INVESTIGATION INTO THE MOLECULAR AND PHYSIOLOGIC RELATIONSHIP BETWEEN PEPTIDE TYROSINE TYROSINE AND N-ACETYLGLUTAMATE SYNTHASE

By

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This work is dedicated to my family.

To my husband, Joe for his
steadfast love and support

and

To my children, Audrey and Eli who
bring much joy to my life

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LIST OF ABBREVIATIONS

ARG1	arginase 1
ASL	argininosuccinate lyase
ASS1	argininosuccinate synthase
ATP	adenosine triphosphate
BLAT	Blast-Like Alignment Tool
BMI	body mass index
bp	base pair
cDNA	complementary deoxyribonucleic acid
C/EBPβ	ccaat-enhancer binding protein β
CPS1	carbamoyl-phosphate synthase 1
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2
DNA	deoxyribonucleic acid
DPPIV	dipeptidyl peptidase IV
fg	femtograms
GAPDH	glyceraldehyde phosphate dehydrogenase
GSP	gene specific primer
HNF-4	hepatic nuclear factor 4
NAG	n-acetylglutamate
NAGS	n-acetylglutamate synthase
NCBI	National Center for Biotechnology Information

NH_4^+	ammonia
nm	nanometers
NPY	neuropeptide Y
ORNT1	ornithine transporter: SLC25A15 (mitochondrial carrier)
OTC	ornithine transcarbamylase
OTCD	ornithine transcarbamylase deficiency
PBS	phosphate-buffered saline
pg	picograms
pge	picogram equivalents
PKU	phenylketonuria
RACE	Rapid Amplification of cDNA Ends
RNA	ribonucleic acid
RT	Reverse transcription
PCR	Polymerase chain reaction
PP	pancreatic polypeptide
PYY	peptide tyrosine (Y) tyrosine (Y) or peptide YY
qRT-PCR	quantitative real time reverse transcription – polymerase chain reaction
TSS(s)	transcription start site(s)
UCD(s)	urea cycle disorder(s)
UCSC	University of California Santa Cruz
UTR	untranslated region

CHAPTER I

OVERVIEW

The size of a genome and the number of genes in it are not indicators of organism complexity. Mechanisms of gene regulation likely play an important role in both the relative complexity of organisms and in generating genetic diversity. Coordinate regulation of genes is one mechanism by which this could occur. Bidirectional promoters provide a unique mechanism of coordinate gene regulation. Until recently they were thought to be rare in mammals; however *in silico* evidence suggests that a class of bidirectional promoters exists within the human genome.

Bidirectional promoters offer a mechanism to coordinate gene expression for genes whose products function cooperatively in the same complex or within a common pathway. However, a significant number of bidirectional gene pairs do not display obvious functional homology. In humans, the non-homologous gene pair N-acetylglutamate synthase (*NAGS*) and peptide tyrosine tyrosine (*PYY*) are divergently transcribed and separated by less than 200 base pairs (bp) consistent with regulation by a bidirectional promoter.

NAGS plays a critical role in nitrogen metabolism supplying the essential co-factor for the first enzyme of the urea cycle which is the primary means of ammonia detoxification in ureotelic organisms. The loss of NAGS function

results in an inability to convert ammonia to urea, leading to a build-up of this toxic molecule. If left untreated, hyperammonemia quickly leads to death.

PYY is secreted by the endocrine L cells of the distal small intestine and colon. It inhibits gastric acid secretion, delays gastric emptying and slows gut motility. It also functions in appetite regulation, inducing feelings of satiety postprandially. Consequently, research on PYY has focused on its potential as an anti-obesity target.

The regulation of this gene pair has not been examined previously. The aim of this work is to investigate both the molecular and physiologic relationship of *NAGS* and *PYY*. Specifically, I hypothesize that *NAGS* and *PYY* are coordinately regulated via common regulatory elements in their shared 5' flanking region. Further, I propose that the coordinate regulation of this gene pair is no coincidence, and there exists an important functional connection between NAGS, PYY, and nitrogen balance.

The following chapters detail my investigation into relationship between *NAGS* and *PYY*. Chapter II provides background on *NAGS* and its role in the urea cycle, the multiple functions of the neuropeptide *PYY*, and the potential for coordinate regulation of this gene pair. Finally, the hypothesis and specific aims are outlined at the end of this chapter.

Chapter III describes the 5' structure of *NAGS* and *PYY* which was determined using multiple experimental approaches including: 5'RACE, non-quantitative RT-PCR and quantitative real time RT-PCR (qRT-PCR). The 5' untranslated region (UTR) of *NAGS* which was previously undefined is revealed

in this chapter. Four potential TSSs exhibiting tissue-specific expression were identified for *NAGS*. Two major TSSs were identified for *PYY*. The data reveal tissue-specific expression patterns for these *NAGS* and *PYY* transcripts suggestive of regulation by alternative promoters.

If *NAGS* and *PYY* are coordinately regulated then the expectation is that they will be concordantly expressed in at least some tissues. To establish if *NAGS* and *PYY* are expressed similarly in human tissues, we measured their expression in a panel of normal human tissues using quantitative real time RT-PCR. Results from these experiments, presented in Chapter IV, demonstrate that if *NAGS* and *PYY* are coordinately regulated it occurs in a tissue-specific manner in the ileum and pancreas. Also summarized in Chapter IV are my efforts to identify an appropriate cell line to further investigate the regulation of this gene pair.

To examine the regulation of *Nags* and *Pyy in vivo* we measured expression of these genes in tissues of mice under different feeding conditions to determine if expression of both genes is increased or decreased under each condition. These experiments and their results are discussed in Chapter V.

Next, I examined the physiologic relationship between PYY and NAGS. To this end, plasma PYY levels were measured in urea cycle disorder patients and another group with an inborn error of amino acid metabolism, phenylketonuria patients. The data demonstrate that PYY levels are increased in both patient populations. A proposed functional relationship that links co-regulation of *PYY* and *NAGS* to the maintenance of nitrogen balance is

discussed. Lastly, Chapter VII summarizes the work presented in this dissertation and highlights future directions to further elucidate the relationship between *PYY* and *NAGS* and its potential link to nitrogen balance.

CHAPTER II

INTRODUCTION

NAGS and the urea cycle

Proteins are essential components of all living things, and therefore it is quite fitting the word protein was derived from the Greek *proteios* meaning ‘of the first rank’. Proteins have a remarkable number of physiological roles, serving as structural components, transporters, antibodies, enzymes, hormones, receptors, and more. Proteins are comprised of polypeptides which consist of linear sequences of amino acids. Thus, amino acids are the building blocks of proteins.

Amino acids are critical not only for synthesis of new proteins, but are also important for the production of a variety of other nitrogen-containing compounds including nucleotides, glutathione, and several neurotransmitters. Additionally, while not a primary source they are used for energy production, with approximately 10% of total energy coming from amino acids. In cases where not enough energy is supplied by diet, endogenous protein stores are broken down and the constituent amino acids can be used in energy production.

The primary sources of amino acids in the body are from consumption of dietary protein and the breakdown of endogenous proteins. When dietary protein is consumed it is broken down in the digestive tract to its constituent parts, and is absorbed primarily as dipeptides, tripeptides, and single amino acids. When amino acids are in excess they are catabolized. The first step in oxidation of

amino acids is deamination, or the removal of the nitrogen-containing amino group. The carbon skeletons are degraded into a number of molecules that are subsequently used in the synthesis of fatty acids and glucose or in the production of energy. The waste nitrogen from the amino group is in the form of ammonia which is toxic, and therefore must be eliminated. The way in which ammonia is detoxified varies in different organisms. Teleost fish primarily excrete ammonia through their gills, while birds and reptiles convert ammonia to uric acid. Mammals and amphibians detoxify ammonia via the urea cycle.

First described by Sir Hans Krebs in 1932, the urea cycle is a biochemical process of ammonia detoxification. Waste nitrogen generated in the metabolism of protein is converted into urea which is water-soluble and can be easily excreted as waste (Figure 2.1) (Krebs and Henseleit 1932; Rezvani 2007). Five primary enzymes and two transporters make up the cycle. The first steps of the cycle occur in the mitochondria and the remaining steps take place in the cytoplasm. First, carbamoyl phosphate synthetase 1 (CPS1) synthesizes carbamoyl phosphate from ammonia (NH_4^+) and bicarbonate (HCO_3^-). Next, carbamoyl phosphate combines with ornithine to form citrulline, a reaction catalyzed by ornithine transcarbamylase (OTC). Citrulline is then exported out of the mitochondria via the ornithine transporter (ORNT1), where it reacts with aspartate to form argininosuccinate, a reaction catalyzed by argininosuccinate synthetase (ASS1). The mitochondrial aspartate/glutamate transporter, citrin, is responsible for transporting aspartate out of the mitochondria for this reaction. Next, argininosuccinate lyase (ASL) cleaves argininosuccinate to form arginine

and fumarate. Finally, arginase (ARG1) cleaves arginine, producing ornithine and urea. Ornithine is transported back into the mitochondria via the ORNT1 transporter to be used in subsequent rounds of ureagenesis.

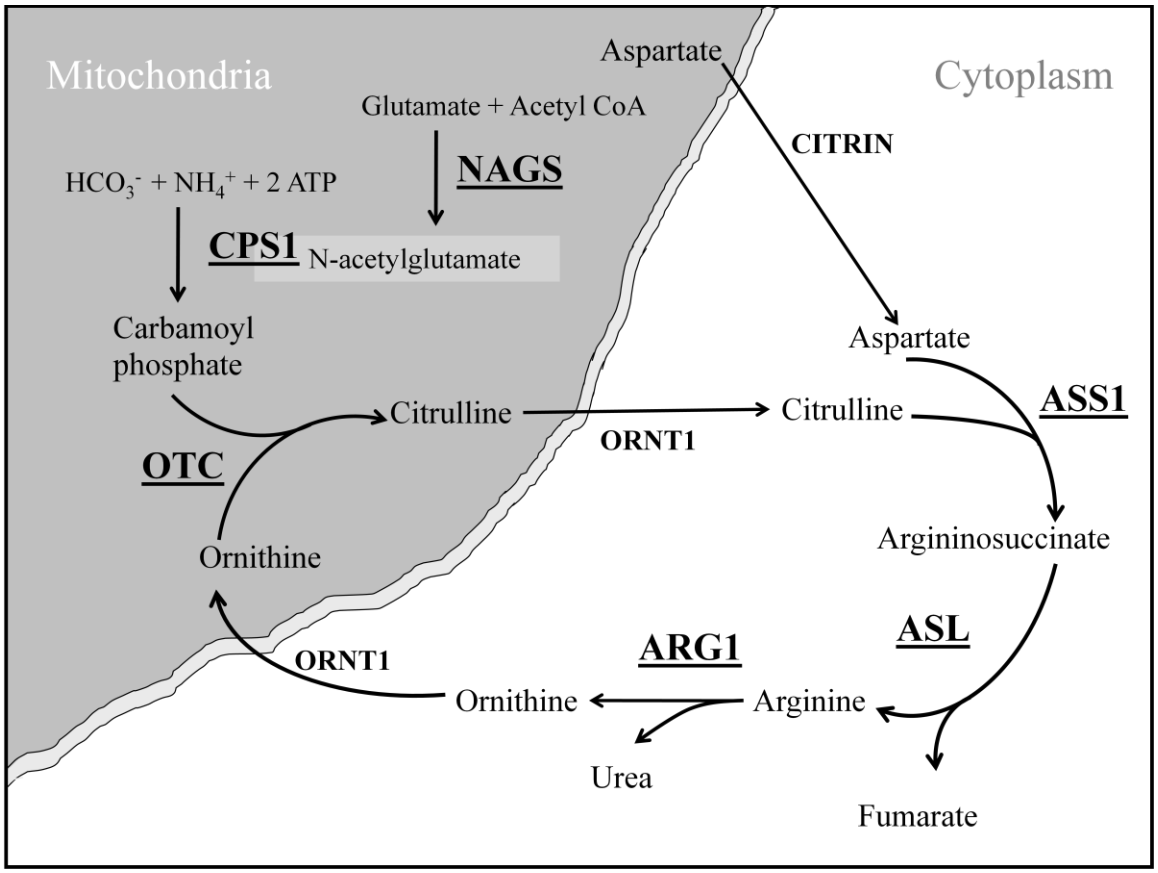


Figure 2.1: The urea cycle. Ammonia is converted to urea in a series of biochemical steps known as the urea cycle. The initial steps take place in the mitochondria and the latter steps occur in the cytosol. NAGS = N-acetylglutamate synthase, CPS1 = carbamoyl phosphate synthetase 1, OTC = ornithine transcarbamylase, ASS1 = argininosuccinate synthetase, ASL = argininosuccinate lyase, ARG1 = arginase 1, ORNT1 = ornithine/citrulline transporter, citrin = aspartate/glutamate transporter

The urea produced from this series of biochemical reactions is either excreted or taken up by the colon to be recycled. Urea travels in the blood from the liver to the kidneys where it can be excreted as waste in urine. However, nearly 25% of the urea passes into the intestinal lumen of the colon where it is hydrolyzed by bacterial ureases to ammonia and carbon dioxide. Studies demonstrate that approximately 80% of this ammonia is recycled and the remaining 20% is again converted to urea. This process is referred to as urea nitrogen salvage. Both the urea cycle and the salvage pathway contribute to the maintenance of nitrogen balance.

As described above, CPS1 catalyzes the first and rate-limiting step of the urea cycle. Activity of CPS1 requires the allosteric activator N-acetylglutamate (NAG). NAG binds CPS1, inducing a conformational change in the enzyme revealing the active site (Colombo, Pfister, and Cervantes 1990). NAG is synthesized from glutamate and acetyl-CoA in a reaction catalyzed by the enzyme NAGS (Figure 2.2), making NAGS necessary for urea synthesis.

