INFLUENCE OF FOOD INTERVENTION ON ENTERIC NERVOUS SYSTEM AND ENTEROENDOCRINE CELLS DEVELOPMENT IN A SMALL FOR GESTATIONAL AGE PIGLET MODEL

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in VMS – Pathobiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2015

Urbana, Illinois

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Abstract

The gut immaturity of small for gestational age (SGA) infants predisposes these infants to gastrointestinal diseases like necrotizing enterocolitis (NEC). A SGA piglet model was used to investigate the influences of birth weight and diet on the development of the enteric nervous system (ENS) and entero-endocrine system (EES) during the first month of life, via multivariate analyses. Newborn SGA or AGA (average for gestational age) piglets were randomized into two diet groups: sow reared (SR, 24h/day with the sow) or formula fed (FF, milk replacer) after colostrum intake. Gut samples (duodenum, ileum, colon, rectum) were collected at postnatal day (PD) 1, 14 and 28, and used for immunohistochemistry (IHC) (n=4/subgroup). Neuronal markers (BIII tubulin (B3T), synaptophysin (SYN), neuropeptide Y (NPY), tyrosine hydroxylase (TH)) and EES markers (Chromogranin A (CoA), somatostatin (SOM), serotonin (SER), NPY, Neurotensin (NeuroT)) were used to assess the development of the ENS and enteroendocrine cells (EECs), respectively.

At PD1, the ENS of AGA animals was almost fully developed compared with PD14 and PD28. For SGA piglets, a delayed development of nerve endings was observed on PD1 associated with a less developed synaptic system in the enteric nervous ganglia, particularly in colon and rectum. The delayed development of nerve terminals and synaptic system in SGA animals was observed up to PD14. While by PD14, the intensity of SYN staining was similar in all groups. At D28 only, FF piglets presented with a lower number of noradrenergic nerve endings in the ENS ($p=0.001$). The expression of NPY remained the same between AGA and SGA groups during the first month of postnatal life.

Along the study the maturation of the EES was shown to be a dynamic process. The number of CoA, SER and NPY positive cells was influenced by the feeding strategy as their cell numbers increased overtime with SR diet ($p<0.001$, $p<0.001$, $p=0.004$, respectively). NeuroT cell numbers varied overtime in the different gut sections ($p<0.001$), and is not influenced by nutritional intervention.

Overall, this study demonstrates that the development of ENS and EES can be influenced by birth weight and/or diet and provides a useful model for studying intestinal maturation in different birth weight infants. Finally, we identified for the first time that the ENS is less developed in SGA piglets, and the maturation of the EES has shown to be a diet-dependent dynamic process.

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List of Abbreviations

AGA: Average for gestational age

ANS: Autonomic nervous system

B3T: Beta III tubulin

bHLH: Basic helix-loop-helix

CNS: Central nervous system

CNTF: Ciliary neurotrophic factor

ChAT: Choline acetyltransferase

CD: Crohn's disease

CCK: Cholecystokinin

Cgs: Chromogranins

CoA: Chromogranin A

DYN: Dynorphin

ENS: Enteric nervous system

EECs: Enteroendocrine cells

ELBW: Extremely low birth weight

E: Embryonic day

EDNRB: Endothelin receptor type B

ENCCs: enteric neural crest cells

EGCs: Enteric glial cells

ENK: Encephalin

EC: Enterochromaffin

ECL: Enterochromaffin-like

FF: Formula Fed

GIT: Gastrointestinal tract

GDNF: Glial cell-line-derived neurotrophic factor

GABA: γ-aminobutyric acid

GRP: Gastrin releasing peptide

GLP-1: Glucagon-like peptide 1

GLP-2: Glucagon-like peptide 2

GIP: Glucose-dependent insulin-releasing polypeptide

HD: Hirschsprung disease

IBS: Irritable bowel syndrome

IUGR: Intrauterine growth retardation

IVH: Intraventricular hemorrhage

IGF-1: Insulin-like growth factor 1

IND: Intestinal neuronal dysplasia

IPANs: Intrinsic afferent primary neurons

IBD: Inflammatory bowel disease

LGA: Large for gestational age

LBW: Low birth weight

LPS: lipopolysaccharide

LDCVs: Large dense-core vesicles

MS: Maternal separation

NEC: Necrotizing enterocolitis

NPY: Neuropeptide Y

NC: Neural crest

NOS: Nitric oxide synthetase

NO: Nitric oxide

NeuroT: Neurotensin

PD: Postnatal day

PP: Pancreatic polypeptide

PYY: Peptide YY

PVL: Periventricular leukomalacia

RET: Rearranged during transfection

5-HT: Serotonin

SGA: Small for gestational age

SR: Sow reared

SYN: Synaptophysin

SOM: Somatostatin

SP: Substance P

SLMVs: Smaller synaptic-like microvesicles

TH: Tyrosine hydroxylase

TLR2: Toll-like receptor 2

TLR4: Toll-like receptor 4

TGF-β: Transforming growth factorβ

UC: Ulcerative colitis

VLBW: Very low birth weight

VIP: Vasoactive intestinal polypeptide

Chapter 1: Introduction

The living environment of neonates changed after birth and this change is actually crucial for the development of some organs, like gut and brain [1]. The majority of the newborns survive and develop well [2]. But for infants born preterm, some organs are more vulnerable to certain diseases as a result of undevelopment [1], and thus they are more susceptible to develop diseases compared with term ones [2]. Even for babies born at term, some of them will face small for gestational age (SGA) conditions which mostly related to intrauterine growth restriction [3, 4]. These neonates tends to develop intestinal diseases like delayed meconium passage, abdominal distension, delay in tolerating enteral feeding, and fatal necrotizing enterocolitis [5, 6] resulting from the immaturity of intestine and immunologic defense [7]. Animal model has long been used to study all kinds of diseases and pig as a useful animal model has been used a lot in pediatric research [8]. The similarity of gut development and physiology between pig and human makes pig a useful model for studying intestinal problems [1, 8].

The enteric nervous system (ENS) is a neuronal network embedded in the whole gastrointestinal tract (GIT) [9, 10]. It has been regarded as a second brain since it functions independently and shares a lot of similarities with the brain like the number of neurons and the type of the neurotransmitters [11]. The ENS play an important role in normal gastrointestinal functions including motility, secretions, blood flow and the immune system with different kinds of neurons [12].

The enteroendocrine cells (EECs) are distributed all along the whole GIT and form the largest endocrine system in the body [13, 14]. The EECs have at least 16 different cell types and scatter along the gastrointestinal wall instead of grouping together [15, 16]. The major function of EECs is regulation of digestive process which is exerted by peptides like cholecystokinin (CCK), GPI, glucagon-like peptide 1 (GLP-1), peptide YY(PYY), neurotesin (NT), somatostatin (SOM) etc. [17] which are all secreted by EECs.

The ENS and EECs are two major components of the GIT that regulate main gastro-intestinal functions. But little is known about the development of both ENS and EECs in the first month of the life and also how food influences their development, maturation and normal functions. In addition it still remains unknown what is the major cause of the high rate of intestinal diseases in the SGA neonates compared with average gestational age (AGA) ones. Since ENS and EECs are two major parts of the GIT we assume that alternations of the ENS and EECs during the postnatal development are involved in the etiology of the SGA newborns. Thus we initiated our studies to investigate the development of the ENS and EECs during the first month of life and at the same time evaluate the effects of a formula diet versus natural milk diet on these two system.

Chapter 2: Literature Review

2.1 Small for gestational age (SGA) and Average for gestational age (AGA)

2.1.1 Definition of SGA, AGA, and other related conditions

Fetuses with low birth weight usually have relative higher morbidity (incidence of diseases) and mortality no matter what gestational age they are at [18]. Even for the infants born at term whose birth weights are at or below the 3rd percentile for their gestational age the mortality and morbidity are increased [18]. It has been reported that more than 20 million infants are born with low birth weight each year and it is much more prevail in developing and least developed countries than industrialized countries [19]. According to the World Health Organization, nearly 69% of all Low birth weight (LBW) infants have intrauterine growth restriction (IUGR) [19]. In general, the low birth weight (LBW) infants are defined as the newborns whose birth weight are less than 2500g [18-20]. The very low birth weight (VLBW) infants are characterized as the neonates whose birth weight are below 1500g and the extreme low birth weight (ELBV) infants are those whose birth weight are less than 1000g [21]. The LBW is related to maternal and environmental factors. It has been shown that underweight women tend to have higher risks of preterm birth and LBW infants than normal weight women [22]. Air pollution also can increase the risk of preterm birth or LBW [23, 24].

Intrauterine growth restriction (IUGR) referred to the infants with a birth weight and/or birth length below the 10th percentile for gestational age (GA), with a pathologic restriction of fetal growth [4, 25, 26]; “retardation” has also been used [25] but the term “restriction” overcomes it, as this reveals a reversible, transient condition [4, 25]. The term “Small for gestational age (SGA) newborns” is defined as those whose weight were below the 10th percentile for their gestational ages [19, 27] which is very similar to the definition of IUGR. The main difference between these two terms is that IUGR refers to fetuses who fail to achieve their genetically determined growth potential in uterus [25, 28-30] and that SGA refers to infants who failed to achieve a standard weight threshold for their gestational age at birth [25]. In general, all IUGR fetuses will be SGA, but not all SGA fetuses are caused by IUGR [3, 4]. In other way, not all

infants whose birth weights below the 10th percentile will show pathologic growth restriction, some of them are small only because of some maternal factors, like race or ethnic group and maternal weight [18, 31]. IUGR can be caused by different factors (Table 1) [4]. It has already been shown that the likelihood of IUGR increased when pregnancy encountered with medical complications, like hypertension, inflammatory bowel disease and hypoxia [4, 19]. Environmental influences and other factors like ethnicity, maternal age and maternal weight, height are also involved in IUGR [4, 19]. Smoking of the mother during pregnancy is the most important cause of IUGR among all the environmental factors. It has been demonstrated that 40% of IUGR in developed countries is caused by smoking of the mother [4]. As regard to maternal age, white middle-class American women among 13 to 17 years old had remarkable high risk of delivering the infants who had low birth weight or who was SGA compared with those among 20 to 24 years old [32].

Table 1: Factors associated with intrauterine growth restriction (IUGR) [4]

Maternal conditions	**Examples**
Medical complications	Hypertension Inflammatory bowel disease
Environmental factors	Smoking Alcohol Drugs
Other factors	Ethnicity Low maternal age Pregnancy weight gain
Fatal conditions	**Examples**
Genetic	Chromosomal abnormalities
Infections	Syphilis Malaria
Malformations	Gastrointestinal defects
Placental factors	**Examples**
Placental abnormalities	Reduced placental blood flow
Metabolism, hormones	Insulin Growth factors
Paternal conditions	**Examples**
Height	Short father tend to have LBW babies

Average for gestational age (AGA) infants are defined as those whose weights were between the 10th and 90th percentile. Infants whose weight were above the 90th percentile are mentioned as large for their gestational age [27]. Battaglia et al. also divided the newborn infants into 3 basic divisions: Pre-term, Term and Post-term. Term has been defined as all infants that were born with gestational ages from the 38th completed week up to but not including the 42nd completed week, all infants born before 38th completed week are recognized as preterm and all born after the 41st completed week as post-tem [27]. The term "premature" actually indicates the same condition as pre-term which refers to the immaturity related to the gestational age of the infants [18]. Table 2 is a summary of all the terms.

Table 2: Terms and definitions (human)

Term	Abbr	Definition
Average for gestational age	AGA	Birth weight between 10th and 90th percentile for their gestational age [27].
Small for gestational age	SGA	Birth weight below the 10th percentile for their gestational age [19, 27]
Large for gestational age	LGA	Birth weight above 90th percentile for their gestational age [27]
Intrauterine growth restriction	IUGR	infants with a birth weight and/or birth length below the 10th percentile for gestational age [4, 25, 26]
Low birth weight	LBW	Birth weight lower than 2500g [18-20]
Very low birth weight	VLBW	Birth weight lower than 1500g [21]
Extremely low birth weight	ELBW	Birth weight lower than 1000g [21]
Term	N/A	Born within 38th to 42nd completed weeks [27]
Pre-term	N/A	Born before 38th completed weeks [27]
Post-term	N/A	Born after 41st completed weeks [27]

2.1.2 Piglet as a model to study IUGR and SGA conditions

The pig has become a popular model for many human biomedical studies, like for endoscopy and laparoscopy techniques in human GI and gynecology surgery [8]. Nowadays this species is especially useful in pediatric research [8, 33] since many critical comparisons can be made between newborn pigs and human infants, especially in maturity level at birth [20]. Humans and pigs are both omnivorous mammals and show remarkable seminaries regarding to a lot

of different aspects, like dental characteristics, eye structure, and cardiovascular anatomy and physiology etc. [34]. As for the gastrointestinal tract (GIT) [8], humans and pigs share similar developmental characteristics, digestive anatomy and physiology, many anatomical and physiological aspects, biochemistry and even pathology [8, 34-36]. The prenatal development of GIT functions occur around the late gestation for both human and pigs [8, 37]. Besides, the digestive enzymes (i.e. trypsin, amylase and lactase etc.) development in pigs are similar to human in fetal and neonatal periods [8].

However, there are still some differences between humans and pigs. For humans, at late gestation most of tissues and organs are at a relative advanced stage of maturity while human muscle and the central nervous system still remain undeveloped even at full term [1]. Pigs has well-developed muscular and nervous systems at birth [1, 38].

It has been noticed that pigs show the highest number of naturally occurring LBW compared with other farm animals [20] which is useful for studying the LBW infants. LBW piglet is probably associated with IUGR [3] since uterine crowding always happens and can lead to reduction of fetal size/weight [39]. Like in human infants, IUGR may have some short or long-term developmental abnormalities on LBW piglets, like growth performance, muscle accretion, and duodenal mucosa morphology [40]. IUGR is mostly caused by inadequate uterine capacity of sow since uterine capacity is the major limiting factor for fetal growth in all species [39, 40]. Moreover, preterm pigs, which are delivered at <95% gestation (<110 d of 116 d gestation), are beneficial for pediatric research since they have close similarity with preterm infants in many aspects like body size, organ development and onset of necrotizing enterocolitis (NEC) [1, 38, 41].

2.1.3 Effects of preterm birth and LBW on gut

Preterm neonate has immature GIT which is unable to sufficiently digest and absorb nutrients [42]. NEC occurs at these newborns when nutrients exceed the digestive capacity of newborns

which usually leads to overgrowth and fermentation [38]. NEC is the most serious GIT disease affecting preterm, VLBW and ELBW neonates and the rate is inversely proportional to gestational age and birth weight [21, 43, 44]. NEC is a course of morbidity and mortality among VLBW and ELBW newborns, but the etiology still remains unclear [21, 43]. However, it has been suggested that the NEC might be caused by the concurrence of a genetic predisposition, intestinal immaturity, and abnormal intestinal microbiota [45]. In IUGR newborns, the thickness of stomach wall and muscularis externa are decreased compared with controls [46]. The gastric pits in IUGR piglets are deeper than those of normal piglets, and decreased wall protection is indicated by hyperplasia around the gastric pits [47].

IUGR also associated with the development and maturation of small intestine which leads to decreasing of food intake and barrier function [46, 47]. Indeed, IUGR is associated with lower length and weight of small intestine [46, 47]. After birth, the absorption area of small intestine in IUGR piglets is reduced due to the decrease of the average number of villi per section of the small intestine and a lower height of intestinal microvillus [40, 46, 47].

2.1.4 Food influences on the development of SGA and AGA newborns

Nutrition is a crucial determinant for the growth and development of organs in the body and developmental changes caused by nutritional programming can become permanent and can lead to certain lifelong problems [8]. After birth neonates lose their nutrients supply from placenta and GIT becomes the major source for their nutrient requirement. During perinatal period, nutrients are needed to match the requirement of various tissues, thus the GIT must be sufficiently developed at birth to provide the digestive functions and defense functions [8] . After birth, the GIT is stimulated by nutritive and non-nutritive substances from colostrum and milk [8] and fasting or malnutrition can lead to intestinal atrophy [48].

