bij deze gewichtscategorie. Bovendien zorgen de door IUGR veroorzaakte veranderingen in de ontwikkeling van de verschillende orgaansystemen voor permanente aanpassingen in de fysiologie en het metabolisme van de neonaat. Deze permanente aanpassingen zorgen voor een hoger risico op het ontwikkelen van endocriene en metabole aandoeningen in het latere leven. Aangezien voldoende voedselopname belangrijk is om de intra-uteriene groeiachterstand postnataal te compenseren en er tevens een link is met de ontwikkeling van metabole en endocriene stoornissen in het latere leven, wordt in

deze thesis dieper ingegaan op de rol van honger-regulerende hormonen. Ghreline, leptine, serotonine en tryptofaan worden bepaald en gecorreleerd aan groei en een aantal metabole parameters in de zoogperiode. Ghreline is tot nu toe het enige geïdentificeerde honger-stimulerende hormoon dat wordt afgescheiden door het spijsverteringsstelsel. De ghreline concentratie in het navelstrengbloed is negatief gecorreleerd met anthropometrische waarden zoals *ponderal index* (kg/m^3) en lichaamsgewicht. Deze resultaten doen vermoeden dat ghreline met zijn orexigene effecten een fysiologische rol kan spelen bij foetale adaptatie voor intrauteriene ondervoeding. Naast ghreline, is het spijsverteringsstelsel de voornaamste bron van serotonine in de circulatie en wordt dit aangemaakt vanuit tryptofaan dat dient opgenomen te worden via de voeding. IUGR kinderen hebben zowel lagere serotonine- als tryptofaan concentraties. Als derde speler wordt het gehalte aan leptine in kaart gebracht, dit omwille van de leptine-ghreline tango. De leptine concentraties zijn in tegenstelling tot zijn appetijt-regulerende antagonist ghreline, positief gecorreleerd met anthropometrische waarden. Pasgeborenen met IUGR hebben significant lagere serumconcentraties van leptine dan pasgeborenen met een normaal groeipatroon.

In deze thesis kijken we dus naar het voorkomen van belangrijke voedselopname regulerende hormonen in de zoogperiode van het varken (**Hoofdstuk 2**). We trachten de aanwezigheid van deze essentiële onderdelen van het neuro- en endocriene functioneren van het spijsverteringsstelsel en voederopname te koppelen aan enerzijds het lichaamsgewicht van de dieren en het metabole profiel (glucose en insuline gehalten) anderzijds. Dit met als uiteindelijke doelstelling na te gaan in hoeverre een intra-uteriene groeiachterstand – te wijten aan een natuurlijk fenomeen – een effect uitoefent op de functionele morfologie van het spijsverteringsstelsel met mogelijks gevolgen op langere termijn.

Er werden staalnames (bloed, serum, weefselstalen van maag, dunne darm en dikke darm) uitgevoerd op foeti van biggen (90-115 dagen drachtduur, berekend via het

meten van de kruin-stuitlengte) enerzijds en biggen van 0, 3, 10 en 28 dagen na de geboorte anderzijds. Binnen de verschillende leeftijdsgroepen werden telkens koppels (nestgenoten, gepaard volgens geslacht) geselecteerd uitgaande van het lichaamsgewicht (small for gestational age ,SGA- biggen: gemiddeld lichaamsgewicht van de nest– 1,5 SD; NW: gemiddeld lichaamsgewicht van de nest ± 0,5 SD). Bij het varken maakt men een onderscheid tussen IUGR en SGA volgens allometrie, meer specifiek de *ponderal index* omdat deze factor ook een goede indicatie geeft over het mortaliteitsrisico bij de biggen. IUGR biggen hebben in tegenstelling tot SGA biggen, naast een laag lichaamsgewicht ook een lage *ponderal index*.