Figure 2.2: The synthesis of N-acetylglutamate (NAG). NAG is synthesized from glutamate and acetylCoA in a reaction catalyzed by NAGS

Deficiencies in any of the primary urea cycle enzymes (CPS1, OTC, ASS1, ASL, and ARG1) or NAGS result in impaired nitrogen metabolism and

together, are known as urea cycle disorders (UCDs). The incidence of each specific disorder is rare, but collectively UCDs have an estimated prevalence of 1 in 30,000 live births (Rezvani 2007). This may be an underestimate as late onset cases caused by partial enzyme deficiencies may go undiagnosed. Ornithine transcarbamylase deficiency (OTCD), which is X-linked, is the most common UCD (Nassogne et al 2005; Rezvani 2007). The remaining UCDs are autosomal recessive.

Patients with a complete urea cycle enzyme deficiency typically present in the newborn period. At first the infant appears healthy, but after the first 24-48 hours the initial symptoms of somnolence and poor feeding appear, followed by vomiting, lethargy, and in some cases seizures (Mian and Lee 2002). Newborns are in state of catabolism; so while they are not ingesting large amounts of protein, they are breaking down endogenous protein, and without a functioning urea cycle cannot process waste nitrogen for excretion. The result is a build-up of ammonia which quickly leads to central nervous system dysfunction, coma, and, if left untreated, death (Rezvani 2007).

UCD patients with partial enzyme deficiencies present outside of the newborn period. These individuals retain varying degrees of residual enzyme activity, and thus still have some capacity to convert ammonia into urea. However, due to the diminished capacity for processing waste nitrogen the urea cycle can be overwhelmed leading to hyperammonemia. Hyperammonemic episodes are generally precipitated by environmental stressors such as fasting,

illness, or trauma, all of which are associated with a catabolic state and thus an increased protein load.

Presentation of symptoms varies in these patients. Symptoms may be chronic or may manifest with an increased intake of protein or during periods of catabolism. Most often they present with gastrointestinal and neurological symptoms such as chronic vomiting, developmental delay, psychiatric illness and in some cases seizures (Mian and Lee 2002). Because patients with partial deficiencies may have periods in which they are asymptomatic the disorder may go undetected for quite some time and multiple hyperammonemic episodes can result in cumulative neurologic damage in these patients.

Partial enzyme deficiency can occur with any urea cycle enzyme, but is most common with OTC (Mian and Lee 2002). The X-linked inheritance pattern of OTCD results in a partially dominant disorder with highly variable clinical phenotypes (McCullough et al 2000; Nassogne et al 2005; Tuchman et al 1998). Male OTCD patients are more severely affected since they have only a single copy of the gene. In females, the OTCD phenotype is extremely variable ranging from asymptomatic to severe depending on the pattern of X-inactivation (Mian and Lee 2002). Interestingly, asymptomatic heterozygous females often have a history of protein avoidance (Maestri et al 1998). This sheds light on potential phenotypes not previously appreciated and implies there may be a mechanism to prevent overconsumption of dietary protein.

Nutritional management is fundamental to the care of patients with UCDs (Fong 1995; Singh 2007). Intake of dietary protein, which is a major contributor

to the waste nitrogen pool, is strictly controlled in these patients. However, protein is critical for normal growth and development; therefore, a sufficient amount must be supplied to meet the minimal growth requirement while limiting intake of toxic substrates (Lee et al 2005; Mofidi and Kronn 2009). Natural sources of protein are severely constrained in these patients and protein requirements are filled via medical foods comprised of single amino acids rather than polypeptides (MacDonald et al 1994; Singh 2007). Furthermore, patients are often supplemented with additional calories from non-protein sources in an effort to promote anabolism and prevent catabolism, which is critical for maintaining metabolic control (Lee et al 2005; Singh et al 2005). Lack of appetite is common in these patients and can lead to under consumption of calories and subsequently catabolism. Preventing catabolism is critical; thus, understanding the etiology of anorexia in these patients is an important component of their nutritional management.

As previously mentioned, NAGS is critical for urea cycle function, supplying CPS1 with its essential co-factor NAG. As a result, NAGS is considered a potential regulator of ureagenesis by varying the amount of NAG available for activation of CPS1. The role of NAGS as a regulator of urea synthesis is controversial, with some groups maintaining it has no role in regulating ureagenesis. The rate of urea synthesis is regulated, in part, by substrate availability, and Cohen *et al* suggest that ammonia and ornithine exert direct effects on CPS1 activity in any given nutritional context (Cohen, Cheung, and Raijman 1987; Waterlow 1999). However, changes in protein intake lead to

alterations in the activity of NAGS in the liver, as well as to an increase in the concentration of NAG which subsequently, would lead to an increase in CPS1 activity and ureagenesis (Caldovic et al 2002a). Therefore, NAGS may well play an important role in controlling nitrogen flux through the cycle.

The complete coding sequence of *NAGS* was first cloned in 2002 by Caldovic *et al* (Caldovic et al 2002a). The initial description of this gene suggested it contains seven exons and spans 4.4 kb which is similar to the structure of mouse *Nags* (Caldovic et al 2002a; Caldovic et al 2002b). The human *NAGS* gene is located on the plus strand of 17q21 in a head-to-head orientation with the gene for peptide tyrosine tyrosine (*PYY*).

Peptide YY

PYY is a 36-amino acid peptide so named for the tyrosine residues located at both its N- and C-termini. PYY, along with neuropeptide Y (*NPY*) and pancreatic polypeptide (*PPY*) make up the NPY family of peptides. These peptides share high structural homology and are all synthesized as polypeptide precursors containing a signal peptide, an active peptide and a carboxy terminal peptide (Minth, Andrews, and Dixon 1986). The signal and carboxy terminal peptides are cleaved leaving a 36-amino acid active peptide. Although these genes are closely related they maintain distinct functions. Their effects are mediated through Y receptors (Y1, Y2, Y4, and Y5), a family of G-protein coupled receptors. The peptides bind these receptors with varying affinities and this may be one mechanism by which they exert their distinct functions.

In addition to the structural homology and the similarity of their precursors, *PYY*, *NPY*, and *PPY* also display high sequence homology (Ekblad and Sundler 2002) suggesting these genes evolved from a common origin. Indeed, *NPY* and *PYY* share high homology at both the protein (70%) and nucleotide levels, and are thought to have arisen as duplications of a common ancestral gene (Hort et al 1995). *NPY*, which is the most highly conserved, is considered to be phylogenetically older than *PYY* (Ekblad and Sundler 2002). *PPY* is the newest family member and is thought to have arisen from a tandem gene duplication of *PYY*.

The importance of *NPY* has been demonstrated in several physiologic situations. It is primarily involved in the regulation of energy homeostasis, stimulating food intake (Pedrazzini, Pralong, and Grouzmann 2003). It is also a vasoconstrictor and plays a role in cardiovascular regulation (Pedrazzini, Pralong, and Grouzmann 2003). *PPY* is expressed primarily in the pancreatic islets of Langerhans and is thought to regulate pancreatic and gastrointestinal function. It also may be important in regulating food intake. Administration of exogenous PPY decreases food intake in both mice (Asakawa et al 1999; Asakawa et al 2003) and human (Batterham et al 2003b).

PYY is unique among its family members in that it functions as both a hormone and a neuropeptide (Soderberg et al 1994). Its expression pattern reflects this dual function. *PYY* is expressed in both enteroendocrine and neuronal cell populations. It is highly expressed in the ileum, colon, and rectum, with lower expression observed in the proximal small intestine and the pancreas

(Leiter et al 1987). Expression of *PYY* has also been reported in a small population of neurons in the brainstem (Ekblad and Sundler 2002).

PYY functions in appetite regulation inducing feelings of satiety postprandially. It also inhibits gastric acid and pancreatic digestive enzyme secretion, delays gastric emptying and slows gut motility (Leiter et al 1987). The ability of PYY to slow intestinal transit makes it a potential mediator of the ileal brake (Korner and Leibel 2003), which is a feedback mechanism to control gastrointestinal transit of a meal to optimize nutrient digestion and absorption (Maljaars et al 2008).

PYY is secreted by the endocrine L cells of the distal small intestine and colon in response to ingested nutrients. Secretion begins approximately 15 minutes after initiation of feeding, long before nutrients reach the *PYY*-expressing cells of the small intestine. This early secretion of PYY is most likely mediated by the vagus nerve (Sheikh et al 1989; Ueno et al 2008; Zhang et al 1993).

There are two circulating forms of PYY: PYY_{1-36} and PYY_{3-36}. The two N-terminal amino acid residues are cleaved from PYY by dipeptidyl peptidase IV (DPPIV) yielding PYY_{3-36}. Approximately 40% of circulating PYY is PYY_{3-36}, which preferentially binds the Y2 receptor and is thought to mediate the satiety function. Administration of exogenous PYY_{3-36}, and PYY_{1-36} to a lesser extent decreases food intake in both mice and humans (Batterham et al 2002; Batterham et al 2003a).

A number of studies show a greater and more sustained release of PYY in response to protein, compared to fats and carbohydrates suggesting it may play

an important role in protein-mediated satiation (Batterham et al 2006; Lomenick et al 2009). Additionally, there is considerable evidence that protein is more satiating than carbohydrates or fats (Batterham et al 2006; Halton and Hu 2004; Latner and Schwartz 1999; Lejeune et al 2006; Lomenick et al 2009; Paddon-Jones et al 2008; Rolls, Hetherington, and Burley 1988; Westerterp-Plantenga et al 1999). The secretion pattern of PYY in response to different macronutrients may explain, in part, why protein is the most satiating macronutrient.

Since its role in appetite regulation was discovered, PYY has garnered much attention as a potential therapeutic target for obesity. Candidate gene association studies reveal that single nucleotide polymorphisms in the *PYY* gene are associated with obesity (Ma et al 2005; Shih et al 2009). However, the relationship between PYY and body mass is inconsistent. Some studies show an attenuated response of PYY in obese individuals compared with normal weight controls (Le Roux et al 2006). On the other end of the spectrum, PYY levels are reported to be increased in patients with anorexia nervosa (Misra et al 2006; Pfluger et al 2007). Some studies demonstrate a negative correlation of fasting PYY levels with BMI (Guo et al 2006; Pfluger et al 2007) other studies demonstrate a positive correlation (Lomenick, Clasey, and Anderson 2008), or no correlation at all (Kim et al 2005). The dysregulation of PYY in both overweight and underweight populations suggests an important role for this peptide in energy homeostasis.

Additionally, abnormal regulation of PYY may play a role in a number of gastrointestinal disorders. It may be a primary effector, and cause disease as in

chronic idiopathic slow transit constipation. Or it may be a secondary effect, resulting from changes brought on by a disease (El-Salhy, Suhr, and Danielsson 2002). Abnormal PYY levels may contribute to symptoms observed in inflammatory bowel diseases such as Crohn's colitis (El-Salhy, Suhr, and Danielsson 2002). Understanding the normal regulation of *PYY* is important in determining what role it plays in obesity, anorexia, and gastrointestinal disorders.

PYY cDNA was first cloned from human colon mucosa in the early 1990s (Kohri et al 1993). The initial description of *PYY* suggested that the gene contained 4 exons. The sequence has since been revised based upon a clone isolated from a cDNA library derived from a brain astrocytoma, and it is now suggested that some isoforms of *PYY* contain 7 exons and span more than 50 kilobases (UCSC genome browser, Feb. 2009 GRCh37/hg19 assembly). The 4 exons originally described by Kohri *et al* map to exons 4-7 of the current *PYY* gene structure. The human *PYY* gene is located on the minus strand of chromosome 17q21 and, as described above is arranged head-to-head with *NAGS*. The arrangement and location of these genes is consistent with coordinate regulation.

Potential coordinate regulation of *PYY* and *NAGS*

Background on coordinate regulation

Neither genome size nor gene number is indicative of organism complexity. Upon completion of the human genome sequence there was a surprising revelation that there are approximately 20,000 to 25,000 genes spread

across our nearly 3.2 billion base pairs of DNA (International Human Genome Sequencing Consortium 2004; Stein 2004). This seems quite low given the genome sizes of other relatively less complex organisms. For example, the rice genome is comprised of approximately 470 million base pairs, yet it contains an estimated 51,000 protein coding genes, twice the estimate for the human genome (Pray 2008). A number of mechanisms are thought to contribute to organism complexity, including: alternative RNA splicing, RNA editing, and complex regulation of genes within a genome.

The regulation of gene expression occurs in a spatial-temporal fashion. Genes are turned on and off in a tissue-specific and time-dependent manner. Gene expression may be regulated at several levels including transcription, mRNA processing, mRNA stability, and translation. Thus, complex regulatory mechanisms exist to ensure that genes are expressed appropriately. These mechanisms likely play an important part in organism complexity.

Koyanagi *et al* propose that one way to generate organism diversity is through the evolution of the co-regulation of gene pairs (Koyanagi et al 2005). Mechanisms for coordinate gene regulation have been described in many species and are especially advantageous in organisms with compact genomes. Traditionally, coordinate regulation was defined as more than one gene responding to a physiologic signal (Grossman, Seelan, and Jaradat 1998). This definition of coordinate regulation is too narrow; it is now known that genes can be coordinately regulated through diverse mechanisms such as locus control regions or via shared *cis* regulatory elements.

Sharing of *cis* regulatory elements has been observed for genes located at the same genomic address particularly those located in clusters like the well-known *Hox* and *β-globin* gene loci. The sharing of *cis* regulatory elements is not restricted to gene clusters. Divergently transcribed genes pairs are located on opposite strands of the genome and are transcribed in opposite directions (Figure 2.2). Consequently, they share a 5' flanking region; thus the possibility exists for shared *cis* regulatory elements (Ikeda et al 2002; Lee and Song 2000; Trinklein et al 2004). This was demonstrated for the divergently transcribed gene pair *CYP1A1* and *CYP1A2* located on human chromosome 15 and separated by approximately 24 kilobases. Regulatory elements located in the shared 5' flanking region, function bidirectionally to positively and negatively regulate expression of both genes (Ueda et al 2006). This suggests that gene location and arrangement are important for coordinate regulation through shared *cis* elements.