Breast feeding and breast milk are important for newborn. The incidence and/or severity of a lot of infectious diseases, like necrotizing enterocolitis (NEC) and gastroenteritis [49] and rate

of mortality and morbidity of neonates can be reduced by breastfeeding or breast milk. Intake of colostrum and milk after birth is crucial for stimulation of GIT growth and function [8, 48, 50], the development of nervous system and the entire neonatal organism [8], supporting the neuroendocrine function [8] and providing passive immunological protection [51, 52] since colostrum and milk contain nutrients (glucose, lactate, ketone bodies, and amino acids) [3, 19], hormones, growth factors [8] and immunoglobulins [50, 52, 53].

In litters, the smallest/LBW piglets are often less lively compared with their littermates. They are unable to compete with their littermates for spaces and has less access to sufficient amount of colostrum and milk from sow which leads to poor nutritional status and immunity [54-58]. Preterm neonates actually face the same situation as LBW piglets, they are exposed to maternal contact, colostrum and breast milk less. Since maternal contact, colostrum and breast milk are critical to stimulate a balanced bacterial colonization of the gut (microbiota) which is important for normal gut function, LBW and preterm term newborns are predisposed to certain diseases like NEC [59-61] and have elevated mortality [53].

For preterm newborns the incidence of NEC is different between colostrum feeding and milk replacer feeding. It has been showed that colostrum feeding group has lower NEC occurrence compared with milk replacer feeding group in both human and pigs [1, 38, 42, 62-64]. For pigs with formula feeding, they have decreased intestinal villous heights, lowed enzyme activities and reduced nutrient absorption [42].

Moreover, the food influence on mother can indirectly influence the birth weight of neonates. It has been demonstrated that underweight women tend to give birth to preterm birth and LBW newborns [22].

2.1.5 Effects of preterm birth and LBW on neurodevelopment in humans

Preterm infants has higher risk for neurodevelopmental disabilities, including cognitive and psychomotor delay [43, 65]. Small for gestational age and LBW can lead to poor long-term neurological outcomes [65] and internalizing and externalizing behavioral problems [66], including autism [67]. Periventricular leukomalacia (PVL) and severe intraventricular hemorrhage (IVH) are the most common central nervous system (CNS) injuries in the preterm infants [43]. PVL occurs in 3-4% preterm infants with LBW and is an important predictor of neurodevelopmental disorders, including cerebral palsy (*damage to the motor control centers of the developing brain*) [68, 69]. Reduced volume of grey matter has been observed in both term and preterm LBW infants [70-72]. Besides brain abnormalities, prematurity is associated with cerebellar hypodevelopment, hemorrhages and infarction [73]. The children born with VLBW showed reduced volumes for thalamus and cerebellar white matter [74]. For children in SGA group, they have smaller total brains, and proportionally smaller regional brain volumes [74]. It has been showed that preterm SGA infants had difference in brain structural measurements at the age of 5 months [75]. SGA term babies are more likely to have cerebral palsy than term AGA infants [76].

2.1.6 Effects of IUGR and SGA on other organs of newborns

The preterm neonates are at risk of developing infections, which often leads to high morbidity and mortality [43]. Early-onset or late-onset sepsis, meningitis or NEC can induce an overwhelming systemic inflammatory response, which will lead to multi-organ failure, brain injury or death [43].

The weight of all internal organs are lower in IUGR neonates compared with normal ones [40, 46], but the relative weights pre unit body weight remains the same between the two groups except the pancreas which is marked smaller in the pigs with IUGR in both absolute and relative terms [8, 46]. In pigs with IUGR, there are fewer acidophilic zymogen granules and cytoplasmic basophilia and decreased lipase activity [8, 46]. The metabolism is also affected

by IUGR [8]. The rate of glucose utilization is reduced in IUGR piglets [77]. Decreased number of muscle fibers and physiological limits on fiber hypertrophy limit the growth potential of some of the skeletal muscle of IUGR pigs [8]. Although the difference is not significant, there is a decreased expression of insulin receptor, growth hormone receptor and IGF-1 (insulin-like growth factor 1) in IUGR pigs which may be associated with lower insulin and growth hormone level in plasma [8, 47].

SGA neonates have reduced linear growth in infancy and excess abdominal fat gain in children which are associated with increased risk of cardiovascular diseases, hypertension, and insulin resistance later in adult life [19]. The studies of SGA pigs demonstrate that LBW has negative effect on glucose metabolism and body composition in juvenile and young adult pigs [8].

2.2 Enteric Nervous System

2.2.1 Definition, structure, and development

A functional gastrointestinal tract (GIT) is essential for food transportation, absorption, digestion and excretion. The major conductor of all these complex processes is the enteric nervous system (ENS). The ENS is a vast and complex network of neurons and glial cells and is embedded in the intestinal walls throughout the GIT [9]. The ENS has been known as the second brain since it is capable of functioning independently when the link (the sympathetic and parasympathetic arms of the autonomic nervous system (ANS)) between the central nervous system (CNS) and the ENS is cut off [11]. In humans, the ENS contains approximately 5×10^8neurons and in mice more than 1.2×10^8 neurons are found [78-80]. The enteric ENS governs most functions of the GIT including motility, secretions, blood flow and the immune system with all these different kinds of neurons [12].

The ENS is derived from the neural crest (NC) [78, 79, 81-83]. The vagal neural crest is the most important source of enteric neurons, while sacral neural crest contributes a small amount of neurons to the distal bowel [78, 79, 81, 83]. A functional ENS in the gut requires vagal and

sacral neural crest cells to go through a number of crucial processes like cell migration, proliferation, and differentiation [83] [79]. Vagal neural crest cells emerge from the neural tube around embryonic day 8.5 (E8.5) in the mouse [84, 85]. As they entered the embryonic mouse gut at E9.5, they are termed enteric neural crest cells (ENCCs) [81, 83]. The rostrocaudal migration of vagal ENCCs to colonize the entire length of the developing gut starts from E9.5 and ends at E13.5-E15.5 in mouse [81, 86]. For human embryos, the rostrocaudal wave of migration lasts from prior to week 4 to week 7 [87]. The ENCCs at the wavefront of migration remain undifferentiated and continue to invade regions lacking ENCCs while those behind the migratory wavefront are at different stage of differentiation with neuronal differentiation happens before glial differentiation [81, 83]. The whole developing process of ENS is mainly regulated by cell surface receptors and their ligands and transcriptional factors, like RET (rearranged during transfection) proto-oncogene/GDNF (glial cell line-derived neurotrophic factor) signaling pathway, EDNRB (endothelin receptor type B) signaling pathway, Sox10 and Phox2B [78, 79, 81, 83].

The development of ENS continues after birth [80]. It has been showed that the enteric mucosal innervation appears only at birth in the pig which indicates that the maturation of mucosal plexus proceed within the first few months after birth [88]. Also, functional synapses and two major classes of neurons are present and can be distinguished electrophysiologically and morphologically at birth [89]. During the postnatal development of the ENS in mouse, electrophysiologically and morphologically properties undergo major changes which could influence changes in gut motility during development [89]. Since the ENS is still plastic in postnatal life, it is subject to both internal and external influences after birth, like intestinal microbiota may affect the structure and normal function of the ENS in mouse [90].

The ENS has two major ganglionated plexuses: the myenteric (Auerbach's) plexus and the submucous (Meissner's) plexus which has both inner and outer parts in large mammals. The myenteric plexus is located between the outer longitudinal and inner circular muscle layers of the muscularis propria all along the digestive tract. The submucous plexus is placed in the submucosa in the small and large intestine which is different from the myenteric plexus. The

inner submucous plexus is seen next to the circular muscle layer and the outer submucous (Schabadasch's) plexus is positioned close to outer circular muscle layer of muscularis propria [10].

Neurons in the ENS can be classified according to their morphology, neurochemistry and electrical properties, and function [10, 91]. Functional classification is more often used by scientists. Neurons in the ENS can be classed as sensory neurons, interneurons and motor neurons based on their different functions [10, 91]. Sensory neurons in the ENS includes both extrinsic and intrinsic primary afferent neurons [10, 91]. The extrinsic afferent primary neurons are mainly vagal and spinal afferents with their cell bodies in nodose and jugular ganglia and dorsal root ganglia, respectively [10, 91]. Intrinsic afferent primary neurons (IPANs) have cell bodies in the gut wall and provide information for the ENS to function autonomously [92]. Extrinsic afferent neurons mainly convey information relevant to energy, fluid and electrolyte homeostasis from the gut to the brain [92]. Interneurons forms interconnecting chains within myenteric plexus [10, 91]. At least one type of ascending (orally directed) and three types of descending (anal directed) interneurons have been identified in the guinea-pig [10, 80, 91]. The ascending interneurons are mainly cholinergic and involved in local motility reflexes [10, 80]. The descending interneurons have a more complex chemical coding [10, 80, 91]. Those contain NOS (nitric oxide synthetase)/VIP (vasoactive intestinal polypeptide) are engaged in local motility reflexes, while Ach (acetylcholine)/SOM (somatostatin) neurons are likely to be related to the direction of the migrating myoelectric complexes (MMC) along the intestine [10, 80, 91]. For motor neurons, there are three major types: muscle motor neurons, secretomotor neurons and neurons innervating entero-endocrine cells [80]. Muscle motor neurons are either excitatory or inhibitory and release neurotransmitters that regulate muscle contraction (substance P) or relaxation (NO (nitric oxide), VIP, NPY and GABA (γ-aminobutyric acid)) [80]. Secretomotor neurons are responsible for secretions and blood flow changes [80]. Major neurotransmitters presence in the ENS are indicated in Table 3.

Table 3: Major neurotransmitters in the ENS [9, 80]

Neurotransmitters	Abbr	Presence
Acetylcholine	Ach	Interneurons
Cholecystokinin	CCK	Secretomotor neurons and interneurons
Dynorphin	DYN	Secretomotor neurons, interneurons, and motor neurons
Enkephalin	ENK	Interneurons and motor neurons
Galanin	N/A	Secretomotor neurons, and motor neurons
Gastrin releasing peptide	GRP	Interneurons and nerve fibers
Neuropeptide Y	NPY	Secretomotor neurons, interneurons, and motor neurons
Nitric oxide	NO	Motor neurons
Substance P	SP	Secretomotor neurons
Vasoactive intestinal polypeptide	VIP	Secretomotor neurons
γ-aminobutyric acid	GABA	Motor neurons

Except enteric neurons, enteric glial cells (EGCs) is another major cellular component of the ENS. EGCs arise during the time ENCCs colonize the gut [81, 83]. EGCs are not only distributed in enteric neuronal structures like ganglia, interganglionic fiber stands and nerve fibers but also can be find in all layers of the gut wall [93]. EGCs outnumber enteric neurons in the ENS [94, 95] and can be divided into at least 4 different subclasses regarding their morphology and localization [96]. EGCs within ganglia are star-shaped (type I), whereas EGCs in interganglion are more elongated (type II) [93]. Mucosal and intramuscular EGCs are type III and type IV, respectively [93]. It has already been showed that EGCs play an important role in the enteric neuronal functions and regulation of gut homeostasis. EGCs is crucial for protecting support and nourishing enteric neurons [93, 94]. In addition, it has been demonstrated that EGCs may regulate the neurochemical coding of enteric neurons [97]. In the transgenic mouse with glia disruption, the proportion of ChAT (Choline acetyltransferase) and NOS neurons in myenteric plexus increased [97]. EGCs also have the ability of neurogenesis in response to chemical injury of the enteric ganglia in adult mouse gut [98]. Besides neuronal function, EGCs also regulate non-neuronal functions, like gastrointestinal motility and intestinal epithelial barrier functions [93]. EGCs are similar to astrocytes of the CNS in both morphological and functional aspects.

2.2.2 Food influences on the ENS

Food/nutriments are the major substances that stimulate the ENS and other systems to work together for the digestion and absorption. Mechanical distention as well as chemical stimulations are main factors inducing ENS and EES activation [99, 100]. The enteroendocrine cells (EECs) of the gut are the main receptor for the chemical stimuli [101]. Food can activate the ENS indirectly by the peptides and hormones secreted by EECs.

It has been showed that the ENS is not fully developed right after birth and the microbiota might influence the postnatal development of the ENS in the mice [90]. Collins et al. showed that on postnatal day 3 the myenteric ganglia of GF mice contained less neurons compared with control group and intestinal motility is decreased in GF mice [90]. Bacterial byproducts like lipopolysaccharide (LPS) or short-chain fatty acids has been showed to affect ENS normal function [102, 103]. Indeed, it has been showed that interactions between ENS and microbe increased neuron survival and intestinal motility in mice [103]. TLR4 (Toll-like receptor 4) activation by LPS turns out to promote survival of enteric neurons [103]. TLR2 (Toll-like receptor 2) signaling controls ENS structure and neurochemical coding and neuromuscular function, and regulates the intestinal inflammation [104]. Diet is actually a determinant of gut microbiota composition [102, 105] thus food might influence the development and normal function of the ENS indirectly by changing the microbiota composition in the gut.

Breast milk influences the development of the ENS during the first postnatal week which indicated by an in vitro study that showed protein extracts of breast milk like glial cell-line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and transforming growth factor β（TGF-β）increased both the neuron survival and neurite growth in rat [106].

2.2.3 Diseases related to abnormal ENS

The ENS is involved in normal gut functions thus any changes in the ENS might cause some diseases related to gastrointestinal dysfunction. There are many specific developmental

ENS diseases, like Hirschsprung disease (HD), hypertrophic pyloric stenosis, intestinal neuronal dysplasia (IND), slow transit disorders, desmosis, myopathies, ganglioneuromatosis, [107] irritable bowel syndrome (IBS), diabetic gastroparesis, laxative abuse colonic neuropathy and opioid induced dysfunction [108]. Actually, enteric neuropathies can be classified into 4 different groups: (1) congenital or developmental neuropathies; (2) sporadic and acquired disorders; (3) disorders secondary to other diseases states; and (4) latrogenic or drug drug-induced disorders [10].

HD, which is also called intestinal aganglionosis is a congenital disorder [10]. The incidence of HD is around 1 per 5000 individual and there are differences in incidence between ethnic groups and there is a 1:4 female: male gender bias [78, 109]. HD is characterized by absence of enteric neurons in the distal bowl and extending for varying distances [9]. Most infants with HD fail to pass meconium and suffer from constipation while healthy infants normally pass meconium within the first 1-2 days after birth [109]. HD happens as an isolated disorder in 70% of patients and for the remaining 30% it is part of the syndrome in which other neural crest derivatives are commonly affected [78, 109]. In over 80% of HD patients, aganglionosis is confined to the rectosigmoid colon, which is termed short-segment HD, in the rest of the patients, aganglionosis can also affect significant length of the colon and even extend into small intestine, which is long-segment HD [78, 109]. The entire small and large intestine are both aganglionic is rare and is termed total intestinal aganglionosis [109]. The absence of enteric neurons in the aganglionic regions in HD result from a failure of ENCCs to colonize the affected gut regions during development which is associated with a delay in the entry into the foregut and a delayed progression of ENCCs along the gut [9, 78, 109]. It has been showed that the occurrence of HD is involved in mutations of RET [78, 109].

IND is also in the first group of enteric neuropathies [10]. There are two types of IND: IND A and IND B [9, 107]. Type A is very rare and occurs in less than 5% cases and is characterized by immaturity or absence of sympathetic innervation [9, 107]. Type B is characterized by malformation of the submucous plexus which results in development of hyperganglionosis and giant ganglia [9, 107]. It has been suggested that the pathological changes occurred with IND

may be part of normal development or a secondary circumstance result from congenital obstruction and inflammatory diseases [110].