De aanwezigheid van ghreline- en serotonine bevattende cellen werd aangetoond door middel van immunohistochemie en de frequentie van voorkomen werd bepaald via stereologische meetmethoden. De aanwezigheid van ghreline en leptine in het serum werden nagegaan via RIA terwijl de serumgehalten aan serotonine, en insuline via ELISA werden bepaald. Tryptofaan en glucose serum waarden werden bepaald via HPLC en een glucometer.

Hoofdstuk 3 bood een antwoord op de deelhypothese dat SGA biggen meer ghreline zouden aanmaken in de zoogperiode als foetale adaptatie voor intrauteriene ondervoeding.

Endocriene cellen waarin actief ghreline immunohistochemisch kon worden aangetoond (GCn) waren, zoals beschreven bij de mens als de rat, verspreid over het hele gastro-intestinale systeem in zowel normale als SGA biggen. Deze cellen vertoonden – in tegenstelling tot wat werd beschreven in de rat- geen verschillende verdeling over de lengte van de klieren van de pars fundica wanneer de big de speenleeftijd had bereikt. We zagen, in tegenstelling tot onze hypothese, dat in pasgeboren biggen met een normaal geboortegewicht meer GCn aanwezig zijn ter hoogte van de pars fundica in vergelijking met de waarden die we aantroffen in hun SGA nestgenoten. Dit stemt overeen met andere studies die aantonen dat het lichaamsgewicht een belangrijke factor is die voedselopname bepaalt. Dit resultaat stemde echter niet overeen met de serum gehalten aan totaal (actief en niet actief)

ghreline, die niet verschilden tussen NW en SGA biggen. Verder onderzoek zal moeten uitklaren of de hogere densiteit van GCn in de klieren van de pars fundica van de NW (d0) biggen ook tot uiting komt in de serum concentraties van actief ghreline in deze biggen.

In **Hoofdstuk 4** werd de distributie van serotonine in het proximale en distale gedeelte van de dunne darm in functie van de leeftijd en van het lichaamsgewicht in kaart gebracht. Bovendien werden serotonine met zijn precursor *free fraction of tryptophan* (FFT), totaal tryptofaan (Trp) en de albumine-gebonden fractie Trp geanalyseerd in het serum van normale en SGA biggen. De volumedensiteit van de serotonine cellen in de dunne darm daalde in functie van de leeftijd van de perinatale biggen, een fenomeen dat verklaard kan worden door een postnatale toename van de tunica mucosa. In tegenstelling tot wat werd waargenomen voor de GCn in de pars fundica vertoonde de distributie van enterochromaffiene cellen in de dunne darm geen lichaamsgewicht-gerelateerde verschillen. De serumgehalten vertoonden echter wel een verschil. SGA biggen hadden lagere serotonine en totaal Trp concentraties vergeleken met hun normale nestgenoten. Bovendien hadden deze SGA biggen hogere FFT/totaal Trp ratios. Deze resultaten komen overeen met humane data en suggereren dat zowel het intestinaal als het centraal serotonine verstoord zijn in SGA biggen aangezien de Trp concentraties een impact hebben op neuronale serotonine synthese. En dus, zoals beschreven in de humane literatuur, is ook bij het varken het serotonine metabolisme verstoord. Dit kan gevolgen hebben op de ontwikkeling van zowel het gastro-intestinaal systeem als het centraal zenuwstelsel.

In **Hoofdstuk 5** werden de serum concentraties van ghreline en serotonine samen met nieuwe data over het serumgehalte aan leptine met elkaar vergeleken en ook in functie van leeftijd en het lichaamsgewicht en ponderal index. Een verstoord glucose metabolisme in functie van het lichaamsgewicht werd meegenomen in de analyse. De achterliggende reden voor dit triumviraat – namelijk ghreline, serotonine en

leptine is dat deze interageren tijdens het moduleren van de voedselopname en tevens betrokken zijn bij de glucose homeostase.