A distinct class of divergently transcribed gene pairs, those separated by 1000 base pairs or less, has been identified. Multiple studies demonstrate that such bidirectional gene pairs are common in the human genome (Adachi and Lieber 2002; Trinklein et al 2004; Yang and Elnitski 2007). Trinklein *et al* designated the region between the transcription start sites for these gene pairs, putative bidirectional promoters. Thus, bidirectional promoters are sequences located between divergently transcribed genes pairs separated by 1000 base pairs or less which may be involved in regulating expression of both genes (Adachi and Lieber 2002; Trinklein et al 2004). Bidirectional promoters have also

been observed in other organisms including: the Vaccinia and SV40 viruses, the prokaryotic bacteria *Escherichia coli*, and other eukaryotes such as *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Adachi and Lieber 2002; Beck and Warren 1988; Knutson et al 2009). Trinklein *et al* determined that greater than 10% of genes in the human genome have a putative bidirectional promoter, suggesting this is a common regulatory mechanism in human (Trinklein et al 2004).

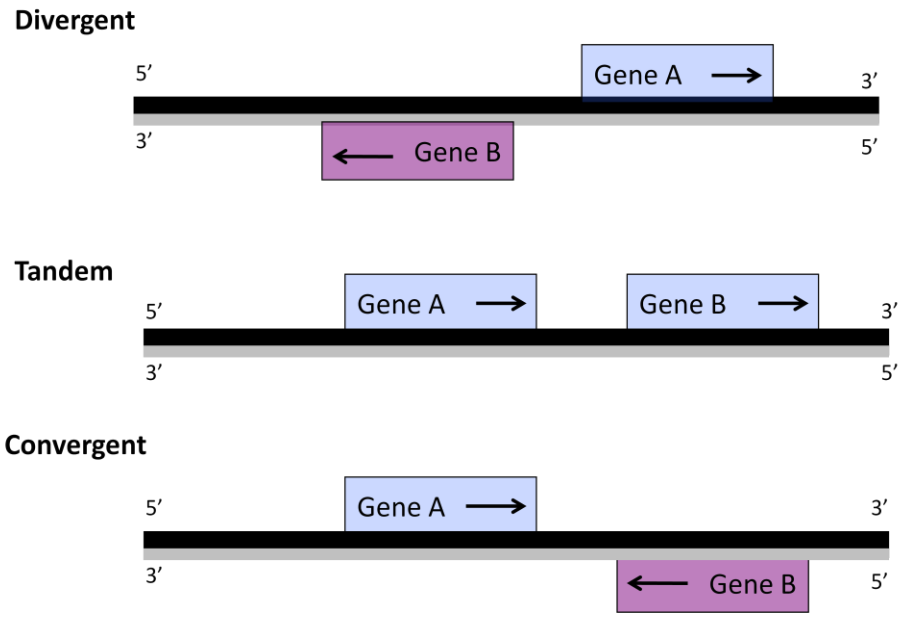


Figure 2.3: Neighboring Gene Arrangements. Neighboring genes on chromosomes may be divergent (head-to-head), in tandem (tail-to-head) or convergent (tail-to tail)

Divergently transcribed gene pairs have more correlated gene expression compared with other gene neighbors including tandem and convergent gene pairs (Figure 2.3) in flies (Herr and Harris 2004), yeast (Kensche et al 2008), and

humans (Lin et al 2007; Trinklein et al 2004). Studies indicate that most bidirectional gene pairs are coordinately regulated (Trinklein et al 2004; Zanotto, Shah, and Jacobs 2007). A reasonable explanation for coordinate regulation is the need for simultaneous gene expression for gene products that function cooperatively in the same complex, or within a common pathway. For example, the α1(IV) and α2(IV) genes are regulated bidirectional promoter (Burbelo, Martin, and Yamada 1988; Heikkila, Soininen, and Tryggvason 1993). Together, the gene products comprise the major form of type IV collagen which is a heterotrimer consisting of two α1(IV) chains and one α2(IV) chain (Burbelo, Martin, and Yamada 1988). Thus, it is beneficial to coordinately regulate this gene pair to ensure that each gene is expressed in amounts needed to successfully form type IV collagen.

It is important to note that a significant number of bidirectional gene pairs do not have an obvious functional connection (Adachi and Lieber 2002). Even if gene products are functionally unrelated, it may still be advantageous to arrange genes in this way simply because it is efficient to do so. CpG islands, which are overrepresented in bidirectional promoters (Adachi and Lieber 2002; Trinklein et al 2004), are associated with open or active chromatin suggesting bidirectional gene pairs are actively transcribed. When the chromatin is in an open conformation both genes are likely to be accessible to the transcription machinery. Also, it is possible that genes in a bidirectional pair need to be expressed at the same time (i.e. during development) or in the same place (cell

type) even if they are functioning in different capacities and this arrangement allows for efficient transcription of these genes.

Rationale for coordinate regulation of *PYY* and *NAGS*

The location and orientation of *NAGS* and *PYY* is consistent with coordinate regulation. In humans, *NAGS* and *PYY* are divergently transcribed and separated by less than 200 base pairs (UCSC genome browser, Feb 2009 GRCh37/hg19 assembly), suggesting they may be regulated by a bidirectional promoter (Figure 2.3). The *NAGS/PYY* gene pair was identified as having a putative bidirectional promoter in computational studies by both Trinklein *et al* and Yang *et al* (Trinklein et al and Yang et al).

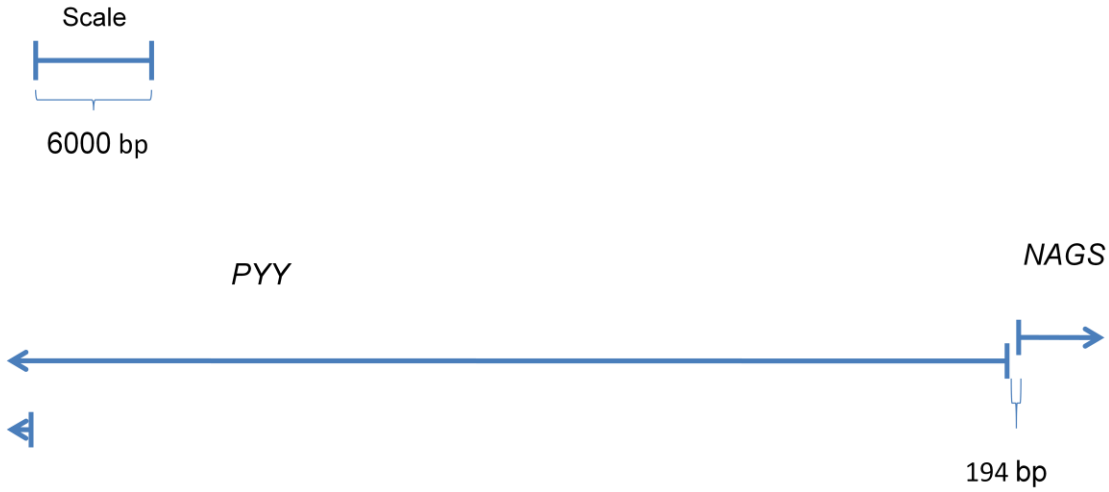


Figure 2.4: The location and arrangement of *PYY* and *NAGS*. *PYY* and *NAGS* are divergently transcribed on Chr 17q21 and are separated by less than 200 base pairs (UCSC genome browser, Feb 2009 GRCh37/hg19 assembly). Data suggest there are two transcriptional start sites for *PYY*, located in exons 1 and 4.

NAGS and *PYY* are both expressed in the small intestine so it is possible they are coordinately regulated in this tissue. Through northern blot analysis of human tissues Caldovic *et al* identified differential transcript sizes for *NAGS* in the liver and small intestine, 2.3 kb and 1.7kb, respectively (Caldovic et al 2002a). The differences in these transcripts have not been characterized. Co-expression of *PYY* and *NAGS* in the small intestine could result in a smaller transcript for *NAGS*, which would be one way to explain the tissue-specific transcripts observed by Caldovic *et al*. This might be due to the use of an alternative promoter and consequently an alternate TSS when both genes are being transcribed simultaneously. On the other hand, it could be due to differential splicing mechanisms or alternative polyadenylation site usage. Characterizing the differential transcripts will be important in determining the mechanism by which they are generated and what influence, if any, co-regulation of *PYY* and *NAGS* has on the alternate transcript usage.

In the context of normal physiology, why might *PYY* and *NAGS* be coordinately regulated? At first glance they do not appear to be functionally related. However, upon further examination a functional connection is revealed. It is known that expression of both *PYY* and *NAGS* is upregulated in response to dietary protein intake. NAGS is essential for successful processing of waste nitrogen, and PYY is a gut peptide that inhibits digestive enzyme secretion, gastric emptying, and gut motility (Leiter et al 1987). PYY also functions in appetite regulation, inducing feelings of satiety postprandially. Studies demonstrate greater and more sustained elevations in PYY concentration in

response to protein compared with other macronutrients. The coordinate regulation of this gene pair may be a mechanism to prevent overconsumption of dietary protein. Ingestion of dietary protein in extreme excess would result in large amounts of waste nitrogen which could potentially overwhelm the capacity of the urea cycle leading to hyperammonemia. PYY induces feelings of satiety and these feelings are sustained longer with higher protein meals allowing time for the urea cycle to clear the waste nitrogen thus preventing the accumulation of ammonia. It's possible the coordinate regulation of *PYY* and *NAGS* is one of the mechanisms regulating nitrogen balance in the body.

The coordinate regulation of these genes could also explain some of the clinical observations in patients with UCDs. As described above, anorexia is common in patients with UCDs (Batshaw and Berry 1991; Brusilow 1985; Summar and Tuchman 2001). The decreased appetite in these patients is normally attributed to cerebral edema resulting from hyperammonemia. However, if *PYY* and *NAGS* are coordinately regulated, then it is possible that increased levels of PYY may be an underlying cause of anorexia. An increase in ammonia levels results in upregulation of the urea cycle enzymes and *NAGS*. Coordinate regulation of *PYY* and *NAGS* would consequently lead to an increase in PYY levels, perhaps as a means to suppress further nitrogen intake. Thus, elevated PYY levels in UCD patients may result in prolonged satiation that manifests as a lack of appetite.

Increased levels of PYY would also contribute to constipation, a common symptom in UCD patients (Summar and Tuchman 2001). While the cause of

constipation in these patients is unknown it has been attributed to hypotonia. However, a rise in *PYY* expression may be the underlying cause of this symptom, as PYY inhibits colonic mobility. Constipation can have deleterious effects in UCD patients because decreased gut mobility increases the urease activity of bacteria in the gut. In this process urea is hydrolyzed to ammonia and carbon dioxide and the ammonia is re-circulated to the liver (Gropper, Smith, and Groff 2009), potentially creating a negative feedback loop in which *NAGS* and *PYY* are upregulated.

To summarize, the location and arrangement of *PYY* and *NAGS* is consistent with regulation via a bidirectional promoter. Understanding the regulation of *NAGS* may be the key to unlocking its role in regulating urea synthesis. PYY functions in multiple capacities and may play an important role in the pathogenesis of gastrointestinal disorders as well as in obesity and diabetes (Ahituv et al 2006; Boey et al 2006a; Boey et al 2006b; El-Salhy, Suhr, and Danielsson 2002; Karra and Batterham 2010). Importantly, upregulation of *PYY* may also contribute to phenotypes associated with urea cycle deficiencies. Determining the mechanisms which govern the regulation of *PYY* and *NAGS* will help to clarify the molecular and physiologic relationship of these two genes. Furthermore, it may contribute to our overall understanding of protein-mediated satiation, the mechanisms involved in the maintenance of nitrogen balance, and the etiology of anorexia in UCD patients and others populations with a diminished capacity for processing waste nitrogen.

Hypotheses and Specific Aims

Hypotheses:

a. The 5' flanking region shared by *PYY* and *NAGS* is a bidirectional promoter that coordinately regulates this divergently transcribed gene pair.
b. Coordinate regulation of *PYY* and *NAGS* results in elevated plasma PYY levels in patients with urea cycle deficiencies, contributing to the anorexia observed in this population.

Specific Aim I: Characterize the 5' structure of human *PYY* and *NAGS* transcripts.

a. Identify the transcription start sites for NAGS

b. Identify and confirm the transcription start sites for PYY

Determining the transcription start sites (TSSs) for *PYY* and *NAGS* is an essential first step in determining if these genes are regulated by a bidirectional promoter. The complete coding sequence for *NAGS* has been cloned, but the 5' untranslated region, and consequently the TSSs have not yet been defined. The TSSs for *NAGS* will be identified using 5' RACE. Evidence suggests at least two transcript isoforms exist for *PYY*, indicating this gene may be regulated by alternative promoters. 5' RACE will be used to identify and confirm the TSSs for *PYY* and quantitative real time RT-PCR (qRT-PCR) will be used to determine the abundance of each transcript isoform.

Specific Aim 2: Determine if *PYY* and *NAGS* are coordinately expressed in a panel of human tissues.

a. *Perform qRT-PCR to identify tissues in which PYY and NAGS are coordinately expressed*
b. *Identify a cell line that expresses both PYY and NAGS*

If *PYY* and *NAGS* are coordinately regulated, then the expectation is they will be expressed concordantly in at least some tissues. Quantitative real-time RT-PCR will be performed to determine if these genes are coordinately expressed in a panel of normal human tissues. Additionally, human cell lines will be screened to identify a line that expresses both *PYY* and *NAGS* at similar levels.