2.3 Enteroendocrine cells

2.3.1 Definition, development and differentiation

The motility of the GIT is mediated by both neural and hormonal networks; the latter includes peptide hormones released from endocrine cells in the gut [15]. The GIT involved in the control of metabolism also via gut peptides secreted by the endocrine cells in the gut [111]. The endocrine cells in the GIT mucosa and the pancreas is termed entero-endocrine cells (EECs) which forms the largest endocrine system in the body according to the number of cells and the variety of the hormones produced [13, 14]. EECs represent only about 1% of all the cells in the mucosa [15, 16, 112, 113] but they have at least 16 different cell types based on their primary products, which consists of more than 30 known peptides [15, 16, 112]. The major difference between EECs and other endocrine cells is that EECs are mostly scattered along the GIT except in rectum which is the only location where EECs are sometimes adjacent to each other or in cluster [113] and they have a very limited life span [111]. Generally, EECs can be grouped into open type and closed type [13, 114]. The open type cells are the ones contact lumen with their microvilli while the closed type are the cells with no directly contact with lumen [13, 114]. Although the cellular morphology varies among different EECs subtype, most of them still share some common features, as for the EECs in the large intestine they have basal processes that prolong to epithelial cells next to them [113]. EECs are characterized by the ability to secrete peptides with signaling capacity [13, 113]. The compounds released by EECs can exert their signaling action by endocrine, paracrine or autocrine action. EECs has either large dense-core vesicles (LDCVs) or the smaller synaptic-like microvesicles (SLMVs) and the latter is similar to neuronal postsynaptic vesicles [113]. Recently it has been showed that about 70% of the EECs peptide-secreting vesicles are included in an axon-like basal process which is called neuropod and this neuropod is surrounded by enteric glia [115].

It has been showed that EECs are developed from pluripotent stem cells in the crypts as all the other epithelial cell lineages, which are endoderm-derived [116]. The pluripotent stem cells migrate and differentiate into one of the four cellular lineages [113]. It has been showed that loss of Notch (transmembrane receptor) cause excess EECs [117] which indicates that Notch may prevent adjacent cells from differentiating into EECs and lead to the scattered distribution of EECs in the GIT [113]. Due to the short life span of around 4-6 days, EECs have an extensive plasticity compared with other endocrine organs [118]. Basic helix-loop-helix (bHLH) is the major transcription factor that controls the continuously differentiation of EECs from pluripotent stem cells in the crypts [17]. In fact, nutrition alters EECs differentiation, like high fat diet decreases the expression of bHLH and thereby reduce the EECs number [119].

It has been mentioned before that there are at least 16 different types of EECs in the GIT and actually most of these types are named after letters of the alphabet. For example, alpha cells made up around 40% of the endocrine cell population in the pancreatic islets and most of the rest of the endocrine cells are beta cells. The former cells store and produce glucagon while the latter ones produce and store insulin. And together, they maintain the blood glucose level. The D cells are found throughout the GIT with the highest frequency in the duodenum [120] and they also comprise 5% of the islet endocrine cells. These cells store and secrete somatostain (SOM). Enterochromaffin (EC) cells distribute all along the GIT and are the most abundant cell in the GIT which constitute about 70% of the EEC population. They have a pyramid shape with a process reaching the luminal surface [120]. EC cells mainly secrete serotonin (5-HT) and contribute to about 80% of the total amount of 5-HT in the body. N cells are neurotensin producing cells and are primarily found in the ileum. Other cell types of EECs contain: enterochromaffin-like [121] cells, epsilon cells, F cells, G cells, I cells, K cells, L cells, M cells, PP cells, S cells and XIA-like cells and PID1 cells (Table 4) [13].

Table 4: Major peptides secreted by EECs

GI hormone	Site of secretion	Actions
Cholecystokinin (CCK）	I Cell	Activates bile and pancreatic secretion [122], acts as a hunger suppressant [13], stimulates gallbladder emptying [15], slows gastric emptying [123], accelerates small intestine transit [124]
Gastrin	G Cell	Stimulates acid production and histamine release, activates gastric and esophageal mucosa growth, and control growth hormone production [13]
Ghrelin	ε Cell/Gr Cell/M Cell	Affects appetite, food intake, fat utilization and body weight [125]
Glucagon	α Cell	Causes liver convert glycogen into glucose[13]
Glucagon-like peptide 1 (GLP-1)	L Cell	Stimulates release of insulin and somatostatin [122], inhibits gastric acid secretion and gastric emptying [126], decrease food intake [126]
Glucagon-like peptide 2 (GLP-2)	L Cell	Co-secreted with GLP-1 and PYY, stimulates mucosal enterocyte proliferation [15]
Glucose-dependent insulin-releasing polypeptide (GIP)	K Cell	Stimulates insulin secretion [13]
Insulin	β Cell	Decrese glucose level in bloodstream,causes storing of glucose as glycogen [127]
Motilin	M Cell	Induces antral phase III activity [128], stimulates pepsin production and PP, SOM releasing [13]
Neurotensin (NeuroT)	N Cell	Stimulates GI motility and secretion, activates intestinal cell growth [129]
Pancreatic polypeptide (PP)	PP Cell/F Cell	Elicits intestinal contriction, inhibits jejunal and colonic motility [13]
Peptide YY (PYY)	L Cell	Inhibits upper GI motility [13]and gastric acid secretion, increases absorption of water and electrolytes in colon [13], activates mucosal enterocyte proliferation, suppresses appetite
Secretin	S Cell	Increases bicatbonate, bile, insulin and gastic pepsin secretion, inhibits gastic acid and glucagon secretion, decreases intestinal motility [13], acts as a neuropeptide in the CNS [130]
Serotonin (5-HT)	Enterochromaffin (EC) Cell	Activates gut motility and increases intestinal transit, stimulates inflammation, involves in vomiting process, abdominal pain, nausea [131]
Somatostatin (SOM)	D Cell	Acts as an inhibitory hormone, stimulates colonic peristalsis [113]

Peptides secreted by EECs are important for digestive process since they can regulate appetite and satiety [17]. Like, there are two EECs secreted peptides involved in interdigestive motility: motilin and ghrelin. It has been showed that in human endogenous motilin and ghrelin both induces antral maximum contraction [128]. And ghrelin also regulates irregular contraction in both human and dogs [132]. During the postprandial state, levels of motilin and ghrelin decline and EECs secreted other peptides to modulate GI motility, including cholecystokinin (CCK), GIP, glucagon-like peptide 1 (GLP-1) and peptide YY(PYY) [15]. It is possible that CCK achieves its functions which are slowing down GI motility, suppressing ghrelin intake, stimulating PYY secretion, and increasing gallbladder contraction by act on vagal afferent [133, 134]. GLP-1, together with PYY mediates the inhibition of upper GI motility [15].

2.3.2 Important peptides secreted by EES

There are various receptors on the EECs which partly regulate secretion of the signaling peptides [13]. Moreover, the release of peptides is mediated by factors in the GI lumen, include food, gut microbiota products, pH and others as well [135]. The impulse from nerve also involves in regulation of releasing of peptide from EECs.

Chromogranins (Cgs) are the main protein content in the vesicular matrix of LDCV and are involved in numerous physiological processes, including vesicle sorting, bioactive peptide generation and accumulation of compounds in LDCV [136]. Some studies show that Cgs is directly involved in the development of neurological diseases such as schizophrenia, epilepsy and neurodegenerative diseases like Parkinson’s disease and Alzheimer’s disease [136]. Chromogranin A (CoA) is often used as a general IHC marker for EECs [113]. With a series of experiments on knockout mice, it has been implicated that CoA plays an important role in catecholamine concentrating and retaining in LDCVs [136]. In addition, CoA is now served as a marker in blood of neuroendocrine tumors and can be used in detection of reoccurrence [137].

Neurotensin (NeuroT) release is induced by fat ingestion and is produced by N cells, most of which are open type [129, 138]. NeuroT is involved in intestinal relaxation [138] and intestinal cell growth stimulation [129]. For some species like rat, cat, dog, NeuroT positive innervation of the GIT has been demonstrated and the NeuroT positive fibers are mainly found in the myenteric plexus of these species [138]. For the guinea pig, there is no conclusion for the occurrence of NeuroT positive fibers in the ENS since various laboratories showed different results.

SOM is expressed in brain, pancreas and GIT. Within the human GIT, SOM expression can be observed in both neurons of myenteric plexus in muscular layer and D cells in mucosa [113, 139]. The secretion of SOM is induced by luminal acidity, fat and mechanical factors and is inhibited by luminal peptones [13]. SOM is the main inhibitory hormone of the GIT which decreases the secretion of all the GI hormone even itself [113]. And it also inhibit the function of immune system including the proliferation of T cells and secretion of pro-inflammatory cytokines [113, 140].

Serotonin (5-HT) is the major secretory product of EC cells and is induced mainly by mechanical stimulation by food in the intestine [141]. 95% of the 5-HT in the body is localized in the GIT [113]. Besides EC cells, it has been mentioned that microbiota is able to produce 5-HT [142]. It has been reported that the 5-HT level is the highest in the rectum [143]. 5-HT induces elevated gut motility and increased intestinal transit [131] which might be regulated by the interaction between EECs and ENS [113, 144].

2.3.3 Diseases related to abnormal EECs

Ulcerative colitis (UC) and Crohn's disease (CD) are both immune-mediated inflammatory bowel diseases (IBD) [145]. It has been showed that mice with mutant T cell receptor spontaneous develop IBD [146]. Along with the inflammation, there is a decrease in the number of EEs expressing CCK, 5-HT, and NeuroT in the colon [147]. But GLP-1 and gastrin expression remains unchanged and colonic EECs remain unaltered [147]. The alternations of

EECs are not only restricted to the inflamed areas. It has been demonstrated that in ileitis there is an increase of number of colonic EECs expressing 5-HT and GLP-2 and the release of 5-HT is elevated at the same time [148]. In both UC and CD patients, there are increase in CoA and 5-HT immunoreactive cell numbers, but the number of PYY immunoreactive cells is significant decreased [149]. Small bowel CD patients exhibited reduced appetite both before and after eating and the decreased appetite may be related to EECs response changes, like increased PYY and ghrelin levels [150].

Lymphocytic colitis (LC) is a disorder belongs to microscopic colitis (MC), which is characterized by chronic diarrhea [151]. In the patients of LC, it has been showed that both CoA, PYY, and 5-HT cell densities were increased in colon [151, 152]. And the change of 5-HT might result from the interaction between immune cells and serotonin cells [151]. With a 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) pig model of ileitis, it has been showed that the number of EECs expressing SOM, NeuroT and 5-HT were increased in ileum [153].

CCK levels are elevated in patient with acute upper gut infection (Giardia lamblia). Healthy people get high dose of intravenous CCK showed nausea and abdominal discomfort which are symptoms of gut infection [154]. It is possible that EECs dysfunction directly contribute to symptoms production [155]. Mice with the intestinal nematode Trichinella spiralis confirmed the up regulation of CCK in gut infection and demonstrated that CCK expressing cells are under control of CD4 T cells [156].

In IBS patients, a reduction of density of secretin and CCK immunoreactive cells was noticed in duodenum and this decline was only observed in IBS-diarrhea patients [157]. And both GIP and SOM cell densities were reduced in duodenum of IBS patients [157] and 5-HT and PYY cell densities were reduced in the colon of IBS patients [158]. Increase of overall plasma CCK levels has been observed in IBS patients as well [159]. Although the plasma level of CoA remained unchanged, a decline of the density of CoA cells was noticed in both the duodenum and colon in patients with IBS [160]. This reduction might imply a decline of the total amount

of intestinal endocrine cells [160]. However, CoA is not a specific marker for certain cell type, it is still unclear where does this change derive from [145].

Chapter 3: Experimental Studies

Introduction

This chapter contains two articles focus on the development of the ENS and EES during the first month of life with a SGA piglet model. Different feeding strategies has been used to study the development of these two critical systems in the GIT. The first article is about the development of ENS and shows that birth weight and/or diet can influence ENS development. The second article mainly talks about the development of EECs and demonstrates that maturation of the EECs is a dynamic process and can be influenced by feeding strategy.

3.1 Development of the enteric nervous system in a sow-reared or formula-fed small for gestational age piglet model.

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3.1.1 Abstract

Due to the transition from uterine to extramatemal environment, the infants with growth retardation and preterm delivery are at high risk of developing intestinal problems ranging from delayed meconium passage to fatal necrotizing enterocolitis. A SGA piglet model was used to investigate the effects of birth weight and diet on the development of the enteric nervous system (ENS). Newborn SGA and average (AGA) piglets were randomized into two groups: sow-reared (SR, 24h/D with the sow) or formula-fed (FF, milk replacer) after colostrum intake. Gut samples were analyzed by immunohistochemistry using neuronal markers at postnatal day (PD) 1, 14 and 28 to assess their respective development. At PD1, marked lower number of nerve endings was observed in gut sections of SGA piglets associated with a marked hypo-development of presynpatic vesicles in the enteric ganalia. Up to PD14 included, the innervation of the colonic musculosa, evaluated by image analysis, was still under developed in SGA piglets. At PD28, FF piglets had less immune-positive noradrenergic nerve endings in the ENS than SR ones. These results demonstrate that birth weight and/or diet can influence ENS development and for the first time identified that the ENS is less developed in SGA piglets. This study highlights the potential interest of using this SGA piglets to study intestinal maturation in low birth weight infants.

Key words: IUGR (intra-uterine growth retardation), pig, development, enteric nervous system.

3.1.2 Introduction

The newborn mammal experiences a transition from a sterile uterine environment to a microbe-rich environment at birth [1]. During gestation, fetuses get nutrients supply from their mothers constantly from maternal blood via placenta; while after birth, infants have to get all the nutrients they need through oral intake via gastrointestinal tract [8] and any problems related to the GIT might result in the morbidity and mortality of neonates. For the infants with fetal growth retardation and premature birth, they tend to develop intestinal problems due to the immaturity of intestine and immunologic defense [7]; these infants frequently develop a delayed meconium passage, abdominal distension, delay in tolerating enteral feeding, and fatal necrotizing enterocolitis (NEC) [5, 6]. At any given gestational age, infants with low birth weight tend to have high risk of developing these diseases and relatively high morbidity and mortality, especially for the preterm infants [18, 161]. It has been showed that infants born at term have increased mortality and morbidity when their birth weight are at or below the 3rd percentile for their gestational age [18], and for the preterm infants whose birth weight are at the same condition, they showed differences in brain structure compared with preterm AGA infants[162]. The natural variance that occurs in birth weights between piglets of the same litter, mostly due to decreased passage of adequate nutrition from sow to some piglets, can be used to model intrauterine growth restriction (IUGR), which is observed in approximately 24% of newborn human infants every year [163].

Human and pigs are both omnivorous and share remarkable similarities regarding to level of maturity at birth, GIT anatomy, physiology and development. Thus, pig is often used as a model to study the field of nutrition and associated domains like gut growth and maturity [8, 20]. Preterm pigs and infants share the similar GIT characteristics and even body size, organ development and many clinical features which makes preterm pig a good model to study GIT immaturity, especially for pediatric research [1]. Regarding the gut maturation little is known about the development of the enteric nervous system (ENS) during the first month of life which is an important key regulator of the gut function including motility, absorption and secretion. And after birth, nutrition plays a critical role in the functional development and maturation of

the GIT [8]. Thus, the goals of our study were to provide a description of the development of the ENS of the gut of SGA piglets compared to AGA piglets and evaluate the effects of a formula diet versus natural milk diet during the first month of life.

3.1.3 Material and methods

Animals, housing, and feeding

Littermate pairs of naturally farrowed, newborn piglets (Sus scrofa domestica), (AGA, mean 1.49 kg [1.47-1.52 kg]; SGA, mean 0.72 kg [0.58-0.9 kg]) were obtained from 5 separate litters from the University of Illinois swine herd. Sow reared (SR) piglets were cross-fostered and maintained with the sow throughout the study. Formula fed (FF) piglets were brought to the biomedical animal facility 48h after birth to allow for colostrum intake. On PD1, 14 and 28, four piglets per group (FF or SR, AGA or SGA) were sacrificed and duodenum, Ileum, ascending colon and rectum samples were fixed in 4% paraformaldehyde for 48h and embedded in paraffin as described in our previous paper [164].

All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

Immunohistochemistry (IHC)

Histological sections (5µm) were dewaxed, rehydrated in water and then used for standard immunohistochemical analyses as previously described [165, 166]. Briefly, each section was incubated with one of the primary antibodies [NPY (NPY, Abcam 30914); Synaptophysin (SYN, Biocare CM371AK); BIII Tubulin (B3T, Promega G7121); Tyrosine hydroxylase (TH, Abcam ab112)]. An adequate secondary biotinylated antibody (Jackson Laboratory) and avidin–biotin–peroxidase complex system (ABC Vector Laboratories, Burlingame, CA, USA) were used to detect primary antibodies. Diaminobenzidine DAB (Zymed, SanFrancisco, CA, USA) was used as chromogens. A slight counter-staining was done using aqueous hematoxylin.