De concentraties van ghreline en leptine waren respectievelijk negatief en positief gecorreleerd met de concentraties aan serotonine, glucose en insuline. Er was echter geen directe negatieve correlatie tussen ghreline en leptine, waarvoor in de literatuur nochtans voldoende indicaties bestaan. Deze correlatie wordt dan omschreven als de 'ghreline-leptine tango'. Deze twee antagonisten hebben daarentegen wel tegengestelde correlaties met serotonine, glucose en insuline. Hieruit kunnen we besluiten dat de biologische functies en regulatie van van ghreline en leptine tegengesteld zijn. Het is inderdaad gekend dat ghreline secretie bevorderd wordt door hypoglycemie terwijl dat leptine secretie geïnduceerd wordt door hyperglycemie.

Deze studie toonde ook aan dat het voornamelijk de *ponderal index* was die de verschillende serum parameters beïnvloedt. Ghreline concentraties waren negatief geassocieerd- terwijl leptine en serotonin concentraties positief geassocieerd waren met de *ponderal index*. Bovendien waren de leptine en serotonin concentraties van SGA biggen lager dan van hun normale nestgenoten. De leptine concentraties werden ook beïnvloed door leeftijd mits de d10 biggen de hoogste leptine concentraties hadden ten opzichte van de andere leeftijdsgroepen. We kunnen hieruit concluderen dat de serologische concentraties van de verschillende appetijt regulerende hormonen overeenstemmen met hetgene wat in humane studies beschreven staat.

De verschillende resultaten werden in **Hoofdstuk 6** nogmaals met elkaar vergeleken en met beschikbare gegevens uit de literatuur waardoor bijkomende pro's en contra's gevonden werden voor het gebruik van de SGA big als model voor humane IUGR processen. Een minpunt aan dit diermodel is dat er een verschillend onderscheid wordt gemaakt tussen IUGR en SGA ten opzichte van de mens. Bovendien wordt de prenatale groeirestrictie bij biggen voornamelijk veroorzaakt door '*intrauterine crowding'* door het hoge ovulatieniveau van de

hyperprolifererende zeug waarbij er een te hoog aantal foetussen zich in de baarmoeder bevindt. Bij de mens komt IUGR en SGA voornamelijk bij enkelvoudige zwangerschappen voor. Maar, IUGR komt bij het varken spontaan voor. Dit in tegenstelling tot andere diermodellen, waarbij IUGR experimenteel moet geïnduceerd worden. Bovendien vertoont de prenataal groeivertraagde big tal van pathologische gelijkenissen die bij het IUGR kind op lange termijn zijn beschreven zoals glucose intolerantie en een verhoogd vetpercentage. Ook catch up groei komt bij varkens voor. We kunnen hieruit besluiten dat de SGA big een isomorf diermodel is voor IUGR mits het dezelfde symptomen als humane IUGR deelt.

De resultaten van deze studie kunnen inzicht geven in de fysiologie van zowel de gedomesticeerde big als de mens. Bovendien heeft deze studie aangetoond dat de big een nuttig diermodel is om de impact van prenatale groei restrictie op appetijt- en energie homeostase te bestuderen.

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Sofie

Curriculum Vitae

Sofie Willemen werd geboren op 14 maart 1986 te Wilrijk. In 2004 beëindigde ze het secundair onderwijs, richting Wetenschappen-Moderne Talen aan het Koninklijk Atheneum in Mortsel en startte ze de studie Biomedische Wetenschappen aan de Universiteit Antwerpen. Ze behaalde haar Master opleiding van Moleculaire en Cellulaire Biomedische Wetenschappen in 2009 met grote onderscheiding. Ze startte in februari 2010 een doctoraatsonderzoek over de perinatale distributie van appetijt regulerende hormonen in het porciene IUGR diermodel bij het department Diergeneeskundige Wetenschappen bij de vaksgroep Toegepaste Diergeneeskundige Morfologie. In 2014 vervolledigde ze haar doctoraatsopleiding. Sofie Willemen is auteur en mede-auteur in international tijdschriften en nam actief deel aan nationale en international congressen.

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