Specific Aim 3: Determine if plasma PYY levels are elevated in patients with urea cycle disorders.

a. *Measure plasma PYY levels in urea cycle disorder patients using an ELISA*
b. *Measure plasma PYY levels in phenylketonuria patients and a control group using an ELISA*

In individuals with diminished capacity for nitrogen processing urea cycle enzymes are presumably upregulated in an effort to clear the waste nitrogen. If *PYY* and *NAGS* are coordinately regulated then the expectation is *PYY* would also be upregulated in these individuals. PYY is secreted and can be measured

in plasma. Therefore, to extend the investigation of the relationship between *PYY* and *NAGS* to human clinical applications plasma PYY levels will be measured in patients with urea cycle disorders. These levels will be compared with plasma PYY levels in a control group and a group of individuals with another inborn error of amino acid metabolism, phenylketonuria patients.

CHAPTER III

DEFINING THE 5' STRUCTURE OF *PYY* AND *NAGS*

Introduction

Human *NAGS* and *PYY* are located in a head-to-head orientation on chromosome 17 and are separated by less than 200 base pairs (UCSC genome browser, Feb 2009 GRCh37/hg19 assembly). Their location and arrangement is consistent with coordinate regulation via a bidirectional promoter. An important first step in analyzing the transcriptional regulation of a gene or gene pair is to identify the promoter region; which, by definition, includes DNA sequences in the vicinity of the transcription initiation site (Carey and Smale 2000). Thus by defining the transcriptional start sites (TSSs), the location of the promoter can be identified.

The human *NAGS* locus is on the plus strand of chromosome 17q21. This gene contains 7 exons and spans approximately 4.4 kilobases (Figure 3.1) (chr17:42082032 – 42086435; UCSC genome browser, Feb 2009 GRCh37/hg19 assembly). It has been less than ten years since Caldovic *et al* cloned *NAGS* from a human liver cDNA library (Caldovic et al 2002a). This clone included the complete coding sequence and the 3'UTR, but lacked the 5'UTR, which prior to the work presented here was still undefined.

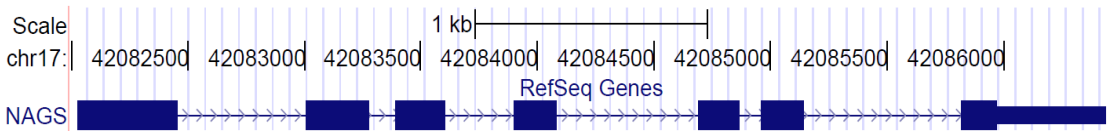


Figure 3.1: *NAGS* gene structure. This figure was generated using the UCSC genome browser. The structure of *NAGS* as currently defined (UCSC genome browser, Feb 2009 GRCh37/hg19 assembly) contains 7 exons (indicated by the solid blocks) and spans approximately 4.4 kilobases.

Human *PYY* is located on the minus strand of chromosome 17q21. Kohri *et al* first cloned *PYY* cDNA from human colon mucosa, and their initial description suggested this gene contains 4 exons and spans 1.2 kilobases (Kohri et al 1993). The sequence has since been revised, and suggests that at least some isoforms of *PYY* contain 7 exons and span more than 50 kilobases (chr17:42081837 – 42081837; UCSC genome browser, Feb 2009 GRCh37/hg19 assembly). I will refer to this as the long isoform of *PYY*. The *PYY* transcript isoform originally described by Kohri *et al* aligns to exons 4-7 of the long isoform of *PYY* (UCSC genome browser, Feb 2009 GRCh37/hg19 assembly). It is important to note, the long isoform also differs from that of mouse, rat, and porcine *Pyy*, all of which contain only 4 exons aligning to exons 4-7 of the long *PYY* isoform. The translational start site, located at the beginning of exon 5, does not differ among the transcripts in these species, and the protein coding exons are highly conserved. Taken together, these data suggest there are at least two *PYY* isoforms in human with TSSs located in exon 1 and exon 4. Moreover, these TSSs are separated by approximately 51 kb suggestive of

regulation by alternative promoters. Furthermore, the long isoform of *PYY* has not been identified in other species suggesting it may be unique to the human lineage.

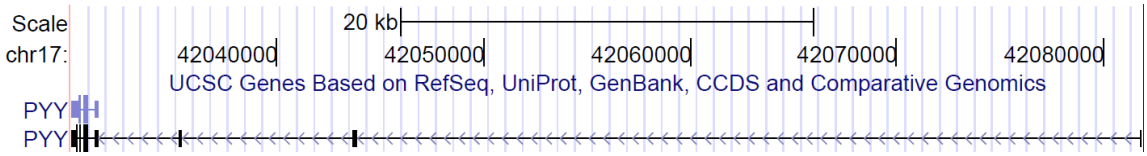


Figure 3.2: *PYY* gene structure. This figure was generated using the UCSC genome browser. The structure of *PYY* as currently defined (UCSC genome browser, Feb 2009 GRCh37/hg19 assembly) contains 7 exons (indicated by the solid blocks) and spans approximately 51 kilobases. Data suggest there is a second transcriptional start site for *PYY* located in exon 4.

The transcripts for *NAGS* and *PYY* have not been fully characterized. Given that the 5'UTR of *NAGS* has not been defined, the transcript sequence is incomplete. Therefore, the TSS(s) and consequently the promoter have not yet been identified. Two *PYY* isoforms have been described in human; the long isoform was isolated from a cDNA library derived from a brain astrocytoma and has not been studied further (NCBI reference sequence NM_004160.4; MGC cDNA clone 52233) (Strausberg et al 2002). Given that genes can have multiple TSSs where TSS selection varies across tissues, it is important to establish the TSSs for the gene in each tissue of interest. I describe here the identification and verification of TSSs for both *PYY* and *NAGS* in various human tissues, the initial

steps necessary to fully elucidate the mechanisms of regulation and expression for this head-to-head gene pair.

Materials and Methods

Human total RNA

Commercially available human total RNA samples were obtained from Clontech, Chemicon, Stratagene and Ambion (based on availability of RNA from desired tissues). All RNA samples were isolated from normal human tissues. Most samples were derived from a single individual, while a few were pooled from multiple individuals. The concentration and quality of RNA was assessed by the manufacturer. Human total RNA samples included: brainstem, colon, ileum, kidney, liver, pancreas, skeletal muscle, small intestine, spleen and testes.

5'RACE

Rapid amplification of cDNA ends (RACE) was performed to map the 5' ends of *PYY* and *NAGS* transcripts in human tissues. The Roche Applied Science 5'/3' RACE Kit, 2nd Generation (catalog # 03353621001) was used. In general, first strand cDNA synthesis was performed with a gene specific primer (GSP1). The cDNA was then purified using the High Pure PCR Product Purification Kit (Roche Applied Science catalog # 11 732 668 001). Next, a poly (A) tail was added to the 5' end of the cDNA with terminal transferase. This was followed by PCR with a nested primer, GSP2, and the oligo(dT)-anchor primer supplied by the manufacturer. Using this PCR product as template, a second

round of PCR was performed using another nested primer, GSP3 and the anchor primer supplied by the manufacturer. Primer sequences are given in Table 3.1.

Next, 5'RACE products were analyzed by agarose gel electrophoresis. Bands were excised from the gel and products were gel purified using the Wizard SV Gel and PCR Clean-up System (Promega catalog # A9281). 5'RACE products were cloned into the TOPO PCR 2.1 vector using the TA-cloning system (Invitrogen catalog # K4500-40), following the manufacturer's instructions. Both the gel-purified products and the PCR reaction were used in the cloning reactions. A minimum of 14 colonies were screened via PCR and ethidium bromide stained agarose gel electrophoresis. 5 mL cultures of bacteria containing clones for each 5'RACE product were grown and plasmid isolations were performed using the QIAprep Spin Miniprep Kit (Qiagen catalog # 27104). Clones were sequenced by the Vanderbilt DNA Sequencing Core. Sequences were analyzed with Sequencher 4.8 (Gene Codes Corporation) alignment software and the University of California, Santa Cruz (UCSC) genome browser BLAST-Like Alignment Tool (BLAT).

Table 3.1: 5' RACE primer sequences for *PYY* and *NAGS*

Primer name	Primer sequence	Primer location
*PYY*RACE1	GTC TGA AGA AGG AGC ATG CAG	exon 2
*PYY*RACE2	GTC CAG GAA TTG GAA TGT GAC C	exon 2
*PYY*RACE3	TTT CCT CTT GGC AGC AG	spans exon 1-2 junction
*PYY*RACE4	GAC CGA TAG TGG GTT CAG TTC C	exon 1
*PYY*race1b	CAG AAG CAC TGT GGT CAA GG	exon 5
*PYY*race2b	CTG CGC ACG AAC ACC ATA G	spans exon 4-5 junction
*PYY*race3b	CTT GTG AAG CAG ACG AGC AG	exon 4
*NAGS*RACE1	CCA TGA CGA CAA CCA ACT CTT G	begins at base +4 from ATG
*NAGS*RACE2	CAA GAG TGG CAG TCT GTC TGG	begins at base -24 from ATG
*NAGS*RACE3	GGG CTC TTA ACT TGC CGT TG	begins at base -60 from ATG

Non-quantitative RT-PCR

Reverse Transcription

Reverse transcription was performed using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen catalog # 18080-051). Following the manufacturer's instructions, 20 µL reactions were prepared using oligo(dT) primer and 2 µg of total RNA. RNA/primer mixtures were prepared and incubated at 65°C for 5 minutes and then placed on ice. Next, cDNA synthesis master mix was added to each tube and reactions were incubated at 50°C for 50 minutes and then 85°C for 5 minutes. To remove RNA template, RNAse H was added to each tube and reactions were incubated at 37°C for 20 minutes. RT-PCR primers for *PYY* and *NAGS* are given in Table 3.2.

PYY

Using 1 µL of RT product from brainstem, ileum, and kidney as template, PCR was performed with four primer sets to amplify discrete regions of *PYY* cDNA: exons 1-6, exons 2-6, exons 4-6 and exons 1-2 (Figure 3.4). Full-length *PYY* cDNA (500pg) containing exons 1-7 (Open Biosystems clone ID 40005903) was used as a positive control, while a water blank served as a negative PCR control. Amplified products were analyzed via agarose gel electrophoresis.

NAGS

Primers were designed to further characterize the 5' exon/intron structure of *NAGS* transcripts identified in *NAGS* 5'RACE liver experiments. Forward primers were designed to the beginning of each of the 5'RACE products identified in the liver. Each forward primer was paired with the third gene specific primer, GSP3, from the 5'RACE experiments.

Using 1 µL of RT product from liver as template, PCR was performed with each primer set. The expected product sizes for these primers pairs were: liver1/GPS3 – 322 bp, liver 2/GSP2 – 302 bp, liver 3/GSP3 – 222 bp. Additionally, another PCR was performed pairing liver 1 forward primer with a reverse primer in exon 1 of *NAGS*, expected product size 780 bp. The amplified products were analyzed via agarose gel electrophoresis and then cloned into the TOPO PCR2.1 vector. Clones were screened and sequenced as described earlier in this chapter (see 5'RACE methods).

Table 3.2: Non-quantitative RT-PCR primer sequences for *PYY* and *NAGS*

PYY primers	Primer Sequence
*PYY*RACE	GGAGGAACTGAACCCACTATCG
PYY ex2	GGA GGT CCC TGG AGA TTT G
PYY ex3	GTC CTA GAG CGA AGC CTG AG
PYY ex4	GGG ATA TAA GCC CCA CAA GG
PYY ex6	GGC CGT CTC TTT TCC CAT AC
NAGS primers	
NAGS smint	CCC AAC GGC AAG TTA AGA GC
NAGS ex 1	GAG TGA TGG CAG GTC TGG AAC
NAGS ex 2	GCC TCC CAG AAG GAA AGA CAG
NAGS liver 1	CGA GAG GGA GGA CCT GGA G
NAGS liver 2	CCT GTG CTC AAA GCC ACC TAC
NAGS liver 3	GGA ACC TAC CTT GGC AGC AG

Quantitative real time RT-PCR

Primers and probes

Quantitative real time RT-PCR (qRT-PCR) was used to determine the presence and amount of *PYY* transcript isoforms in a panel of human tissues. Three Applied Biosystems TaqMan® gene expression assays for *PYY* were used. The first assay (ID # Hs01062281_m1) probe spans exons 1-2 and will specifically detect the *PYY* isoform containing exon 1. The second assay (ID # Hs01062282_m1) probe spans exons 2-3 and will thus detect *PYY* transcripts that begin in exon 1 or in exon 2. It will not differentiate between those which contain exon 1 and those that do not. The final assay (ID # Hs00373890_g1) probe spans 6-7 and will detect all *PYY* transcripts.

First strand cDNA synthesis

First strand cDNA synthesis was performed using the SuperScript™ III First Strand Synthesis System (Invitrogen, catalog #18080-051). Following the manufacturer's instructions, 20 µL reactions were set up using oligo(dT) primer and 4 µg of total RNA. First, RNA/primer/dNTP mixtures were prepared, incubated at 65°C for 5 minutes and then placed on ice. Next, 10 µL of cDNA synthesis master mix was added to each tube and reactions were incubated at 50°C for 50 minutes followed by an incubation at 85°C for 5 minutes. 1 µL of RNAse H was added to each tube and reactions were incubated at 37°C for 20 minutes.