Nonspecific binding was controlled by omitting the primary antibody, and using other primary antibodies as isotypic controls.

Morphological analyses

For SYN and TH IHCs, a scoring system that reflected the intensity of immunolabeling was used as follows; 1: minimal, 2: slight, 3: moderate, 4: marked, 5: intense. All grading was done by a trained investigator who was blinded to the treatment.

The quantification of B3T was done on the ascending colon only using “Image J” (NIH software). Six microscopic pictures per animal (Obj 40) of the mucosa and musculosa were used for this analysis. Each image (containing a 255-grey tone) was transformed into an 8-color scale (Figure 3A). Based on the comparison with initial images, it was established that the black to purple colors (values 0-191) were associated with a specific labeling of the B3T IHC (figure 3). The number of pixels associated with the each value (0-191) was then reported in an Excel table and the ratio with the total number of pixels in the selected area was established for each image. The mean percentage of positive pixels was then established for each group for the mucosa and musculosa.

Statistical Analyses

Statistical analyses were performed using STATA 9.1, StataCorp LP, College Station, TX.

The associations of body weight, percentage of positive pixels for B3T, diet, size of piglets, and site within the intestine, including 2- and 3-way interactions, were examined by ANOVA models. Post-hoc analyses were performed using the Mann–Whitney U-test with Bonferroni correction. Logarithmic transformations (for body weight, percentage of positive pixels for B3T) were necessary to provide normality and homogeneity of variance (homoscedasticity) of residuals, and both were assessed graphically and by the Shapiro–Wilk, and the Breusch–Pagan and Cook–Weisberg tests, respectively. Distribution and residual were considered as normal and homoscedastic, respectively, when $p>0.05$ for the Shapiro–Wilk and the Breusch–Pagan/Cook–Weisberg tests. Normality and homoscedasticity of residuals could not be achieved despite transformations for the ANOVA studying the association between body

weight gain and day of analysis, diet, size of piglets, and site within the intestine. This ANOVA was therefore not performed, and only the associations between body weight gain and diet and size of piglets were examined by Mann–Whitney U-tests.

In addition, the relationships between intensity of staining for SYN, NPY, and TH in muscular and submucosal plexus, and day of analysis, diet, size of piglets, and site within the intestine were assessed by ordered logistic regressions. Post-hoc analyses were performed using the Fisher exact test with Bonferroni correction. The last step in the analysis was to estimate the correlations between body weight, weight gain, and intensity of staining for SYN and NPY in muscular and submucosal plexus. Correlation were considered poor if <0.3, mild if 0.31-0.6, good if 0.61-0.8, excellent if >0.8.

For all the analyses performed, tests used were two-tailed and p values less than 0.05 were considered as significant, except when Bonferroni correction was applied (association between intensity of staining for TH and type of food, $p<0.0125$ to be significant; relationship between percentage of positive pixels for B3T and size of the piglets, $p<0.016$ to be significant).

3.1.4 Results

Body weights and body weight gains (Figure 1)

Evolution of body weight from PD1 to PD28 was dependent on the size (SGA or AGA, $p<0.001$) and on diet. The effect of diet was variable between PD14 and PD28 as shown by the significant interaction of Diet*Day ($p<0.001$). SGA piglets remained smaller than AGA piglets with the same feeding strategy all throughout the study ($p<0.001$). However, in both SGA and AGA piglets, body weight gain was influenced by diet (for both FF and SR, $p<0.001$). The magnitude of body weight gain was similar between small and average piglets in both FF and SR groups (Figure 1B). On PD14, the body weight gain of FF piglets was lower compared to SR animals. However, FF piglets almost compensated for this difference on PD28 (Figure 1B).

Development of the ENS

Beta III tubulin

Nerve cells bodies forming muscular and submucosal plexuses as well as all nerve fibers innervating the mucosa and muscular layers were identified using B3T IHC (Figure 2B and 2D). On PD1, the ENS was already fully developed when compared to PD14 and PD28 in AGA animals. The innervation of the large intestine was denser than in the small intestine sections. Between PD1 and PD28, the quantification of the nerve endings in the musculosa showed a progressive decrease in percentage that was associated with the development of the myofiber size and/or number (Figure 3B).

In SGA piglets, the development of the nerve terminals in the musculosa and mucosa was delayed (Figure 2A and 2C) as shown by the significant interaction of Day*Size ($p=0.006$). This was particularly apparent in the small and large intestines of the two smallest animals of the group. The quantification by image analysis of the nerve endings in the colonic musculosa and mucosa only did show a significantly lower ENS development in the musculosa from PD1 (5.4% in SAG piglets versus 10.6% of positive pixels in AGA piglets). This delay of development was observed up to PD14 (4.8% in SAG piglets versus 8.5% of positive pixels in AGA piglets; $p=0.005$). By PD28, there was no more difference between SGA and AGA piglets (Figure 3B).

Synaptophysin

SYN marker was used to identify the presynaptic vesicles. On PD1 in AGA piglets, the synaptic system was relatively well developed (marked labeling) in all animals from the duodenum to the rectum (Figure 2F, star, myenteric ganglia， Figure 4). In SGA animals, on PD1, the synaptic system was clearly less developed particularly in the colon and rectum (Figure 4). In the 2 smallest piglets, no to mild labeling (small intestine) and no labeling (large intestine) were observed in both myenteric (Figure 2E, star) and submucosal plexuses (Figure 2E, arrow) ($p<0.001$). By PD14, the intensity of the SYN IHC was similar (marked to intense) in all groups and in all intestinal sections.

Sympathetic innervation of the gut

TH

The sympathetic noradrenergic innervation of the gut was investigated using an anti-TH (TH) antibody. On PD1, the intensity of the IHC labeling was similar between SGA and AGA piglets (Figure 5). On Day 28 only, both FF SGA and AGA piglets presented with a less intense labeling in the small intestine and colon (Figure 5, A - B) (p=0.001).

NPY

NPY immunoreactivity was presented in duodenum and rectum of all the groups through PD1 to PD28. On PD1, SGA and AGA piglets showed the same expression of NPY in all the regions we had for IHC. After PD14, a decrease of NPY expression was noticed in FF AGA to some extent. Between PD1 and PD28, the intensity of the IHC labeling was increased regardless of diet. In ileum and colon there were no/slightly expression of NPY. In general, there was not much differences between AGA and SGA during the first month of postnatal life as regarding to the expression of NPY.

3.1.5 Discussion

To our knowledge, the current study is the first to demonstrate a marked delay in maturation of the enteric nervous system in SGA piglets, as well as the effects of diet on the maturation of the noradrenergic sympathetic innervation during the first month of life.

Enteric nervous system

In SGA piglets, a delay in the maturation of the ENS was clearly identified and quantified in the colon when compared to AGA piglets at PD1. A similar observation was noticed in other gut sections in SGA piglets, but this was not quantified in our study. This significant delay in ENS maturation was noticed up to D14, and SGA animals only compensated this difference between D14 and D28. On D1, this difference between SGA and AGA piglets was particularly marked in the 2 lightest animals (ie 0.57 and 0.58 kg) when compared to AGA piglets. Synaptophysin, a maker of presynaptic vesicles, also confirmed the delayed maturation of

synapses in SGA piglets. As for B3T, there was marked to severe decreased or lack of immunoreactivity in the muscular and submucosal plexuses in the 2 smallest piglets, particularly in the large intestine sections. This delayed ENS maturation in SGA piglets probably reflects a more general immaturity of the nervous system as shown in our recent publication; SGA piglets presented with less gray matter, smaller internal capsule and reduced white matter development and connectivity [164] as reported in human infants [74, 75, 167]. Most ENS developmental abnormalities are associated with absence of myenteric neurons (Hirschsprung's disease) due to an abnormal migration of neurons from the neural crests [9], or intestinal neuronal dysplasia (hypoganglionosis, ganglioneuromatosis), retarded neuronal maturation, ganglionitis and metabolics disorders (mitochondriopathies and storage diseases) [107]. Delayed maturation of the submucosal and myenteric plexuses is a common cause of chronic constipation during the first year of life in infants; it is characterized by no, or a weak positive SDH reaction in immature ganglia [107]. In our study we also identify a decreased number of nerve endings as well as delayed synaptic formation which might be considered a less severe form of delayed maturation of the ENS in SGA piglets. Electrophysiological studies need to be done on these SGA piglets to correlate this delayed maturation to gut motility/contractibility function. Indeed, immature intestinal motility is suspected to predispose infants to NEC as the ENS is one of the key regulators of the gut functions, modulating motility, exocrine and endocrine secretions, micro-circulation and immune reactions [5]. Immaturity of the ENS may also have consequences on the brain maturation during this critical period in neonates. Indeed bidirectional vagal-dependent communication between the brain and the gut influences stress, pain and brain neurochemistry (for review see [168]).

Even if the ENS seems to be relatively well developed in AGA piglet on PD1, there is some evidence that the development and maturation of the ENS continue beyond birth in normal conditions in pigs [169]. An increase in expression of neuronal nitric oxide synthase and vaso-intestinal peptide during the first 3 weeks of life has also been noted in normal rats which indicated that the neurochemical differentiation is accomplished during the first month of postnatal life[170]. Similarly, in our study, the ENS does not seem to be completely developed at PD1 in AGA piglets as the intensity of the IHC labeling was lower for SYN when compared

to piglets at PD14. We also identified a slight increase in number and intensity of TH nerve endings in the muscular plexuses from PD1 to PD28 in SR animals (Figure 4) which also implies that the maturation of ENS in piglets is not achieved at birth and is accomplished during the first month of postnatal life.

Feeding strategy influences

On PD28 in FF piglets the TH staining is decreased particularly in the small intestine of both SGA and AGA piglets. A similar decrease in synthesis of an inhibitory neurotransmitter associated with a decreased neuronal activity was identified in a model of ocular toxicity in rats [166]. In our case a similar decreased gut activity might be suspected in FF animals compared to SR piglets leading to a lower expression of enzymes involved in the synthesis of noradrenaline. The expression of NPY, which is a neuropeptide abundant in the brain and expressed in enteric and sympathetic neurons acting as a neurotransmitter [171] in duodenum and rectum of FF AGA decreased after PD14. In brain, NPY involved in many cognitive processes such as appetite regulation, memory, and seizure and it might be used in therapies for Parkinson's disease [172]. In mammalian intestine, it presents in both myenteric and submucosal plexus and is important for gastrointestinal motility, secretion, microbiota and immune system [171]. In previous studies about presence and distribution of NPY, it has been showed that the NPY immunoreactivity was in all regions of GIT and in upper gut was the highest in both rat and guinea pig [173]. Surprisingly, in our study, the expression of NPY was only noticed in duodenum and rectum both in SGA and AGA regardless of die. The changes of expression of TH and NPY might represent an adaption of the regulatory sympathetic nervous system in FF animals. Our study also showed that the body weight gain of FF animals was lower compared to SR ones through PD1 to PD14.

The changes related to the different feeding strategies indicated in our study might resulted from various factors. Diet itself can lead to the changes. Colostrum and mike from sow contain hormones and growth factors that are critical for neonates to maintain their neuroendocrine function and regulate the development and function of GIT, since the maturation continued after birth [8]. And colostrum and milk from sow also provide some immunoglobulins like IgG,

IgA and IgM which can enhance disease resistance of newborns [8]; normal weight, IUGR, preterm pigs with colostrum feeding all showed lower incidence of NEC compared with FF ones and similarly, the preterm infants showed the same phenomenon [1, 42, 174]. Maternal separation (MS) is also a possible factor since the FF animals were reared in animal facility. It has been mentioned that MS can cause neurotransmitter 5-HT (serotonin) and noradrenaline alternation in CNS which might related to irritable bowel syndrome (IBS) and permanent increase in anxiety-related behaviors in rat (for review see [175]). Thus it is possible that the MS altered the formation of Sympathetic innervation in the gut. And indeed FF animals were reared in a “cleaner” environment compared to SR piglets.

In conclusion, we demonstrated in our study a delay in maturation of the ENS regarding the number of nerve endings and the development of the synaptic system in SGA piglets. This delayed maturation is likely an important factor regarding the development of intestinal problems of which NEC is the most severe outcome. And feeding strategy can influence the development of nervous system to some extent.

Statement of financial support: This work was supported by the University of Illinois Center for Nutrition, Learning and Memory and HD069899.

3.1.6 Figures

Figure 1

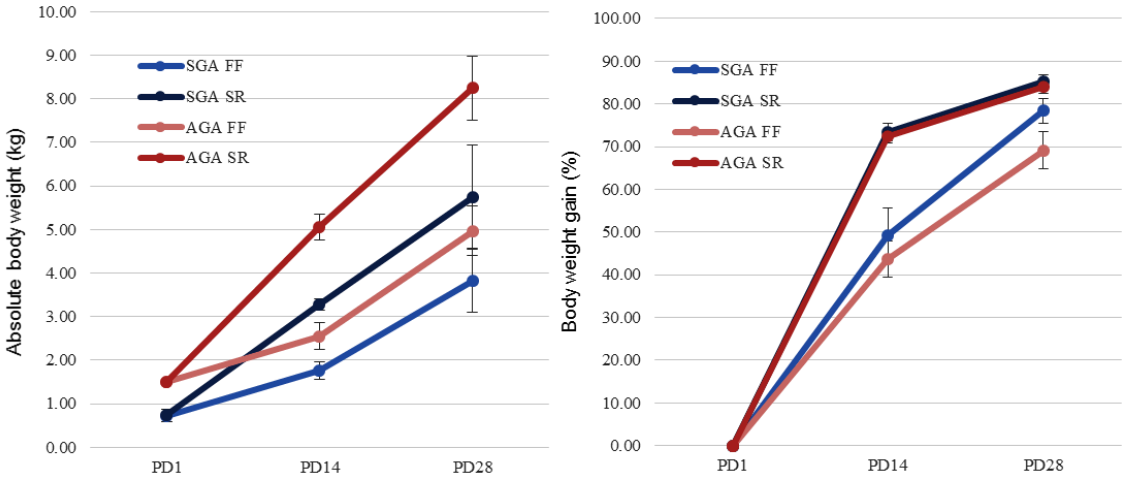

Figure 1. Absolute Body weight (A) and body weight gains (%) (B) of sow reared (SR) and formula fed (FF) SGA and AGA piglets at PD1, PD14 and PD28.