Standard curve preparation

Standard curves were prepared using full-length *PYY* cDNA (Open Biosystems clone ID 40005903) for absolute quantitation of mRNA transcripts. Standard curve templates consisted of ten-fold dilutions of full-length plasmid cDNA ranging from 5 fg to 500 pg. To prepare plasmid cDNA for standard curves, 5 mL cultures of bacteria containing plasmid with full-length *PYY* cDNA were grown overnight in a 37°C shaking incubator. Plasmid isolations were then performed using the QIAprep Spin Miniprep kit (Qiagen catalog #27104). Plasmid DNA was eluted in nuclease-free water. DNA quality and concentration were assessed via spectrophotometric readings at 260, 280 and 230 nm. A 5 ng/µl dilution was prepared, and then serially diluted in 1:10 steps to a final concentration of 5 fg.

qRT-PCR set-up

All samples and standards were assayed in triplicate. Reactions were performed in a total volume of 10 µL and included: 5 µL TaqMan Gene Expression Master Mix, 2X (Applied Biosystems cat #4369016), 0.5 µL TaqMan gene expression assay for the gene of interest, 0.5 µL of TaqMan gene expression assay for an endogenous control gene, and 4 µL of cDNA/nuclease free water mixtures. Standard curve cDNA/nuclease-free water mixes consisted of 1 µL of standard cDNA and 3 µL of nuclease-free water. The cDNA/nuclease-free water mixtures for the RNA samples consisted of 0.5 µL cDNA and 3.5 µL nuclease free water which corresponds to 100 µg of RNA per reaction. The endogenous control gene assay was not included in the standard curve reactions, therefore, 0.5 µL of nuclease-free water was added to make up the volume.

qRT-PCR analyses were performed using Applied Biosystems Prism® 7900 HT Sequence Detection System and the accompanying software SDS 2.3 (Applied Biosystems, Inc., Foster City, CA). The following standard thermal cycler protocol was used: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The ramp rate for temperature change was 100%.

The SDS 2.3 analysis software generated an equation of the line for each set of standards. The highest concentration standard was dropped for each assay because it crossed the threshold before the set baseline. The square of the correlation coefficient (r^2) was ≥ 0.99 for each standard curve. These curves

were used to calculate the quantity of each *PYY* mRNA transcript in the panel of human tissues. The calculated quantities are equivalent to picograms (pg) of plasmid. Therefore, the expression (quantity) of transcript in the samples assayed is given the unit designation pg equivalents, or pge.

Results

5'RACE *PYY*

The aim of these experiments was to confirm the TSSs for *PYY* in selected human tissues. 5' RACE was performed using normal human ileum, brainstem, and liver total RNA samples. The ileum was a primary tissue of interest because *PYY* is highly expressed there and *NAGS* is also reported to be expressed in the small intestine. Thus, it is possible these genes are coordinately regulated in this tissue. The brainstem was selected because *PYY* is reportedly expressed in a small population of neurons in the brainstem (Ekblad and Sundler 2002; Glavas et al 2008). Moreover, the long isoform of *PYY* was identified from a brain astrocytoma cDNA library. Therefore, this is a reasonable tissue in which to confirm the long isoform TSS. Liver total RNA was chosen because *NAGS* is highly expressed in this tissue. I wanted to determine if *PYY* transcripts were also expressed in the liver, and to identify the TSSs of any such transcripts.

I was unable to amplify *PYY* in any of these tissues using the 5'RACE GSPs (*PYY*RACE 1, 2, and 3) located in exons 1 and 2. Recall that Kohri *et al* identified a *PYY* transcript similar to *Pyy* in other species that has a TSS in exon 4. Thus, it is possible the major transcript isoform of *PYY* begins in exon 4. The

translational start site for *PYY* is at the beginning of exon 5; therefore, this region is always expected to be included in the transcript. Bearing all this in mind I designed a new series of 5' RACE GSPs (*PYY*RACE1b, 2b, and 3b) located in exons 4 and 5. These primers are expected to amplify a product if the TSS is located in either exon 4 or exon 1.

5'RACE of *PYY* in the ileum using these primers produced a single robustly amplified product between 100 and 200 bp (Figure 3.3). Sequence analysis revealed the TSS location to be approximately 36 bases into exon 4. This is approximately 10 base pairs upstream of the TSS identified by Kohri *et al* from human colon (Kohri et al 1993). I was unable to amplify *PYY* using the re-designed RACE primers in brainstem or liver, suggesting *PYY* is either not expressed, or is expressed at extremely low levels in these tissues.

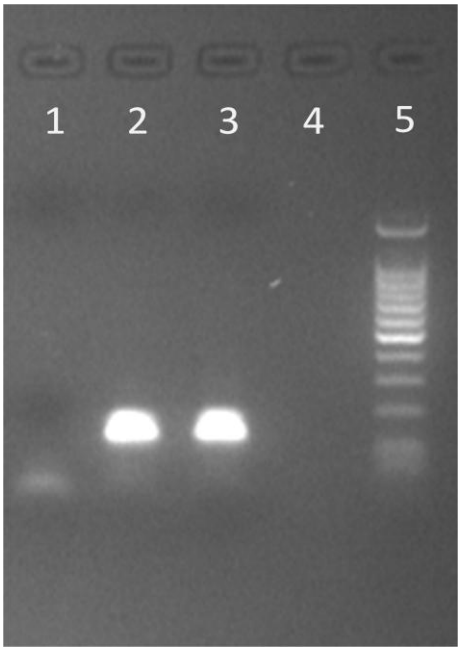


Figure 3.3: *PYY* 5' RACE products in human ileum. 5' RACE was performed on human ileum total RNA. Final amplified products were run on a 1.5% agarose gel containing ethidium bromide. Lane 1: H2O blank, Lanes 2 and 3: *PYY* 5' RACE ileum, Lane 4: empty and Lane 5: 100 bp ladder (Promega). Note: The 500 bp fragment in the ladder is at increased intensity.

RT-PCR *PYY*

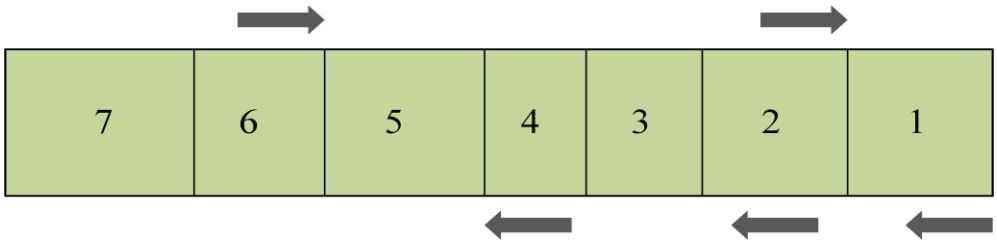
Given that I was unable to amplify exons 1-3 in 5'RACE experiments, primers were designed to amplify discrete regions of the *PYY* cDNA. The goal of these experiments was to determine if exons 1-3 of the *PYY* long isoform are genuine by amplifying these regions from human total RNA samples using non-quantitative RT-PCR. PCR was performed on first strand cDNA from human brainstem, ileum, and kidney using four primer sets. Products were analyzed via agarose gel electrophoresis (Figure 3.4). The expected product sizes for the primer pairs are as follows: 743 bp (exon 1forward/exon 6 reverse), 472 bp (exon 2 forward/exon 6 reverse), 331 bp (exon 4 forward/ exon 6 reverse) and 183 bp (exon 1 forward/exon 2 reverse).

All primer pairs successfully amplified products of expected sizes when using the positive control template (*PYY* cDNA exons 1-7). Primers spanning exons 2-6 and 4-6 amplified products of appropriate size in all three tissues tested. The relative abundance as indicated by the intensity of bands on the ethidium stained agarose gel (Figure 3.4) suggest the *PYY* isoform containing exon 2 is most abundant in the kidney compared to brainstem or ileum.

Primers spanning exons 1-6 and exons 1-2 did not amplify a product in ileum or kidney, indicating the *PYY* transcripts in these tissues do not contain exon 1. A possible product was amplified when brainstem cDNA was used as template for primers spanning exons 1-6 as indicated by an extremely faint band on the agarose gel. Such a faint band after 40 cycles of amplification is indicative of extremely low abundance of *PYY* transcript(s) containing exon 1 and

thus, this product may be of doubtful relevance. It is possible the product was amplified from a single or a few molecules of a transcript containing exon 1 which could have been the result of an antisense transcription event. Additionally, no product was amplified from brainstem cDNA with primers spanning exons 1-2. As demonstrated by the results with primers spanning exons 2-6, exon 2 is included in a *PYY* transcript in the brainstem. Therefore, if *PYY* exon 1 is genuine, I would expect to amplify a product from this tissue using primers spanning exons 1-2. Taken together, these data suggest there are TSSs for *PYY* in exons 2 and 4, and if exon 1 is genuine the *PYY* long isoform is expressed at extremely low levels in these tissues.

a)



b)

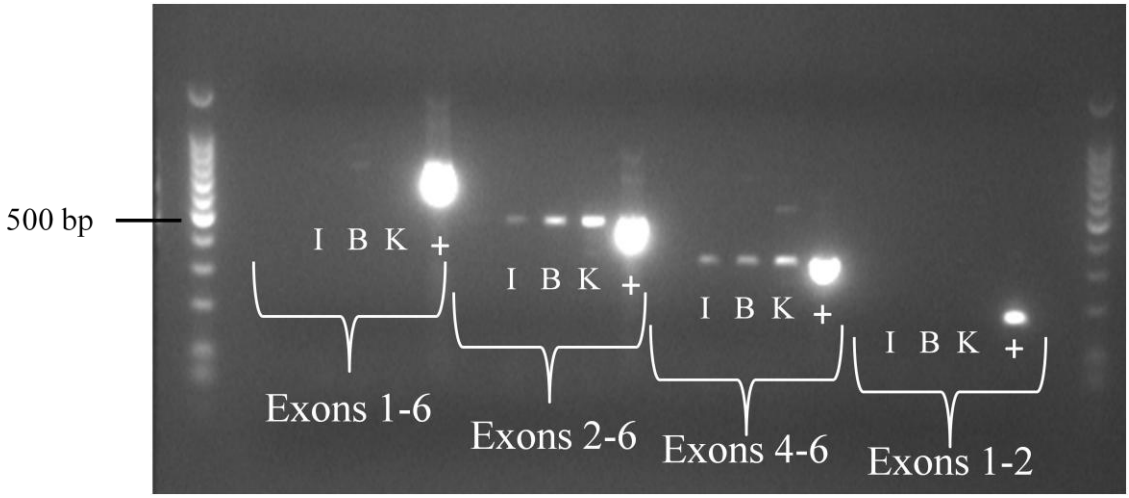


Figure 3.4: RT-PCR analysis of *PYY* in human tissues
Reverse transcription was performed on human ileum, brainstem and kidney total RNA using oligo(dT) primers. This was followed by PCR with 4 primer sets spanning different regions of the *PYY* transcript (exon 1-6, exon 2-6, exon 4-6 and exon 1-2).

a) Location of RT/PCR primers. *PYY* cDNA is diagrammed; boxes represent exons and arrows indicate location of primers.

b) RT-PCR products visualized on a 1.5% agarose gel. *PYY* plasmid cDNA clone containing exons 1-7 was used as a positive control. Samples were loaded as follows for each primer pair: PCR blank, ileum (I), brainstem (B), kidney (K) and *PYY* plasmid (+). A 100 bp ladder was loaded in lanes 1 & 24.

qRT-PCR

Since it is possible that a *PYY* transcript containing exon 1 is expressed in the brainstem and potentially other tissues not included in this RT-PCR experiment quantitative real time RT-PCR was performed on a panel of normal human tissues to further explore the expression of *PYY* transcripts containing exon 1. In both 5' RACE and non-quantitative RT-PCR experiments I was ultimately unable to amplify exon 1 of *PYY*. RT-PCR data suggests there is a transcript which contains exon 2. To determine the presence and amount of *PYY* transcripts qRT-PCR was performed on a panel of normal human tissues (Figure 3.5). The *PYY* transcript including exon 1 was not expressed at appreciable levels in any of the tissues assayed. Indeed, the Ct values for this assay were greater than 32 in all tissues examined (Table 3.1), representative of extremely low expression. The transcript containing exon 2 was expressed primarily in the kidney and pancreas-at a level of 0.005 pge in both tissues. qRT-PCR data reveal this transcript was the major isoform expressed in the kidney, consistent with results from non-quantitative RT-PCR.

The *PYY* TaqMan gene expression assay with the probe spanning exons 6-7 will detect all transcripts. qRT-PCR data indicate that *PYY* isoforms in tissues of the gut (ileum, small intestine and colon) do not contain exons 1 or 2. Taken together with the 5' RACE data, as well as reports in the literature ((Kohri et al 1993), these data indicate that the *PYY* transcript containing exons 4-7 is the major isoform expressed in the gut. It was expressed most highly in the ileum (0.216 pge) and colon (0.398 pge). In the pancreas, it was expressed at

approximately the same level as that of the transcript containing exon 2. This was estimated by subtracting the quantity of *PYY* transcript as determined by the TaqMan assay spanning exon 2-3 (0.005 pge) from the quantity of *PYY* transcript determined from the TaqMan assay spanning exon 6-7 (0.009 pge).

Overall, the data indicate the *PYY* long isoform containing exon 1 is not expressed in the panel of tissues examined here. Furthermore, there is a *PYY* transcript containing exons 2-3 suggesting there is a TSS located somewhere in exon 2. Finally, the short isoform of *PYY* containing exons 4-7 appears to be the main isoform in most tissues.