Figure 2

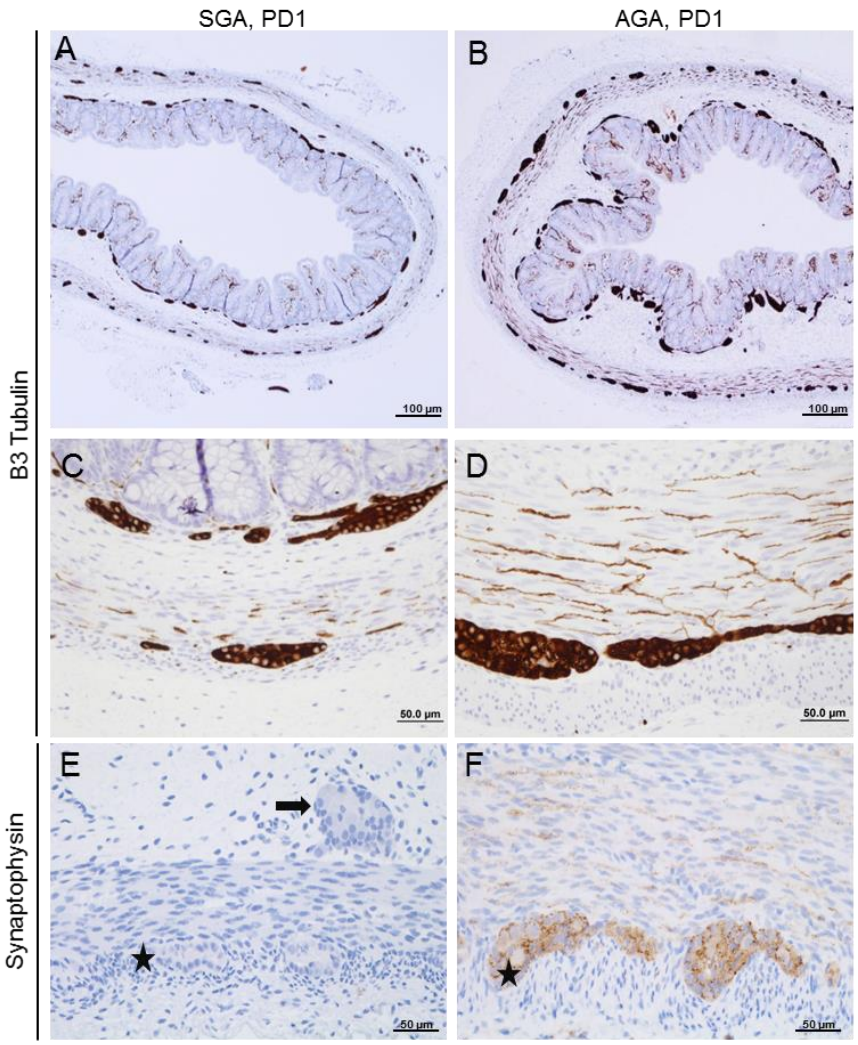

Figure 2. Beta-III Tubulin and Synaptophysin IHC, colon, PD1. The number of nerve endings in the musculosa and mucosa of AGA piglets (B and D) was higher when compared with SGA piglets (A and C). A moderate to marked synaptophysin immunoreactivity was detected in the myenteric ganglia (F, start) in AGA piglets (F, start); weak or labelling was identified in both myenteric (E, Start) and submucosal ganglia (E, arrow) of SGA piglets.

Figure 3

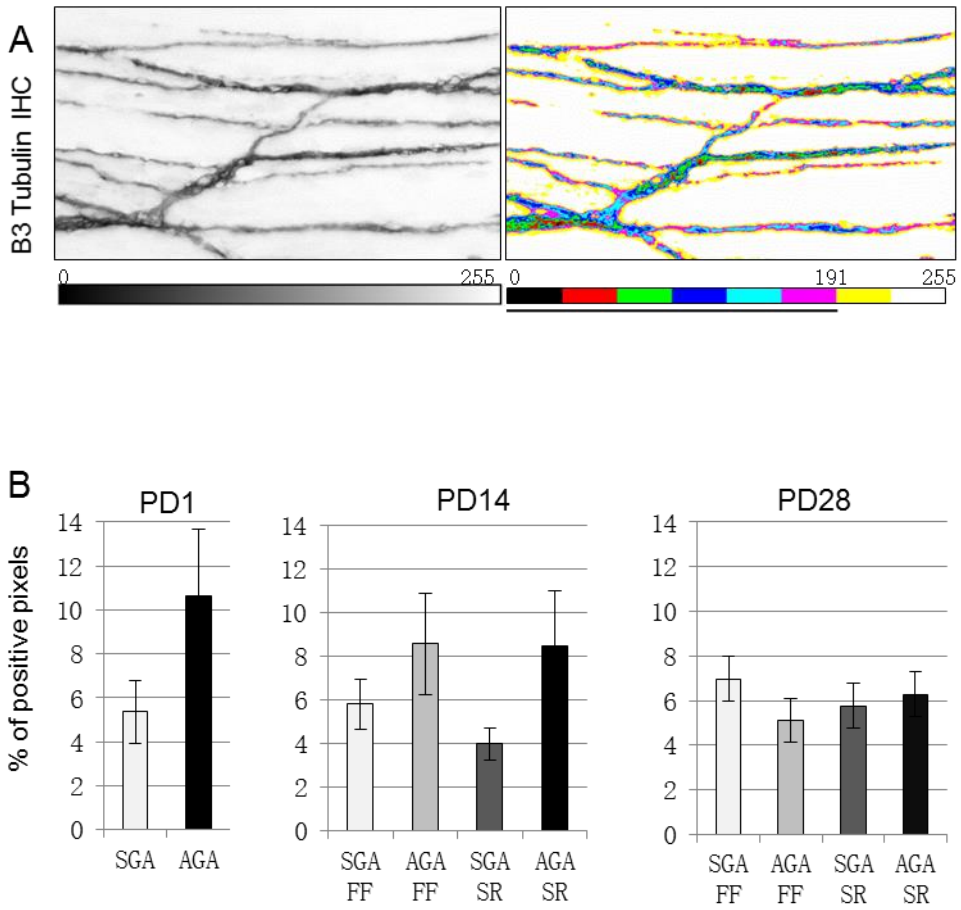

Figure 3. Colon, BIII Tubulin quantification. Evaluation of the percentage of positive pixels in the muscular layer of the colon. (A) Once transformed into an 8 bit grey scale, each image was changed into an 8-color scale. The specific labeling was associated to pixels numbers 0 to 191 (corresponding to the violet color). (B) The percentage of positive pixels was calculated for each area and the mean reported in histograms for PD1, 14 and 28.

Figure 4

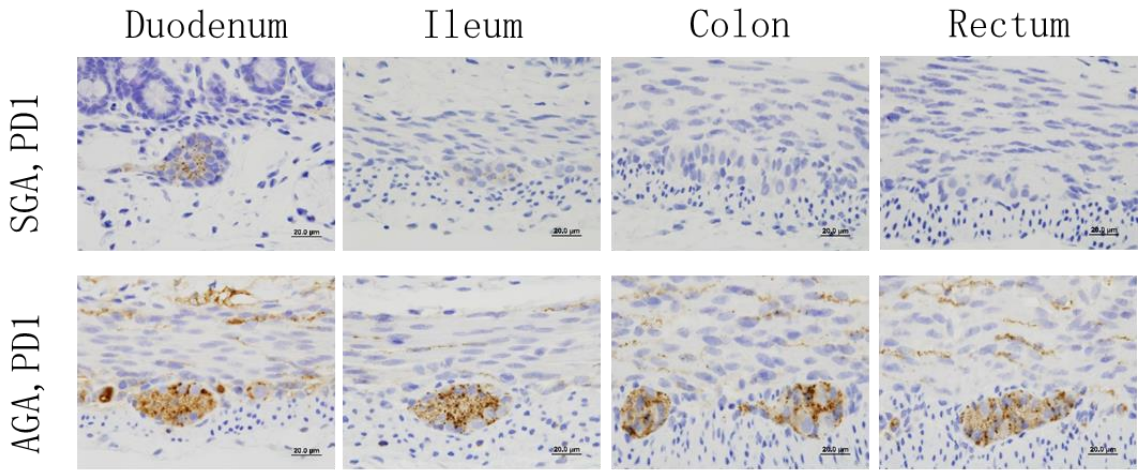

Figure 4. Synaptophysin IHC, synaptic system of SGA and AGA at PD1. On PD1 in AGA piglets, the synaptic system was relatively well developed in all gut sections. In SGA animals, the synaptic system was clearly less developed (less/no staining) particularly in the colon and rectum

Figure 5

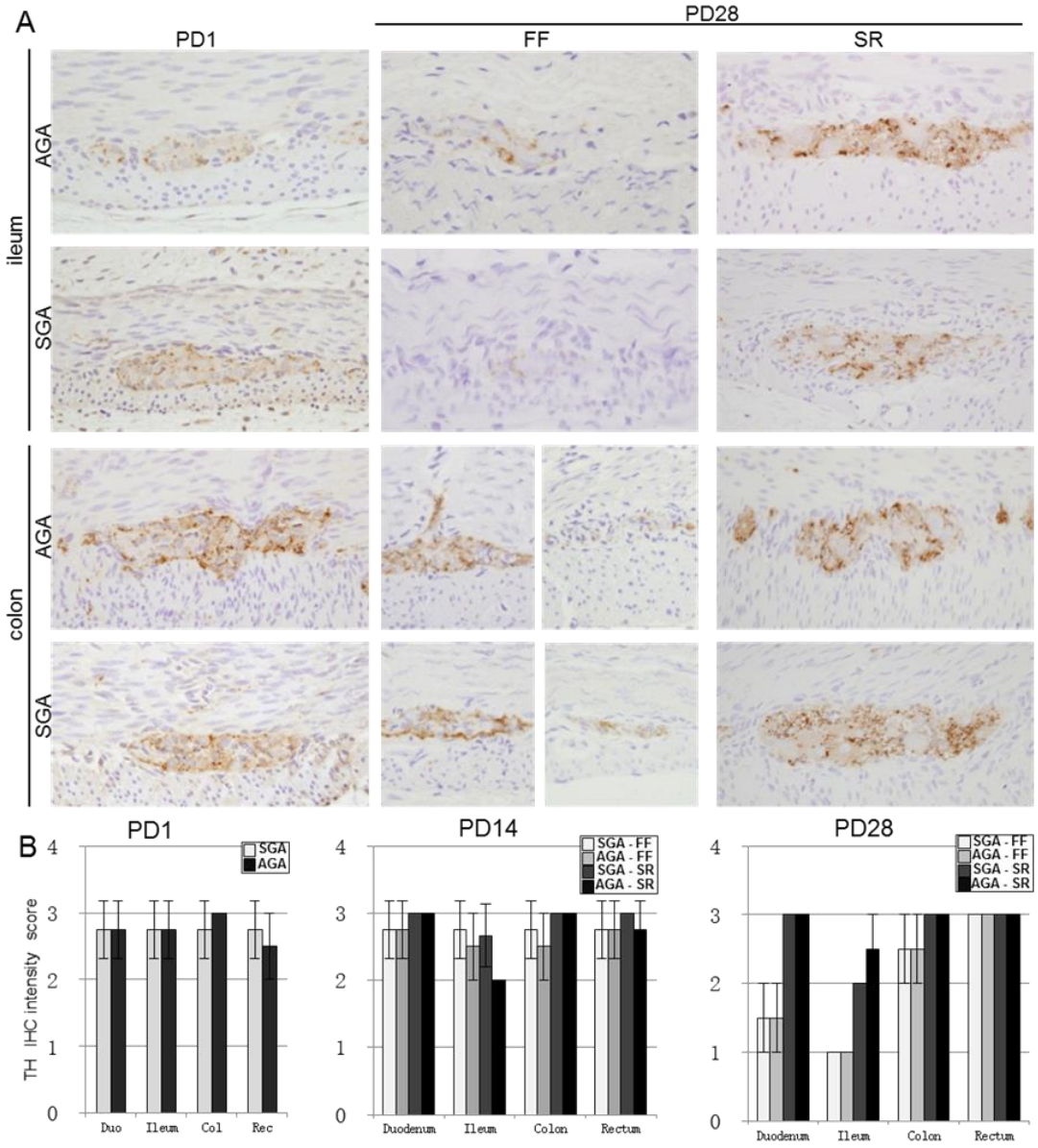

Figure 5. Tyrosine hydroxylase IHC, myenteric ganglia of the ileum and colon at PD1 and PD28. From PD1 to PD28 a slight increase in immunoreactive nerve terminal was observed in SR animals in the small intestine mainly. At PD28, SGA and AGA FF piglets presented with a lower immunoreactivity in the ileum and variable reactivity in the colon (A). Graphs (B) illustrate the intensity of labelling in each group.

Figure 6

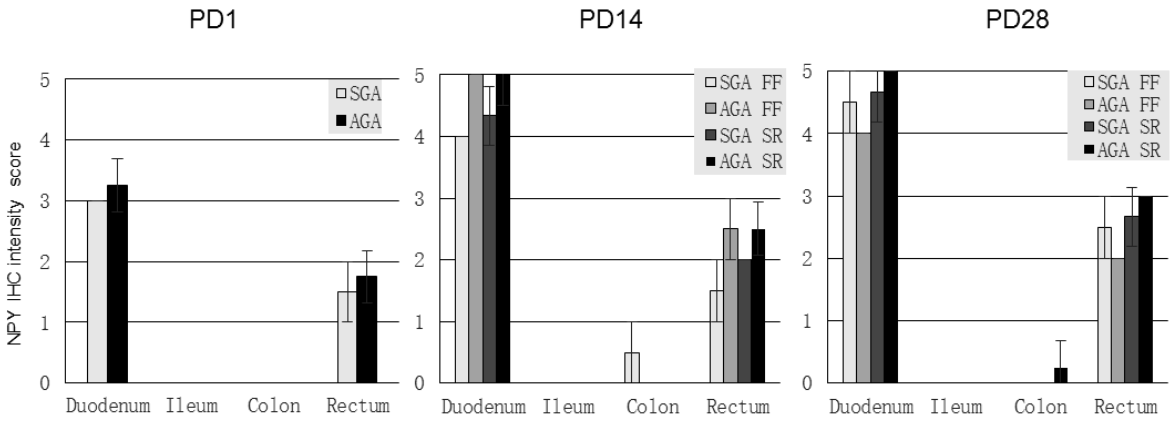

Figure 6. Neuropeptide Y IHC. The expression of NPY was increased in duodenum and rectum from PD1 to PD 28 regardless of food influence. In ileum and colon there were no/slightly staining of NPY. The expression of NPY was not significant different between SGA and AGA during the first month of life.

3.2 Development of the entero-endocrine system in a sow-reared or formula-fed small for gestational age piglet model.

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3.2.1 Abstract

Small for gestational age (SGA) infants are at risk of developing fatal necrotizing enterocolitis likely due to gut immaturity. A SGA piglet model was used to investigate the effects of birth weight and diet on the development of the enteric nervous system (ENS) and entero-endocrine system (EES).

Newborn SGA and average for gestational age (AGA) piglets were either sow-reared (SR) or formula-fed (FF). Gut samples were analyzed by immunohistochemistry using neuronal and EES markers at postnatal day (PD) 1, 14 and 28 to assess their respective development.
At PD1, marked lower number of nerve endings associated with an hypo-development of presynpatic vesicles was observed in SGA piglets. Up to PD14 included, the innervation of the colonic musculosa, evaluated by image analysis, was still under developed in SGA piglets. Across the study, the maturation of the EES was shown to be a highly dynamic process, influenced by the feeding strategy as 4 out of the 5 cells types analyzed were increased in number with the SR diet.

We identified for the first time that the ENS is less developed in SGA piglets, and the maturation of the EES was shown to be a diet-dependent dynamic process.

Key words: IUGR (intra-uterine growth retardation), pig, development, enterochromaffin cells, entero-endocrine cells.

3.2.2 Introduction

In human, intra-uterine growth retardation (IUGR) is observed in up to 24% of newborns every year in developing countries [163]. IUGR increases the risk of perinatal morbidity and mortality, may predispose patients to develop metabolic syndromes [176] [177] or brain disorders such cognitive abnormalities later in life [178]. IUGR can lead to premature birth or of birth of babies with a lower body weight that are called small for gestational age (SGA). Fetal growth retardation and premature births are particularly associated with an increased risk of intestinal problems; these infants frequently develop a delayed meconium passage, abdominal distension, delay in tolerating enteral feeding, and sometimes fatal necrotizing enterocolitis (NEC) [5, 179]. The vast majority of infants who develop the disease are born preterm, and the risk of developing this condition is inversely related to birthweight [161]. The condition remains not well understood but the immaturity of the gastro-intestinal tissue and immune system are likely important key factors. In particular, abnormal intestinal blood flow, gut motility, digestive ability, intestinal barrier function, and delayed immune system development are suspected to be key factors in the development of the disease [5, 7, 180-182].

When IUGR is caused by placental abnormalities or maternal disease, the growth aberration is usually the consequence of inadequate substrates for fetal metabolism and, to a greater or lesser degree, decreased oxygen availability [176]. In pigs, the natural variance that occurs in birth weights between piglets of the same litter, mostly due to decreased passage of adequate nutrition from sow to some piglets (likely due to uterine crowding), can be used to model IUGR [183]. Both humans and pigs are omnivorous, and present with similar gut development and physiology suggesting that pig is a good model to study the gut physiopathology, particularly for pediatric research [8, 184]. Even if some authors did not identify SGA piglets at risks of developing NECs [63], preterm piglets have been developed to study the neonatal physiopathology of NEC [42, 184]. In piglets, studies in IUGRs showed a delay in maturation of the intestinal mucosa, SGA animals presenting with smaller villi and crypts [185] and lower digestive capacities [46] [63].

Regarding the gut maturation little is known about the development of the enteroendocrine system (EES). The EES is the largest endocrine organ in the body [186, 187], and it is composed of isolated cells in the gastro-enteric epithelium and represents 1% of these cells. There are at least 14 to 16 different cell types that produce peptides and amines. The EES is an important key regulator of the gut functions including motility, absorption, secretion of enzymes, cell proliferation, mucus production, appetite, inflammation... [13].

It has been shown that nutritional interventions are important for gut maturation. Several studies have shown that prebiotics and probiotics, as well as some nutrients (e.g. oligosaccharides, gangliosides, lactoferrin, fibers and vitamins) influence the gut maturation in neonates by modulating mucosal epithelial barrier function and maturation, digestive capacities, local inflammatory response and susceptibility to pathogens [184, 188-192]. The goals of our study was to provide a description of the development of the EES using selected markers in SGA piglets compared to AGA piglets and evaluate the effects of a formula diet *versus* natural milk diet during the first month of life.

3.2.3 Material and methods

Animals, housing, and feeding

Littermate pairs of naturally farrowed, newborn piglets (*Sus scrofa domestica*), (AGA, mean 1.49 kg [1.47-1.52 kg]; SGA, mean 0.72 kg [0.58-0.9 kg]) were obtained from 5 separate litters from the University of Illinois swine herd. Sow reared (SR) piglets were cross-fostered and maintained with the sow throughout the study. Formula fed (FF) piglets were brought to the biomedical animal facility 48h after birth to allow for colostrum intake. On PD1, 14 and 28, four piglets per group (FF or SR, AGA or SGA) were sacrificed and duodenum, Ileum, ascending colon and rectum samples were fixed in 4% paraformaldehyde for 48h and embedded in paraffin, as described in our previous paper [164].

All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

Immunohistochemistry (IHC)

Histological sections (5µm) were dewaxed, rehydrated in water and then used for standard immunohistochemical analyses as previously described[165, 166] Briefly, each section was incubated with one of the primary antibodies [Chromogranin A (CgA, rabbit polyclonal, Immunostart 20086, mouse monoclonal Abcam ab80787); Serotonin (SER, mouse monoclonal, Abcam Ab16007); Somatostatin (SOM, rabbit polyclonal, Dako a0566); Neuropeptide Y (NPY, rabbit polyclonal, Abcam 30914), Neurotensin (NeuroT, rabbit polyclonal, Thermo 36128)]. An adequate secondary biotinylated antibody (Jackson Laboratory) and avidin–biotin–peroxidase complex system (ABC Vector Laboratories, Burlingame, CA, USA) were used to detect primary antibodies. Diaminobenzidine DAB (Vector) was used as chromogens. A slight counter-staining was done using aqueous hematoxylin. Nonspecific binding was controlled by omitting the primary antibody, and using other primary antibodies as isotypic controls.

For the analysis of the co-expression of different markers, fluorescent secondary antibodies were used (Jackson Laboratory, Alexa Fluor® 594-red Goat Anti-Rabbit IgG ref 111-585-003, Alexa Fluor® 488-green Goat Anti-Mouse IgG ref 115-545-146). For each double immunolabeling, PD1, PD14 and PD14 piglets were used, from both FF ans SR groups, and evaluation were made in at least 3 different areas per gut sections.

Morphological analyses

The quantification of EECs in the enteric mucosa was performed as follows: CgA and SOM (mean number of cells in 2x Obj10 fields); SER (mean number of cells in 3x Obj10 fields); NPY and NeuroT (total number of cells in 4 x Obj10 fields) for each gut sections for each animal.

The measurement of the large intestinal mucosa thickness and small intestine crypt length and villi height was performed using a Nanozoomer (Hamamastsu).

Statistical Analyses

Statistical analyses were performed using STATA 13.1, StataCorp LP, College Station, TX. The associations of body weight, number of the different types of enteroendocrine cells, with day of analysis, diet, size of piglets, site within the intestine, and thickness of the mucosa, including 2- and 3-way interactions, were examined by repeated measures ANOVA models. Post-hoc analyses were performed via pairwise comparisons of predictive margins with Bonferroni correction. Square root transformations (for SER, NeuroT, and CgA enteroendocrine cells) and logarithmic transformations (for body weight, NPY and SOM enteroendocrine cells) were necessary to provide normality and homogeneity of variance (homoscedasticity) of residuals, and both were assessed graphically and by the Shapiro–Wilk, and the Breusch–Pagan and Cook–Weisberg tests, respectively. Distribution and residual were considered as normal and homoscedastic, respectively, when $p>0.05$ for the Shapiro–Wilk and the Breusch–Pagan/Cook–Weisberg tests. Normality and homoscedasticity of residuals could not be achieved despite transformations for the ANOVA studying the association between body weight gain and day of analysis, diet, size of piglets, and site within the intestine. This ANOVA was therefore not performed, and only the associations between body weight gain and diet and size of piglets were examined by Mann–Whitney U-tests.

The last step in the analysis was to estimate the correlations between body weight, weight gain, and the number of the different types of enteroendocrine cells. Correlation were considered poor if <0.3, mild if 0.31-0.6, good if 0.61-0.8, excellent if >0.8.

3.2.4 Results

Body weights and body weight gains

Evolution of body weight from PD1 to PD28 was dependent on the size (SGA or AGA, p<0.001) and diet. The effect of diet was variable between PD14 and PD28 as shown by the significant interaction of Diet*Day (p<0.001). SGA piglets remained smaller than AGA piglets all throughout the study regardless of the diet (p<0.001). However, in both SGA and AGA piglets, body weight gain was influenced by diet (for both FF and SR, p<0.001). The magnitude of body weight gain was similar between small and average piglets in both FF and SR groups. SR piglets were growing faster than FF ones (figure 7B). On PD14, the body weight gain of FF piglets was lower compared to SR animals. However, FF piglets almost compensated for this difference on PD28 (Figure 7D).

Maturation of the enteroendocrine system

The number and the distribution of enteroendocrine cells in different gut sections were variable from PD1 to PD28. This was noticed for all markers studied (Figure 8).

Chromogranin A (CgA) is located in secretory vesicles in the dense core granules of some neurons, endocrine cells, as well as in most enteroendocrine cells [193].

Overall, there were more CgA cells in the small intestine than in large intestine all along the study. At PD1, AGA animals presented with slightly more CgA positive cells in the duodenum (p=0.035), ileum (p=0.01) and rectum (p=0.01) compared to SGA piglets. At PD14, SGA piglets still had less CgA cells in the duodenum (p=0.001). A diet effect was identified at PD14 in the duodenum only; FF piglets had more CgA positive cells in the duodenum compared to SR animals (p<0.001). At PD28, FF piglets presented with a marked decrease in cells number in the duodenum (p=0.012), but there was no change in the ileum (p=0.071, trend), colon (p=1) or rectum (p=1). Contrarily, in both SGA and AGA animals with SR diet, the number of CgA cells was rather stable from PD14 to PD28 in the ileum, colon and rectum (p=1 for the sites), except in the duodenum that presented with a slight increase in CgA cells (p=0.002).

The ***serotonin*** cell (SER) numbers of were significantly different among the different sites of the intestine independently of the day of analysis (P<0.001). On PD1, there were less cell in the duodenum compare to ileum and colon (p<0.001) but not rectum (p=1), and there was no

difference between SGA and AGA piglets. On PD14, FF piglets had more SER cells in the overall intestine (p=0.01) and on PD28, SR piglets had more SER cells in the gut (p<0.001). In fact, there were no significant overall changes in SER cell numbers in the gut of SR animals from PD14 to PD28 (p=1), but an overall decrease in cell number in FF animals (p<0.001). Compared to PD1, there was an increase in SER cell numbers in the duodenum at PD14 (p<0.001) and PD28 (p=0.001). Differently, from PD1 to PD14 and to PD28 there were less SER cell number in the ileum (p<0,001) colon (p<0,001), and rectum (p=0.003 and p<0.001, respectively). The size of piglet did not affect the number of SER cells at any day (p=0.06).

The number of ***somatostatin*** (SOM) positive cells was significantly different among the different sites of the intestine independent of the day of analysis (P<0.001). There was no difference in SOM positive cell number between SGA and AGA piglets on PD1 (p=0.4). On PD14, SGA piglets suddenly presented with less SOM cells in the duodenum compared to AGA piglets (p<0.001), and there were no more differences on PD28 (p=1). On PD14, there were no differences between FF and SR animals in any of the 4 gut sections (p=1 to p=0.055). Differently, on PD28, compared to FF animals, SR piglets presented with more SOM cells in the duodenum (p<0.001), but no change in cell number were noted in the ileum (p=1), colon (p=1) and rectum (p=1).

From PD1 to PD14, an increase in SOM cell number was noted in the duodenum of FF piglets (p<0.001) but there was no significant difference in SR piglets (p=1). From PD1 to PD28, an increase in SOM cell number was noted in the duodenum of SR piglets (p<0.001), but there were no more significant differences with FF piglets (p=1). In the Ileum, colon and rectum, a progressive decrease in SOM cell number were noted with both diets; indeed, from PD1 to PD14 a decrease in cell number was noted in the colon (p<0.001) and from PD1 to PD28 in the ileum (p<0.001), colon (p<0.001) and rectum (p=0.016).

Between PD14 to PD28, there was an increase in SOM cell number in the duodenum only of SR piglets (p=0.017), and a decrease in FF piglets in the duodenum (p=0.001) and ileum (p<0.001). There were no other significant changes in other gut sections with either diet (p=1 to p=0.108) from PD14 and PD28.

The number of ***neuropeptide Y*** (NPY) positive cells was significantly different among the different sites of the intestine independent of the day of analysis ($p<0.001$). There were more positive cells in the ileum and rectum compared to the other sites. At PD1, there were overall more NPY cells in AGA compare to SGA piglets ($p=0.002$). On PD14 there were no evidence of diet effect on NPY cell numbers ($P=1$). On PD28 there were significantly more NPY cells in SR animals than in FF piglets ($p<0.001$). From PD14 to PD28, there was an overall increase in NPY cell number in SR animals ($p=0.002$). In FF animals the variation of NPY cells in the gut was not statistically significant from PD14 to PD28 ($p=0.196$).

For ***Neurotensin*** (NeuroT), On PD1, there was no difference in cell number between SGA and AGA animals ($p=0.9$). There were significantly more cells in the ileum than in the duodenum ($p=0.001$) and more cells in the small intestine than in the large intestine ($p<0.001$). The same significant distribution was observed on PD14 and on PD28.

From PD1 to PD14 there were no differences in the duodenum ($p=0.4$), but a decrease in NeuroT cells was noted on PD28 ($p<0.001$). In the ileum, a slight decrease in NeuroT cells was observed from PD1 to PD14 ($p=0.025$) and this change was stabilized from PD14 to PD28 ($p=0.3$). From D1 to D28, there was an overall decrease in NeuroT cell number in AGA ($p=0.003$) but no change in SGA piglets ($p=1$).

Good to excellent correlations between CgA and SER ($r=0.72$), CgA and SOM ($r=0.90$), and SER and SOM ($r=0.77$) were observed. There were weak correlations between CgA and NeuroT ($r=0.31$), SER and NeuroT ($r=0.3$) and NeuroT and SOM ((r=20). NPY was not correlated at all to any other EEC markers.

Characterization of enteroendocrine cells on co-expression of markers

Co-expression of EEC markers was analyzed to better characterize the changes observed. Both monoclonal and polyclonal anti-CgA antibodies identified the same cell types in all gut sections (Fig. 10a). All SER cells expressed CgA in all part of the gut (Fig. 10b). All SER

cells also expressed SOM in the crypts of the small intestine (Fig. 11b) but only 50% of them co expressed both markers in the villi (Fig. 11a). None on the SER cells co-expressed NPY (Fig. 11c) or NeuroT (Fig. 11d). Approximately 65% of SOM cells also expressed CgA (Fig 10c). In the large intestine sections all NPY cells co-expressed CgA, but in the ileum, only 50% of the cells co expressed both markers (Fig. 10d). Similarly in the large intestine, all NeuroT cells expressed CgA but only 50% of these cells expressed it in the small intestine (Fig. 10e). There was no evidence of co-expression variability between PD1 and PD28, or any obvious diet effect as well for all these markers.

Development of the mucosa

To evaluate the potential influence of the development of the mucosa on the development EEC system, measurement of the small intestine crypt length and villi height, and large intestine mucosa thickness were included in the statistical analysis.

On PD1, there were no differences in villi length ($p=1$), crypt height ($p=1$) between SGA and AGA piglets. However, in the colonic and rectal mucosal, SGA animals presented with an thicker mucosa compared to AGA piglets ($p=0.005$).

On PD14, villi in the duodenum were longer in SR animals ($p=0.01$), but not in the ileum ($p=0.99$). This difference was maintained in PD28 in the duodenum ($p=0.01$). All along the study crypts size did not change in the ileum from PD1 to PD28 ($p=1$) but increased in size in de duodenum from PD1 to PD14 ($p=0.004$) and to PD28 ($p<0.001$) independently of the food. Similarly the thickness of the mucosa increased overtime in the colon and rectum from PD1 to PD28 in SR animals ($p=0.005$). Indeed, at PD14, SR animals had a thicker mucosa than FF animals ($p=0.002$) as well as PD28 ($p<0.001$).

Some weak correlations were noted between crypt size and SER ($r=0.35$), and crypt size and SOM ($r=0.45$). A possible contributing effect of the development of the large intestine mucosa on NPY cells number was noted, likely due to higher number of NPY cells in the rectum and thicker mucosa in the SR piglets at PD28. This effect was not observed in the colon (low number of NPY cells at D28, and thicker mucosa) and in the ileum (higher number of NPY

cells in SR piglets, but no food effect was observed in villi and crypt size). Overall no evident correlation was identified between the development of the mucosa (in the small and large intestine) and EEC type numbers.

3.2.5 Discussion

Gut endocrine system appear to follow a tissue specific constitutive differentiation but our results clearly show that this process is extremely dynamic and influenced by the diet/mode of nutrition.

Enteroendocrine cells express 14 to 16 different markers in the gastrointestinal tract in mammals that regulate most of the gut functions and regulate appetite [194]. In our study, only 5 markers have been studied. CgA that is located in secretory vesicles of some neurons and endocrine cells, and are part of the large dense core vesicles that contain SER, SOM, NeuroT among other products [194]. CgA is not only a constitutive protein of these vesicles, but it can be cleaved and form various biologically active peptides such as vasostatin-1, vasostatin-2, chromacin and 8 others peptides [195]. All these peptides regulate cardiovascular functions, immune system and tissue repair, calcium homeostasis, carbohydrate metabolism. In particular, vasostatins and chromacin have antibacterial and antifungic activities by penetrating through their membranes [196] [195]. 5-HT is synthesized, stored and released from a subset of enteroendocrine cells, called enterochromaffin cells in the intestinal mucosa, and is involved in gut motility, activation of inflammation, vomiting process, abdominal pain, nausea, cell proliferation and secretion [197] [198]. Differently, SOM is produce by some EEC and act primarily as an inhibiting factor balancing the effects of SER, cholecystokinin, gastrin, and decreasing cell proliferation [199]. NPY promote inflammation in the gut (activation of dendritic cells/ macrophages), potentiate the vasoconstriction effects of noradrenaline, inhibit motility and secretion, and have direct antimicrobial effects against various bacteria [199]. NeuroT regulate digestive process and appetite regulation, lipids assimilation, modulate gut motility, cell proliferation, secretion, and

is pro-inflammatory factor [199]. Other parts of the gastrointestinal tract as well as other marker such as cholecystokinin, gastrin and motilin could also have been studied in our experiments to complete the analysis. However, the selected markers used still represent important ones regulating many gut functions, and likely reflect important changes observed in our study.