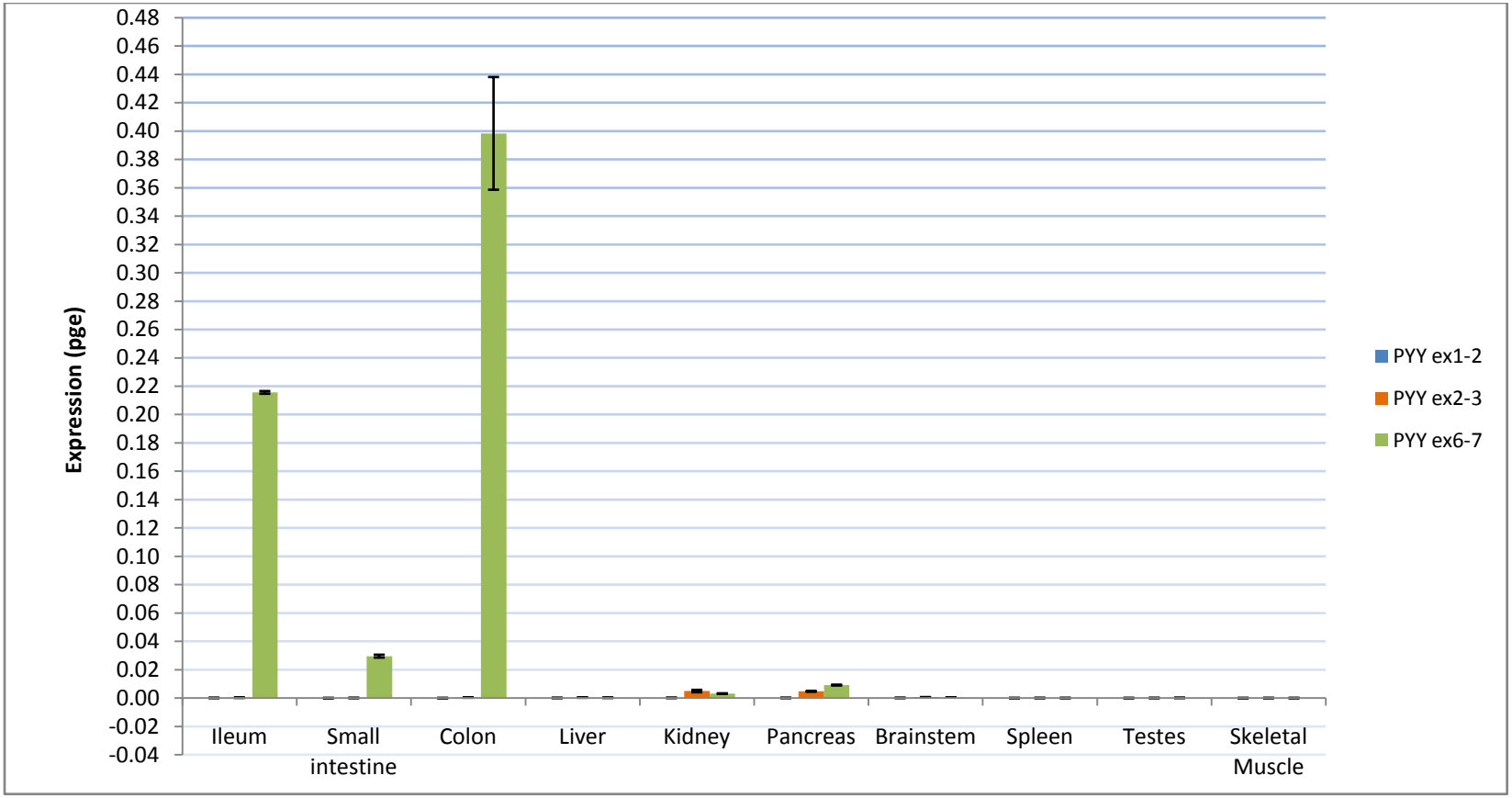


Figure 3.5: Comparison of *PYY* transcripts in a panel of human tissues.
qRT-PCR was performed in triplicate on a panel of human total RNA samples. Plasmid *PYY* cDNA was used to generate a standard curve for each assay, and the quantity of mRNA was calculated based on the standard curve. The expression (quantity) of transcript is given the unit designation pg equivalents, or pge. This graph shows the mean quantity of each transcript. Error bars represent the standard deviation of the triplicate measures.

	PYY assay ex 1-2		*PYY* assay ex 2-3		*PYY* assay ex 6-7	
Tissue	**Ave Ct ± St dev**	**Ave Qty ± St dev**	**Ave Ct ± St dev**	**Ave Qty ± St dev**	**Ave Ct ± St dev**	**Ave Qty ± St dev**
Ileum	32.87 ± 0.28	<0.001 ± <0.001	31.43 ± 0.41	<0.001 ± <0.001	21.56 ± 0.01	0.216 ± 0.001
Small intestine	35.61 ± 0.48	<0.001 ± <0.001	32.91 ± 0.48	<0.001 ± <0.001	24.36 ± 0.04	0.030 ± 0.001
Colon	35.03 ± 0.73	<0.001 ± <0.001	30.54 ± 0.46	<0.001 ± <0.001	20.71 ± 0.14	0.398 ± 0.040
Liver	32.85 ± 0.21	<0.001 ± <0.001	30.67 ± 0.47	<0.001 ± <0.001	31.62 ± 0.17	<0.001 ± <0.001
Kidney	32.35 ± 0.22	<0.001 ± <0.001	26.56 ± 0.23	0.005 ± 0.001	27.49 ± 0.06	0.003 ± <0.001
Pancreas	32.62 ± 0.15	<0.001 ± <0.001	26.60 ± 0.10	0.005 ± <0.001	26.01 ± 0.04	0.009 ± <0.001
Brainstem	32.77 ± 0.59	<0.001 ± <0.001	29.71 ± 0.23	0.001 ± <0.001	30.59 ± 0.10	<0.001 ± <0.001
Spleen	34.95 ± 1.78	<0.001 ± <0.001	33.37 ± 0.95	<0.001 ± <0.001	33.56 ± 0.56	<0.001 ± <0.001
Testis	34.44 ± 1.35	<0.001 ± <0.001	33.20 ± 0.72	<0.001 ± <0.001	32.36 ± 0.66	<0.001 ± <0.001
Skeletal Muscle	34.67 ± 0.60	<0.001 ± <0.001	34.12 ± 1.34	<0.001 ± <0.001	34.52 ± 0.90	<0.001 ± <0.001

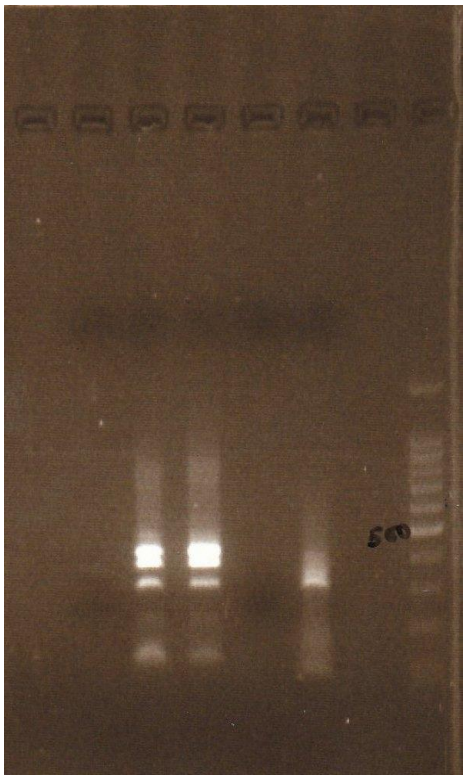
Table 3.3: Quantitative real-time RT-PCR average Ct and quantity values of *PYY* transcripts in normal human tissues. All values reflect the mean of triplicate measurements ± the standard deviation

5'RACE *NAGS*

The aim of these experiments was to identify the TSSs for *NAGS* in selected human tissues. 5' RACE was performed using normal human liver and ileum total RNA samples. Liver was selected because it is a primary site of *NAGS* expression. *NAGS* is also expressed in the small intestine, and while expression has not been localized to a specific region, *PYY* is highly expressed in the ileum. If the hypothesis of coordinate regulation is correct then *NAGS* may also be highly expressed there.

A total of four products were amplified in 5'RACE experiments of *NAGS* in human liver and ileum suggesting three different TSSs in the liver and one in the ileum (Figure 3.6). Transcripts are diagrammed in Figure 3.7. Respectively, the TSSs for liver 1, 2 and 3 transcripts are located 5,353 base pairs, 430 base pairs, and 320 base pairs from the translational start site. All of these transcripts overlap with exon 1 of the *PYY* gene. Liver transcript 1 contains a novel upstream exon that is 60 base pairs long. This is followed by an intron spanning 4968 base pairs. Thus, the 5' untranslated region (UTR) for liver transcript 1 is 385 bases. In liver transcripts 2 and 3 the length of the 5'UTR is 430 and 320 bases, respectively. A distinct TSS, 153 base pairs from the translational start site was identified in the ileum. These data suggest *NAGS* is regulated by alternative promoters in these tissues.

a)



b)

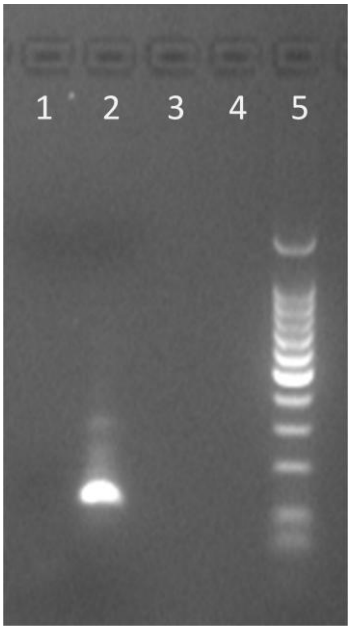


Figure 3.6: *NAGS* 5' RACE products in liver and ileum. 5' RACE was performed on human liver (gel a) and ileum (gel b) total RNA. Final amplified products were run on a 1.5% agarose gel containing ethidium bromide.

Gel a: Lane 1: H_2O blank, Lanes 2 and 3: *NAGS* 5' RACE liver, Lane 4: empty, Lane 5: 100 bp ladder

Gel b: Lane 1: H_2O blank, Lanes 2: *NAGS* 5' RACE ileum, Lane 4: empty, Lane 5: 100 bp ladder

Promega 100 bp DNA ladder was used. It ranges from 100 bp to 1500 bp and the 500 bp fragment in the ladder is at increased intensity.

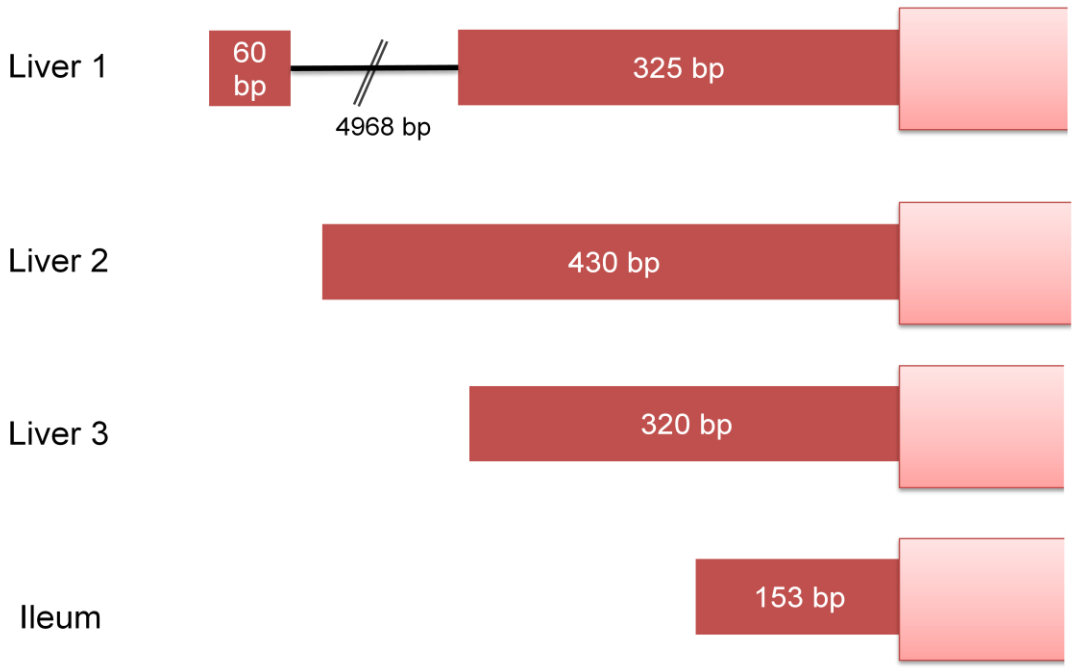


Figure 3.7: *NAGS* TSSs identified in human liver and ileum
Diagram of TSSs identified for *NAGS* using 5'RACE. Three TSSs were identified in the liver and one TSS was identified in the ileum. The solid bars represent the 5'UTR and the shaded bars represent the beginning of the coding sequence. Introns are indicated by solid black line.

RT-PCR *NAGS*

RT-PCR was performed to further characterize the 5' structure of *NAGS* transcripts in the liver. Primers were designed at the beginning of each TSS identified in 5'RACE experiments. Each of these primers was paired with the *NAGS*RACE3 primer used in the 5'RACE experiments. Using human liver first strand cDNA as template in the PCR, single products of the expected sizes were amplified with the liver 2/*NAGS*RACE3 and the liver 3/*NAGS*RACE3 primer pairs (Figure 3.8). As expected both products aligned to the region 5' of *NAGS*. Unexpectedly, multiple products were amplified using the liver 1/*NAGS*RACE3

primer pair. Several attempts were made to optimize this primer pair by raising the annealing temperature during PCR amplification to increase primer specificity. Each time, multiple products were still amplified. These products were cloned and sequenced to determine if they represent *NAGS* transcripts.

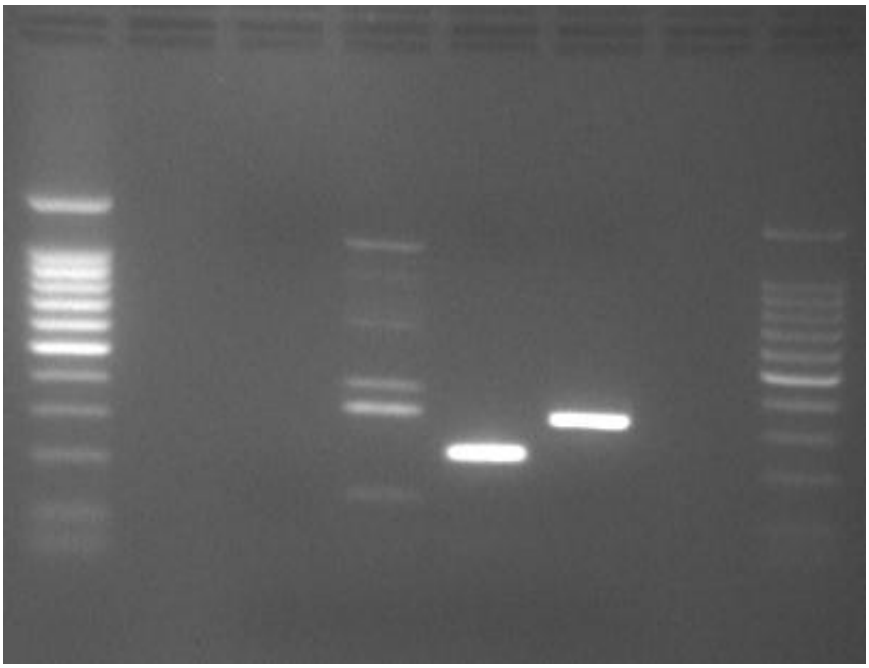


Figure 3.8: RT-PCR of *NAGS* liver transcripts 1, 2, 3.
RT-PCR was performed with forward primers located at the beginning of the transcriptional start sites identified in 5' RACE of *NAGS* in human liver. Lane 1: 100 bp ladder (Promega), Lane 2: empty, Lane 3: H2O blank, Lane 4: liver transcript 1 RT-PCR, Lane 5: liver transcript 3 RT-PCR, Lane 6: liver transcript 2 RT-PCR, Lane 7: empty, and Lane 8: 100 bp ladder (Promega). Note: The 500 bp fragment in the ladder is at increased intensity.

Two additional alternatively spliced transcripts were identified (Figure 3.9). These alternatively spliced isoforms originate from the previously unidentified upstream exon of liver 1 transcript. The first alternatively spliced isoform (liver 1 isoform 2) contains a longer intronic region, 5159 bp compared to 4968 bp, and

thus has a shorter 5'UTR of only 194 bases. The second alternatively spliced isoform (liver 1 isoform 3) contains a second previously unidentified exon upstream of the translational start site. This isoform consists of a 60 base pair exon followed by a 1367 base pair intron, then another 121 base pair exon and an intron spanning 3671 base pairs. The 5'UTR for this isoform is 315 base pairs long. It is important to note that the band on the gel corresponding to the liver 1 isoform 1 is the most intense suggesting it is more highly expressed than alternatively spliced liver isoforms 2 and 3.

A third potential splice variant was identified in a separate RT-PCR experiment. Using liver first strand cDNA as template, a single product of 300 bp was amplified by PCR using the liver 1 forward primer and a reverse primer in exon 1 of *NAGS*. This was not the expected size. Sequence analysis revealed that this product aligned to *NAGS*, but represented another potential transcript isoform. This isoform (liver 1 isoform 4) includes the 60 base pair novel upstream exon and then an intron spanning 5459 base pairs (Figure 3.9).

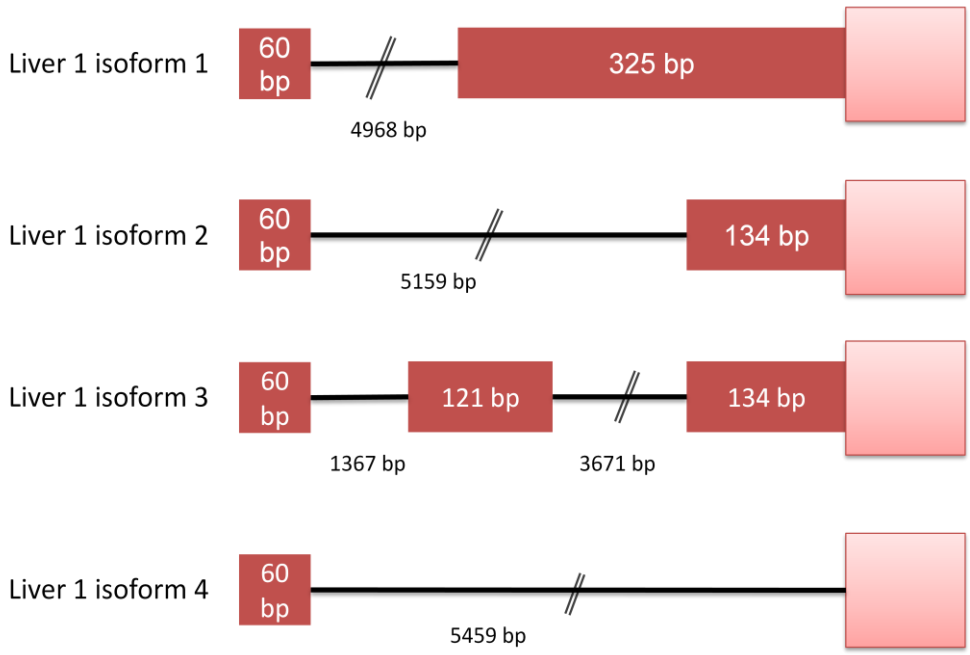


Figure 3.9: *NAGS* liver 1 transcript is alternatively spliced. Diagram of alternatively spliced isoforms of *NAGS* liver 1 transcript. These splice variants were identified through RT-PCR and sequence analysis.

Discussion

My experimental results reveal that the *PYY* transcript containing exons 4-7 is the major isoform in most tissues, particularly in the ileum and colon where *PYY* is most abundantly expressed. A novel *PYY* transcript initiating in exon 2 was identified. This isoform is expressed at highest levels in the pancreas and kidney. In fact, it appears to be the primary transcript in the kidney, while in the pancreas its expression is approximately equal to that of the *PYY* exon 4-7 isoform. *PYY* transcripts containing exon 1 were not expressed at appreciable levels in any of the tissues we tested. This transcript was originally identified

from a cDNA library (NIH_MGC_98; Mammalian Genome Collection clone BC041057.1) derived from a grade IV brain astrocytoma. More than likely, it represents a very minor transcript that is perhaps expressed more strongly in a specific neuronal cell type, or under certain conditions.

Three TSSs for *NAGS* were identified that suggest three types of liver transcripts. The liver 1 transcript contains a previously unidentified upstream exon, and multiple alternatively spliced isoforms were observed originating from the TSS at this novel exon. These splice variants likely represent minor transcript isoforms as they were not amplified in the 5' RACE experiments and they were not robustly amplified in the RT-PCR.

Liver transcripts 1 and 2 completely overlap the liver 3; and while not strictly quantitative, the results from agarose gel electrophoresis suggest this is the least abundant transcript. Thus, it is possible that liver 3 product does not represent a true TSS. During the initial reverse transcription step, the reverse transcriptase can pause or terminate at low frequency before reaching the 5'end of the transcript (Carey and Smale 2000). Such early termination events may occur more often in GC-rich regions where RNA secondary structures form. These truncated reverse transcription products can be preferentially amplified during subsequent rounds of PCR, and thus appear to be a major TSS. Therefore, it is possible the liver 3 transcript identified in 5' RACE experiments does not represent a genuine TSS. Overall, the results of my experiments reveal a complex 5' structure for *NAGS* liver transcripts.

A single TSS for *NAGS* was identified in the ileum that was distinct from the TSSs identified in the liver. This suggests that *NAGS* is differentially regulated in these tissues. This is reminiscent of the tissue specific regulation of the urea cycle enzyme ornithine transcarbamylase (*OTC*). In the small intestine, the *OTC* promoter is activated through binding of HNF-4 (Takiguchi and Mori 1995). In the liver, activation of the promoter requires binding by both HNF-4 and C/EBPβ (Takiguchi and Mori 1995). We identified tissue-specific transcripts in the liver and small intestine suggesting that *NAGS* is regulated by alternative promoters in these tissues. The transcription factor HNF-4 is enriched in liver and small intestine and therefore, as with *OTC*, it may be important for the regulation of *NAGS* in both of these tissues. It is likely that other tissue-selective transcription factors may play a role in regulating *NAGS* via these alternative promoters.

Taken together my results indicate that both *PYY* and *NAGS* are regulated by alternative promoters in a tissue-specific manner. These experiments further reveal that the ileum TSSs for *NAGS* and *PYY* are separated by approximately 50 kb, which indicates they are not under the control of a bidirectional promoter. Recall, that by definition, a bidirectional promoter is the DNA sequence between divergently transcribed genes that are separated by 1000 base pairs or less. Although *PYY* and *NAGS* are not regulated by a bidirectional promoter, they may still be coordinately regulated via shared *cis* regulatory elements. The *CYP1A1* and *CYPIA2* genes are divergently transcribed and separated by an approximately 23 kb intergenic region (Ueda et al 2006). Located in this

intergenic space are regulatory elements that function bidirectionally to positively and negatively regulate expression of both *CYP1A1* and *CYP1A2* (Ueda et al 2006). Thus, it is possible that the intergenic region between *PYY* and *NAGS* contains regulatory elements that are specific to each gene, as well as elements that are common to both genes.

In conclusion, the TSSs for *PYY* and *NAGS* were characterized in multiple tissues. Multiple TSSs were identified suggesting these genes are regulated by alternative promoters. Furthermore, these data demonstrate that the TSSs for *PYY* and *NAGS* are separated by greater than 30 kb and 50 kb in the kidney and ileum, respectively, which indicates they are not under control of a bidirectional promoter in these tissues. Importantly, while the regulatory landscape has been redefined, this does not rule out the possibility of coordinate regulation in *cis* as these genes still share a 5' flanking region.

CHAPTER IV

HUMAN EXPRESSION OF *PYY* AND *NAGS*

Introduction

PYY and *NAGS* are divergently transcribed and, as revealed in Chapter III, their TSSs are separated by an intergenic region of approximately 51 kb in the ileum (Figure 4.1). Since these two genes share a 5' flanking region it is possible they have *cis* regulatory elements in common, as do the divergently transcribed gene pair *CYP1A1* and *CYP1A2* (Ueda et al 2006). If *PYY* and *NAGS* are coordinately regulated, then the expectation is they will be coordinately expressed in at least some tissues.

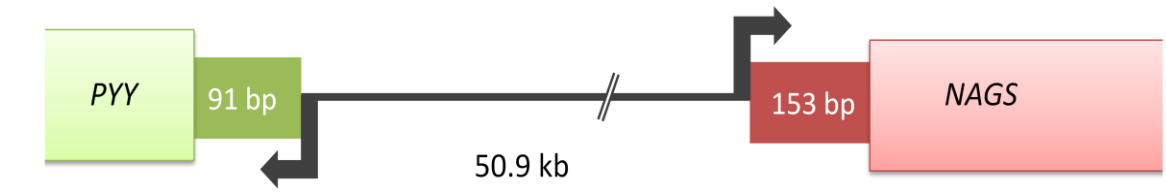


Figure 4.1: *PYY* and *NAGS* TSSs are separated by more than 50kb in the ileum. *PYY* and *NAGS* are divergently transcribed and thus share a 5' flanking region. In the ileum their TSSs are separated by 50.9 kb. The TSS and direction of transcription for each gene are indicated by the arrows; solid blocks represent 5' UTR and shaded boxes represent coding sequence.

PYY is unique among its family members in that it functions as both a hormone and a neuropeptide (Soderberg et al 1994); its expression pattern reflects this dual function. *PYY* is expressed in both enteroendocrine and neuronal cell populations (Ekblad and Sundler 2002; Leiter et al 1987). It is

secreted by endocrine cells of the colon, ileum, and pancreas (Leiter et al 1987). Adrian *et al* determined that *PYY* is expressed at very low concentrations in the proximal small intestine, including the duodenum and jejunum (Adrian et al 1985). It is expressed at higher concentrations in the ileum with increasing expression throughout the colon, and is most highly expressed in the rectum (Adrian et al 1985). It is also expressed in peripheral neurons, specifically those of the enteric nervous system, as well as in a small population of neurons in the brainstem (Ekblad and Sundler 2002). Expression has also been observed in the ovary and testis (Ekblad and Sundler 2002; Leiter et al 1987).

NAGS is critical for function of the urea cycle and therefore, it is highly expressed in the liver. It is also expressed in the small intestine, along with the urea cycle enzymes CPS1 and OTC (Caldovic et al 2002a; Takiguchi et al 1989). Expression of *NAGS* has been observed in the adult kidney (Haberle et al 2003), and at low levels in fetal lung, pancreas, placenta, heart, and brain (Caldovic et al 2002a). Studies of mouse *Nags* reveal similar expression patterns with the addition of expression in the spleen and testis (Caldovic et al 2002b).

Based on previous reports both *PYY* and *NAGS* are expressed in the small intestine. However, no studies have examined the expression of these two genes concurrently in this or any tissue. It is important to emphasize that divergently transcribed gene pairs may be coordinately regulated in a tissue specific manner. Trinklein *et al* demonstrated that putative bidirectional promoters located between divergently transcribed genes pairs display bidirectional activity differentially in various cell types (Trinklein et al 2004).

Across the four cell lines tested, 22% of bidirectional promoter sequences showed bidirectional activity in half of the cell lines and unidirectional activity in the other half (Trinklein et al 2004). Given that coordinate regulation may be tissue specific it is important to establish in what tissues, if any, *PYY* and *NAGS* are coordinately expressed.

Materials and Methods

Quantitative real time PCR (qRT-PCR)

Primers and probes

Quantitative real time RT-PCR was used to determine the presence and amount of *PYY* and *NAGS* transcripts in a panel of human tissues. The following TaqMan gene expression assays were used: *PYY* (Hs00373890_g1) probe spans 6-7 and *NAGS* (Hs00400246_m1) probe spans exons 4-5.

Human total RNA

Commercially available human total RNA samples were obtained from Clontech, Chemicon, Stratagene, and Ambion (based on availability of RNA from desired tissues). All RNA samples were isolated from normal human tissues. Most samples were derived from a single individual, while a few were pooled from multiple individuals. RNA purity and concentrations were assessed by the individual manufacturer. Human total RNA samples included: brainstem, colon, ileum, kidney, liver, pancreas, skeletal muscle, small intestine, spleen, and testes. With the exception of skeletal muscle which serves as a negative control,

these tissues are all known or reported sites of expression for *PYY*, *NAGS*, or both.