On PD1, differences between SGA and AGA piglets have been identified, suggesting that SGA EEC system was not fully mature in SGA animals. For example SGA piglets presented with slightly less CgA cell number in the small and large intestines compared to AGA piglets. At PD14 SGA piglets still had less CgA cells in the duodenum. Similarly SGA piglets presented with less NPY cell number in the gut at PD1. Variation of the distribution of the EEC number is a dynamic process during the gestational and early postnatal periods in human [200] and uterine growth retardation may likely lead to a delay development as shown in our study.

The evolution of number and types of EECs in the gut seems to be a very dynamic process as shown in our study. This has been also reported in human fetuses and neonates; the number and types of EECs in the stomach dramatically change with marked variations between the first trimester of gestation and the early post-natal period [200]; this is likely a general feature in animals as a similar observation was also reported in fish [201]. EES is also likely more adaptive than previously believed, indeed, in humans with different types of bariatric surgical procedures to treat obesity, significant changes in ghrelin, peptide YY and glucagon‑like peptide 1 occur [202]. The distribution and proportion of EECs is also likely influenced by the diet as shown in our study. Similarly in the rat, it has been demonstrated that diet and microflora can influence SER cells distribution [203], as well as proglucagon producing EEC number [204]. In our study, significant variations in number of CgA, 5-HT, SOM and NPY cells were noted between FF and SR piglets. Interestingly, the more significant changes were observed in the duodenum for CgA, 5-HT and SOM, and in the ileum and rectum for NPY. The diet effect is likely playing an important role, however, as our piglets were raised in different environment, the microbiome of SR piglets was likely to be more diverse compared to FF animals that were in housed in a clean room. It has been demonstrated that spore forming bacteria from the mouse and human microbiota promote de novo synthesis of

5-HT by EEC, and increase the number of 5-HT cells in the colonic mucosa, modifying gut motility and plasma level of 5-HT [205]. Interestingly, these changes were observed in the colon only but not in the small intestine. In our study, the number of 5-HT cell number was higher in both small and large intestine sections in SR piglets suggesting that adaptation in 5-HT cell number can be more variable and that other type of bacteria and/or milk factors might also contribute to these changes. Similarly, an increase in gastrin cell number was observed in mice exposed to proton pump inhibitor omeprazole showing the potential for adaptation of the EEC types in the gastrointestinal tract [206]. In addition, in our experiment stress and anxiety due to separation from the sow might also have contributed to the change observed in the FF groups.

The correlations observed between CgA, 5-HT and SOM markers were partially due to co-expression of these markers by the same EECs, but not only. Indeed all 5-HT cells were CgA positive, but approximately 65% of SOM cells also expressed CgA. In addition even if all 5-HT cells co-expressed SOM, a subpopulation of SOM cells did not express 5-HT, especially in the villi of the small intestine. This might suggest that the highest expression of SOM was compensating the highest expression of 5-HT in SR animals at PD28. Indeed, there was a possible regulating physiological response as these two hormones are antagonist. 5-HT is a major factor involved in motility, secretory and vasodilator reflexes under physiological conditions, and acts as a proinflammatory mediator in pathological conditions [207]. By opposition, SOM is largely inhibitory, decreasing endocrine and exocrine secretions, gut motility and inhibiting cell proliferation and secretions of most gastrointestinal hormones [13, 208]. These data also show that individual EEC cells can indeed express multiple hormones as previously demonstrated [111] [209]

Entero endocrine progenitor cells (from the same lineage) in the crypts of the mucosa are also capable to segregated into more or less specialized cell types expressing one or two or more peptide hormones [111]

Unexpectedly, the co-expression of CgA/NPY and CgA/NeuroT was different in the large and small intestine. It is possible that different EEC type expressing other secretory granules (i.e. CgB or other secretogranins) also produce NPY or NeuroT in the small intestine, or a switch may also occur during the maturation of the EECs in the small intestine. These data show that individual EEC cells can indeed express multiple hormones.

It has been suggested that human and swine present with similar gut development and physiology suggesting that the pig is a good model to study the gut physiopathology, particularly for pediatric research [8, 184]. Our data also reinforce this concept as the distribution of 5-HT, CgA, SOM and NeuroT on PD28 was very similar to the data observed in humans [194], reinforcing the concept that gut endocrine system appear to follow a tissue specific constitutive differentiation.

The presence of NPY EEC is not reported in the gut to our knowledge; NPY expression is described in neuroendocrine pancreatic cells in neonates but not in adults [210], and a similar early life transitory expression of NPY is possible in the small intestine of piglets. A cross reactivity with peptide YY, that is a closely related anti-secretory hormone, is also conceivable in our study, although there is very low chance of cross reactivity according to the supplier of the antibody we used. The presence of NPY EEC in the intestinal mucosa of our piglets needs then to be confirmed by other methods.

Regarding the development of the mucosa, there were no differences in crypt length or villi height between SGA and AGA, but interestingly SGA animals presented with a thicker mucosa in the large intestine compared to AGA piglets. Some authors reported very similar results on crypt and villi size in the small intestine of SGA and AGA piglets at birth [63], but other authors have shown that duodenal height of villi was significantly higher in AGA animals [40]. These differences might be due to genetic factors (e.g. different breeds) but more likely to an abnormal mucosal maturation that is observable later as reported by Che *et al* that described differences in villi and crypt sizes on day 2 of suckling [63].

CgA is a marker of secretory granules that is expressed in some neurons and many endocrine cells in the body [193]. Regarding EECs, CgA is expressed by many cell types including 5-HT, SOM, NeuroT cells but not by NPY cells [194]. Strong correlations between CgA, 5-HT and SOM cells were noted in this study. This is likely due to the fact that 5-HT and SOM producing cells express CgA but also because of the similar evolution of cell number in different sections of the GI tract along the study. The marked correlation between 5-HT and SOM positive cell numbers probably reflect the interactions between these two antagonist hormones. Indeed 5-HT is a major factor involved in motility, secretory and vasodilator reflexes under physiological conditions, and acts as a proinflammatory mediator in pathological conditions [207]. By opposition, SOM is largely inhibitory, decreasing endocrine and exocrine secretions, gut motility and inhibiting cell proliferation and secretions of most gastrointestinal hormones [13, 208]. So the similar evolution of the number of SOM cells and 5-HT cells is possibly due to a balanced regulatory phenomenon. Differently, the distribution of NeuroT producing cells in the intestine, which also express CgA, seems to be much less dependent of diet; a diet effect was only observed in the duodenum (More NeuroT cells in SR animals, trend). In the early post-natal life, both duodenum and ileum produce NeuroT but on PD28 the ileum was the main site of production as described in human adults [194]. Many other markers (e.g. Cholecystokinin) can also be analyzed to better characterize the maturation of the EEC system.

In conclusion, the number and distribution of EEC types appear to be very dynamic during the first month of life and can be influenced by of the feeding strategy likely influencing gastrointestinal main functions.

Statement of financial support: This work was supported by the University of Illinois Center for Nutrition, Learning and Memory and HD069899.

3.2.6 Figures

Figure 7

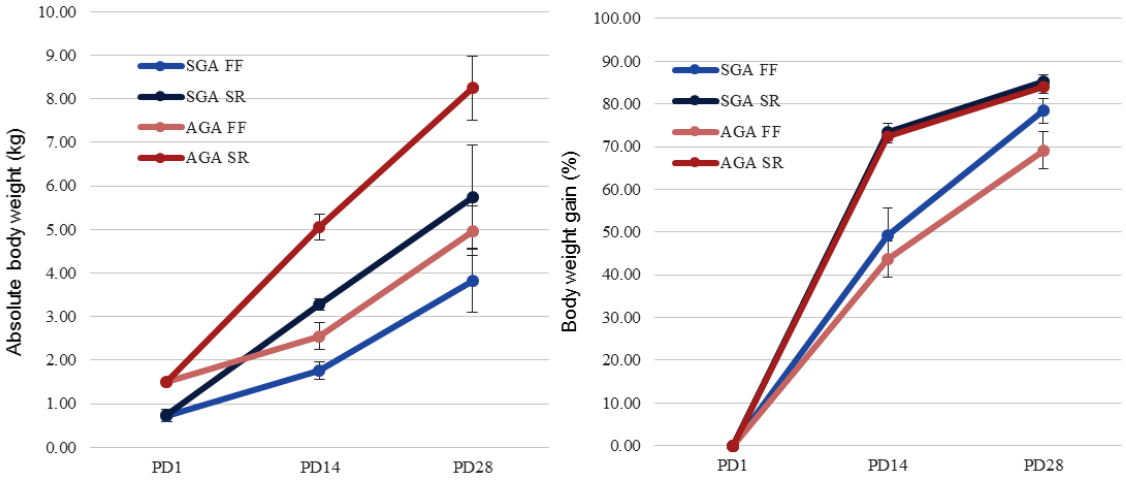

Figure 7. Absolute Body weight (A) and body weight gains (%) (B) of sow reared (SR) and formula fed (FF) SGA and AGA piglets at PD1, PD14 and PD28.

Figure 8

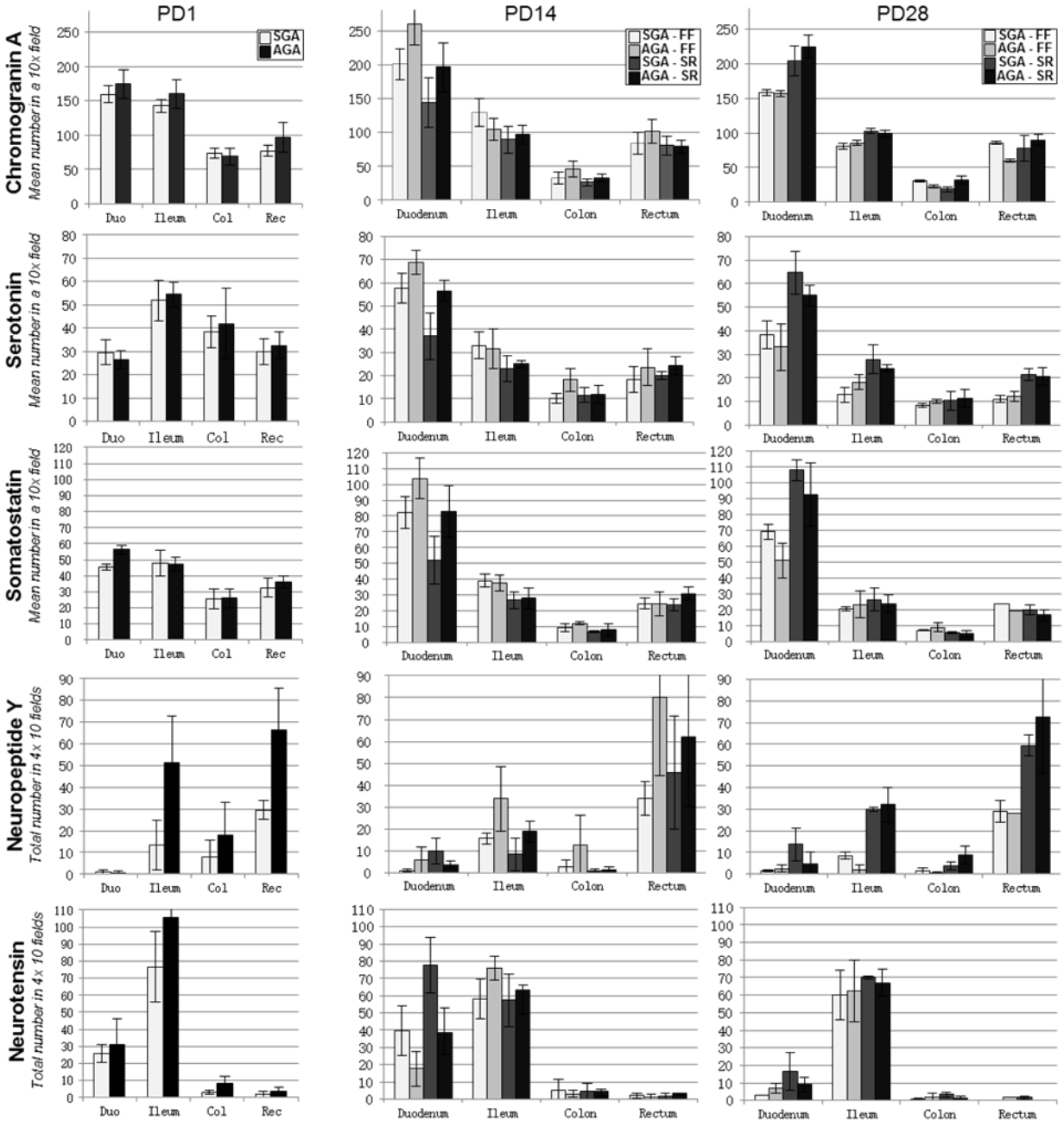

Figure 8. Enteroendocrine cells marker IHC results (duodenum, ileum, colon and rectum) at PD1, 14 and 28. For chromogranin A, Serotonin, and Somatostatin histograms illustrate the mean number of positive cells per 10x field. For Neuropeptide Y and Neurotensin, histograms illustrate the total number of positive cells in 4 fields.

Figure 9

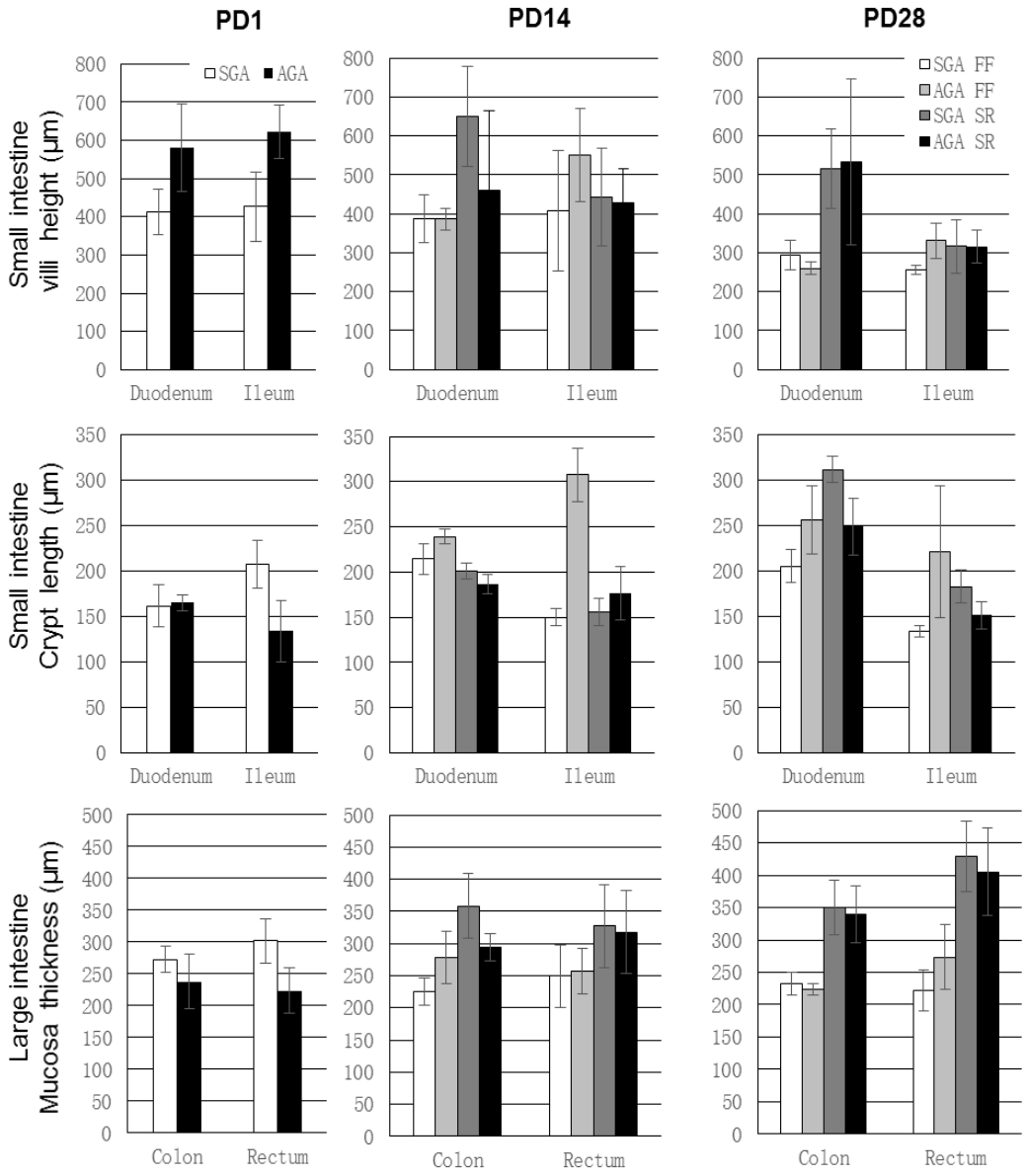

Figure 9. Development of the mucosa. Evaluation of the villi length (first raw), crypt length (second raw) and large intestine mucosal thickness (third raw)

Figure 10

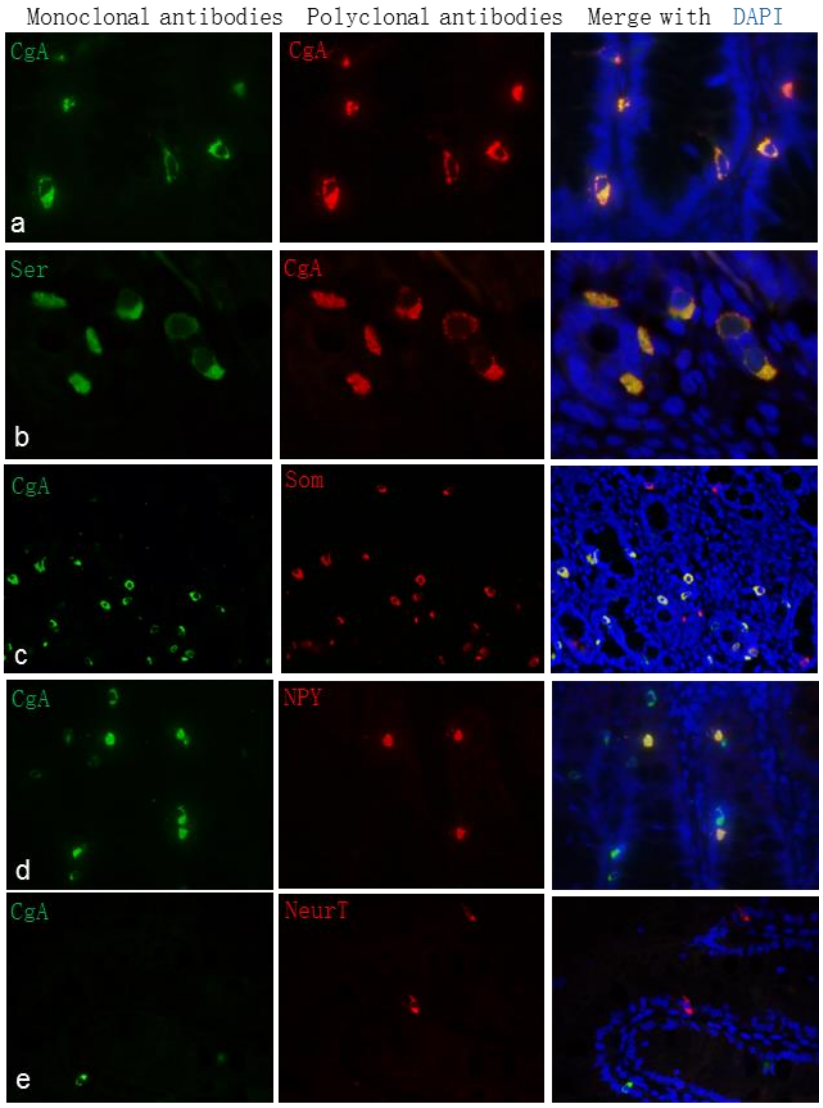

Figure 10. Imunofluorescence. Evaluation of the expression of CgA in SER, SOM, NPY and NeurT cells.

Figure 11

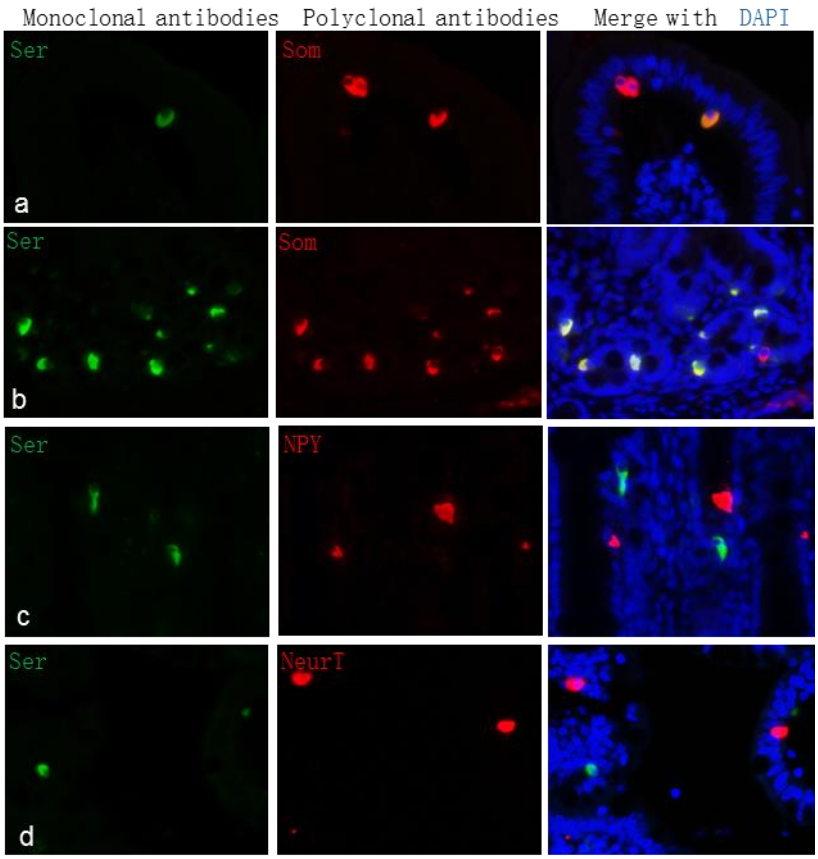

Figure 11. Imunofluorescence. Evaluation of the co-expression of SER and SOM, NPY and NeuroT.

3.3 Appendix

Antibodies	Expression
Chromogranin A (Immunostart 20086) Rabbit	EECs
Doublecortin (Ab18723) Rabbit	Nerve fibers
Enkephalin (Chemicon MAB350) Mouse monoclonal	Neurons
GFAP (Dako Z0334) Rabbit	Glial cells
Iba1 (Ab5076) Goat	Microglial cells
Peripherin (Ab4666) Rabbit polyclonal	Nervous system
Neuropeptid Y (Ab30914) Rabbit monoclonal	EECs, sympathetic nervous system
Neurotensin (Thermo 36128) Rabbit	EECs
Serotonin (Ab16007) Mouse monoclonal	EECs
Somatostatin (Dako a0566) Rabbit	EECs
Synaptophysin (Dako sy38) Mouse monoclonal	Synapses
Tubulin BIII (Promega G7121) Mouse monoclonal	Nervous system
Tyrosine hydroxylase (Ab112) Rabbit	Sympathetic nervous system
NeuN (Ab177487) Rabbit	Neurons

Appendix

Meeting: Experimental Biology (EB)

Date: April 26-30, 2014

Location: San Diego, CA

Title: Development of the ENS and EES in SGA and AGA piglets during the first month of life

Abstract

In humans, fetal growth retardation is associated with an increased risk of intestinal problems (e.g. delayed meconium passage, abdominal distension, delay in tolerating enteral feeding, and necrotizing enterocolitis). These conditions may be related to deficits in gut development. A novel piglet model was developed to investigate the effects of birth weight and diet on the development of the enteric nervous system (ENS) and neuroendocrine system (serotonin, neurotensin, neuropeptide Y) via multivariate analyses. Newborn SGA or AGA piglets were randomized into two diet groups: sow reared (SR, 24h/day with the sow) or formula fed (FF, milk replacer) (n=4/group). Gut samples were collected at postnatal day (PD)1, 14 and 28, and used for immunohistochemistry. Along the study, innervation of the colon in SGA piglets was less developed compared to AGA piglets (p=0.007), except at D28. Serotonin and neuropeptide Y secreting cell numbers increased overtime with SR diet (p=0.0004 and p=0.004, respectively). The distribution of neurotensin cell numbers in different gut levels were highly variable overtime (p=0.0002). In conclusion, birth weight and diet influence ENS/neuroendocrine system development. This model may be useful to study intestinal maturation in low birth weight infants.

Development of the ENS* and EES* in SGA** and AGA** piglets during the first month of life

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*ENS : enteric nervous system, EES : entero-endocrone system

**SGA : small for gestational age, AGA : average for gestational age

Abstract

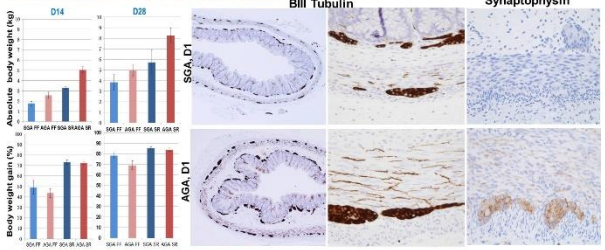
Program Number: 1017.1

In humans, fetal growth retardation is associated with an increased risk of intestinal problems (e.g. delayed meconium passage, abdominal distension, delay in tolerating enteral feeding, and necrotizing enterocolitis). These conditions may be related to deficits in gut development. A novel piglet model was developed to investigate the effects of birth weight and diet on the development of the enteric nervous system (ENS) and neuroendocrine system (serotonin, neurotensin, neuropeptide Y) via multivariate analyses. Newborn SGA or AGA piglets were randomized into two diet groups: sow reared (SR, 24h/day with the sow) or formula fed (FF, milk replacer) (n=4/group). Gut samples were collected at day (D) 1, 14 and 28, and used for immunohistochemistry. Along the study, innervation of the colon in SGA piglets was less developed compared to AGA piglets (p=0.007), except at D28. Serotonin and neuropeptide Y secreting cell numbers increased overtime with SR diet (p=0.0004 and p=0.004, respectively). The distribution of neurotensin cell numbers in different gut levels were highly variable overtime (p=0.0002). In conclusion, birth weight and diet influence ENS/neuroendocrine system development. This model may be useful to study intestinal maturation in low birth weight infants.

Results

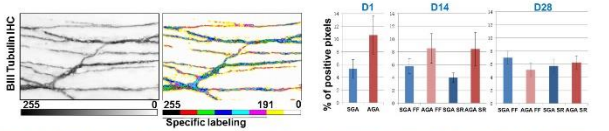
1. Body weights and body weight gain

SGA piglets remained smaller than AGA piglets all along the study regardless of the food. However, in both SGA and AGA piglets, body weight gains was influenced by the food. The magnitude of body weight gain was similar between SGA and AGA in both FF and SR groups. On D14, the body weight gain of FF piglets was lower compared to SR animals. However, FF piglets almost compensated for this difference on D28.

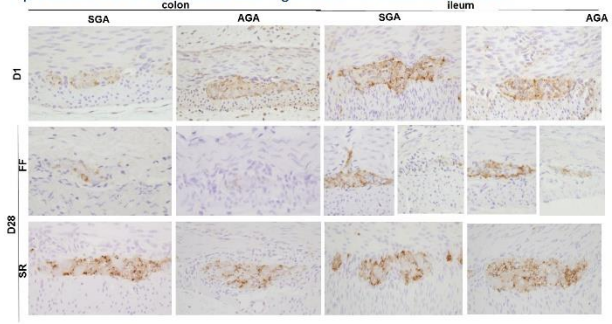


2. Development of the ENS

- Nerve cells bodies forming muscular and submucosal plexuses and all nerve fibers innervating the mucosa and muscular layers were identified using B III Tubulin IHC. On D1, the ENS of AGA was already fully developed compared to D14 and D28. In SGA piglets, the development of terminals in the musculosa and mucosa were delayed. But by D28, there was no more difference between SGA and AGA piglets.
- Evaluation of the percentage of positive pixels in the muscular layer of the colon. Once transformed into an 8 bit grey scale, each image was changed into an 8-color scale. The specific labeling was associated to pixels numbers 0 to 191 (corresponding to the violet color). (B) The percentage of positive pixels was calculated for each area and the mean reported in histograms for D1, 14 and 28.



- SYN marker was used to identify the presynaptic vesicles. On D1, for AGA piglets, the synaptic system was relatively well developed. But for SGA piglets, the synaptic system was less clearly less developed.
- The sympathetic noradrenergic innervation of the gut was investigated using an anti-TH(TH) antibody. On D1, the intensity of the IHC labeling was similar between SGA and AGA piglets. On D28 only, both FF SGA and AGA piglets presented with a less intense labeling in the small intestine and colon.

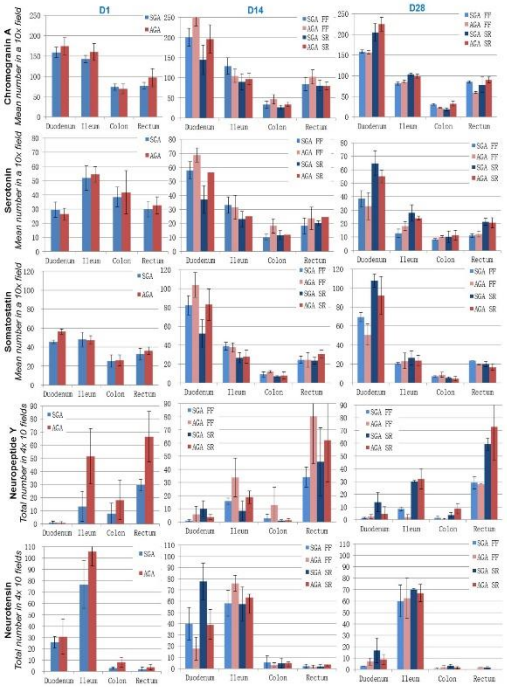


3. Maturation of the EES

The entero-endocrine system (EES) is the largest organ in the body. The enter-endocrine system is composed of isolated cells in the gastro-enteric epithelium and represents 1% of these cells. It is composed of different cell types that produce peptides and amines. EECs play important roles in gut function.
The number and distribution of enteroendocrine cells in different gut sections were variable from D1 to D28.

- **Chromogranin A (CgA)** cells was different among the different sites of the intestine independently of the day of analysis. The number of CgA cells decreased between D14 and D28 in FF piglets and increased in SR animals.
- **Serotonin (SER)** : From D14 to D28, an overall decrease in SER cells was noted in FF animals, and increase in number in SR piglets.
- **Somatostatin (SOM)**: there was a change in the distribution of somatostatinergic cells from D1 to D28 (P=0.012). And this change tended to be different between FF and SR animals.
- **Neurotensin (NeurT)**: there was a variation in the number of positive cells at D1, D14 and D28, but this variation was different among the sites (Day*Site: P<0.001). The type of feeding influenced the number of NeurT Cells at D14 and D28 (P=0.014).
- **Neuropeptide Y(NPY)**:The distribution of positive cells was influenced by the food. FF animal had more positive cells at D14, while SR piglets had more positive cells at D28.

- Excellent **correlations** between CgA and SER (r=0.8), CgA and SOM (r=0.92), and SER and SOM (r=0.84) were observed.



Conclusion

- There is a delay in maturation of the ENS in SGA piglets(i.e. lower number or density if nerve fibers in the muscularis). This delayed ENS maturation in SGA piglets probably reflects a general immaturity of the nervous system. ENS is one of the key regulators of the gut functions. Thus the undeveloped ENS could be an important factor for many gastrointestinal diseases (i.e. necrotizing enterocolitis).
- The number and distribution of EEC types are dynamic and can be influenced by feeding strategy. And the dynamic evolution of number and types of EECs in the gut has also been reported in human fetuses and neonates. The distribution and proportion of EECs probably also evolve during and after the weaning period and during adulthood.

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