First strand cDNA synthesis

First strand cDNA synthesis was performed using the SuperScript™ III First Strand Synthesis System (Invitrogen, catalog #18080-051). Following the manufacturer's instructions, 20 µL reactions were set up using oligo(dT) primer and 4 µg of total RNA. RNA/primer mixtures were prepared and incubated at 65°C for 5 minutes and then placed on ice. Next, 10 µL of cDNA synthesis master mix was added to each tube and reactions were incubated at 50°C for 50 minutes and 85°C for 5 minutes. Finally, 1 µL of RNAse H was added to each tube, and reactions were incubated at 37°C for 20 minutes.

Standard curve preparation

Standard curves were prepared using full-length *PYY* and *NAGS* cDNAs (Open Biosystems clone IDs 40005903 and 5441270, respectively) for absolute quantitation of mRNA transcripts. Standard curves consisted of ten-fold dilutions of full-length plasmid cDNA ranging from 500pg to 5×10^{-6} pg.

To prepare plasmid cDNA for standard curves, 5 mL cultures of bacteria containing plasmid with full-length *PYY* or *NAGS* cDNA were grown overnight in a 37°C shaking incubator. Plasmid isolations were performed using the Qiagen plasmid miniprep kit. Plasmid DNA was eluted in nuclease-free water. DNA quality and concentration were assessed via spectrophotometric readings at 260, 280 and 230 nm. A 5 ng/µl dilution was prepared. This was then serially diluted 1:10 to a final concentration of 5×10^{-6} pg.

qRT-PCR set-up

All samples and standards were assayed in triplicate. Reactions were performed in a total volume of 10 µL and included: 5 µL TaqMan gene expression 2X master mix (Applied Biosystems cat #4369016), 0.5 µL TaqMan gene expression assay for the gene of interest, 0.5 µL TaqMan gene expression assay for an endogenous control gene, and 4 µL of cDNA/nuclease free water mixtures. Standard curve cDNA/nuclease-free water mixes consisted of 1 µL of standard cDNA and 3 µL of nuclease-free water. The cDNA/nuclease-free water mixtures for the unknown samples consisted of 0.5 µL cDNA and 3.5 µL nuclease-free water, which corresponds to 100 µg RNA per reaction. The endogenous control gene assay was not included in the standard curve reactions; therefore, 0.5 µL of nuclease-free water was added to make up the volume.

qRT-PCR analyses were performed using Applied Biosystems Prism® 7900 HT Sequence Detection System and the accompanying software SDS 2.3 (Applied Biosystems, Inc., Foster City, CA). The following standard thermal cycler protocol was used: 50°C 2 minutes, 95°C 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The ramp rate for temperature change was 100%.

The SDS 2.3 analysis software generated an equation of the line for each set of standards. The highest concentration standard was dropped for each assay because it crossed the threshold before the set baseline. The r^2 was ≥ 0.99 for each standard curve. These curves were used to calculate the quantity

of *PYY* and *NAGS* mRNA transcripts in the panel of human tissues. The calculated quantities are equivalent to picograms (pg) of plasmid. Therefore, the expression (quantity) of transcript in the unknown samples is given the unit designation pg equivalents, or pge.

Cell line screens

The following cell lines were obtained and cultured per vendor recommendations: CaCo2, HT29, HuTu 80, and Fhs 74 Int. With the exception of CaCo2 and HT29 lines, cells were treated with a variety of compounds reported to induce expression of either *PYY* or *NAGS* including insulin-like growth factor 1 (IGF-1) which was shown to increase promoter activity of rat Pyy (Wang et al 2004). Cells were also treated with dexamethasone, a potent glucocorticoid. There is considerable evidence, primarily from cultured hepatocytes, demonstrating increases in expression of urea cycle enzymes in response to glucocorticoids including dexamethasone (Morris, Jr. et al 1987; Morris, Jr. 2002; Morris, Jr. and Kepka-Lenhart 2002; Takiguchi and Mori 1995). Amino acids were also used to treat cells because protein increases expression of both *NAGS* and *PYY in vivo* (Batterham et al 2006; Morris, Jr. 2002).

RNA was isolated from cells using a phenol-chloroform based extraction procedure. Growth media was removed from the cells which were then lysed with the phenol-based TRIzol® reagent (Invitrogen cat #15596-018) and RNA was isolated per the manufacturer's protocol. The concentration and purity of RNA was assessed via spectrophotometric readings at 260, 280 and 230 nanometers. Non-quantitative RT-PCR was used to screen CaCo2 and HT29

cells for expression of *PYY* and *NAGS*. Quantitative real-time PCR was performed (as described above) to determine the expression of *PYY* and *NAGS* in treated and untreated cells.

Results

Quantitative real-time RT-PCR on human tissues

qRT-PCR analyses revealed distinct expression patterns for *PYY* and *NAGS* (Figure 4.2). *PYY* was expressed most highly in the colon and ileum with a mean quantity of 0.398 ± 0.040 pge and 0.216 ± 0.001 pge, respectively (Table 4.1). It was expressed at lower levels in the small intestinal sample (0.030 ± 0.001 pge), kidney (0.003 ± <0.001 pge), and pancreas (0.009 ± <0.001 pge). Very low expression was observed in the brainstem ($3.5 \times 10^{-4} \pm 2.5 \times 10^{-5}$pge). Expression of *PYY* was not detectable in liver, spleen, testes, or skeletal muscle. The observed expression pattern of *PYY* is consistent with previous reports (Adrian et al 1985; Krasinski et al 1990; Leiter et al 1987).

NAGS was most highly expressed in the liver and ileum with a mean quantity of 0.232 ± 0.054 pge and 0.243 ±0.021 pge, respectively. It was also expressed in the small intestinal sample (0.070 ± 0.003 pge) and the kidney (0.093 ± 0.012 pge). Low levels of expression were observed in the colon (0.016 ± 0.010 pge), pancreas (0.006 ± 0.000 pge), and brainstem (0.014 ± 0.002 pge), with lowest expression in the testes (0.001 ± 0.001 pge). *NAGS* expression was not detectable in spleen or skeletal muscle.

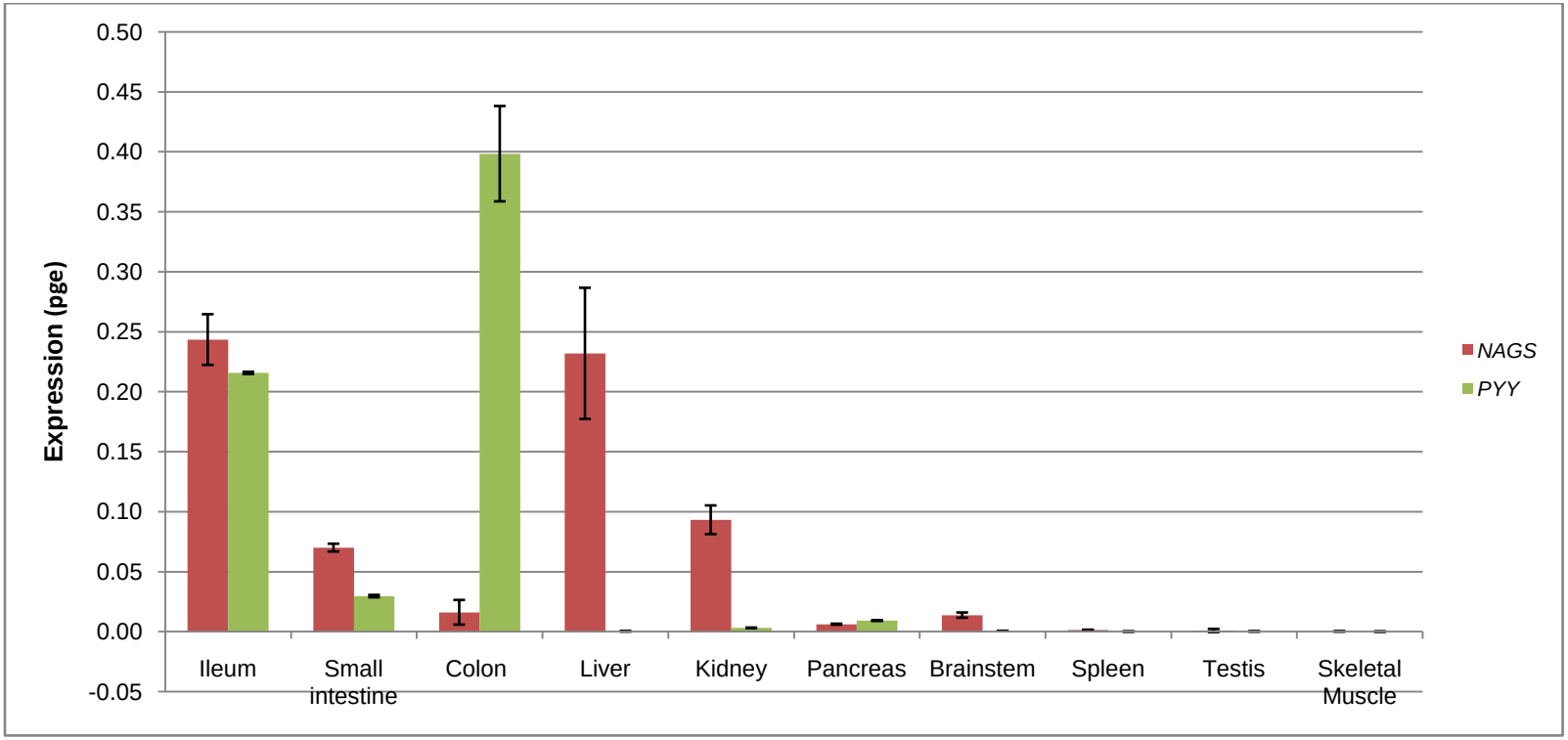


Figure 4.2: Expression of *NAGS* and *PYY* in a panel of normal human tissues.
Quantitative real-time RT-PCR was performed on each sample in triplicate. Plasmid cDNA was used to generate standard curves for each gene and the quantity of mRNA was calculated based on the standard curve. The expression (quantity) of transcript is given the unit designation pg of plasmid equivalents, or pge. The average quantity is graphed and error bars represent the standard deviation of triplicate measures.

	PYY		*NAGS*	
Tissue	**Ave Ct ± St dev**	**Ave Qty ± St dev**	**Ave Ct ± St dev**	**Ave Qty ± St dev**
Ileum	21.56 ± 0.01	0.216 ± 0.001	21.53 ± 0.12	0.243 ± 0.021
Small intestine	24.36 ± 0.04	0.030 ± 0.001	23.23 ± 0.06	0.070 ± 0.003
Colon	20.71 ± 0.14	0.398 ± 0.040	25.40 ± 0.80	0.016 ± 0.010
Liver	31.62 ± 0.17	<0.001 ± <0.001	21.62 ± 0.35	0.232 ± 0.054
Kidney	27.49 ± 0.06	0.003 ± <0.001	22.84 ± 0.17	0.093 ± 0.012
Pancreas	26.01 ± 0.04	0.009 ± <0.001	26.54 ± 0.09	0.006 ± <0.001
Brainstem	30.59 ± 0.10	<0.001 ± <0.001	25.46 ± 0.21	0.014 ± 0.002
Spleen	33.56 ± 0.56	<0.001 ± <0.001	28.55 ± 0.17	0.001 ± <0.001
Testis	32.36 ± 0.66	<0.001 ± <0.001	30.88 ± 2.67	0.001 ± 0.001
Skeletal Muscle	34.52 ± 0.90	<0.001 ± <0.001	32.02 ± 0.51	<0.001 ± <0.001

Table 4.1: Quantitative real-time RT-PCR (qRT-PCR) average Ct and quantity values for *PYY* and *NAGS*.
Expression of *PYY* and *NAGS* was determined in a panel of normal human tissues using qRT-PCR.
All values reflect the mean of triplicate measures ± the standard deviation.

Cell culture experiments

Next, I wanted to examine promoter function using an *in vitro* cell culture system with two goals in mind. The first goal was to determine if expression of *PYY* and *NAGS* responds in the same way under different treatment conditions. For example, IGF-1 is reported to increase expression of *PYY (Wang et al 2004)*; if cells are treated with IGF-1 to increase *PYY* expression, would there also be a similar increase in *NAGS* expression? If expression of both genes increases or decreases in response to the same treatment then this would provide some evidence the two genes are coordinately regulated. The second goal was to identify *cis* regulatory elements in the shared 5' flanking region that are necessary for regulation of these genes using transient transfection reporter gene assays.

An absolute requirement for these experiments is a cultured cell line that endogenously expresses the gene or genes from which the promoter is derived. Thus, the first step was to identify a cell line that endogenously expresses *PYY* and *NAGS*. Cell lines were selected based on human tissue expression of *PYY* and *NAGS*. The gene expression studies presented above reveal that *PYY* and *NAGS* are similarly expressed in the ileum and pancreas which makes cell lines derived from these tissues attractive candidates for examining regulation of this gene pair. Moreover, the ileum is physiologically relevant to the idea that *PYY* and *NAGS* are coordinately regulated in response to dietary protein intake. The number of available small intestinal cell lines is small and none are specifically derived from the ileum. Therefore, we examined cell lines derived from other

tissues as well, screening the following cell lines: CaCo2 (ATCC #HTB-37), HT29 (ATCC #HTB-38), HuTu 80 (ATCC #HTB-40), and Fhs 74 Int (ATCC #CCL-241).

PYY is highly expressed in the colon and while *NAGS* expression is significantly lower in this tissue, I chose to screen two cell lines derived from human colon – CaCo2 and HT29. These immortal cell lines are derived from colon adenocarcinomas and display morphology of epithelial cells. Non-quantitative RT-PCR results reveal that neither CaCo2 nor HT29 express *PYY* or *NAGS*.

The remaining two cell lines, HuTu 80 and Fhs 74 Int, are both small intestinal lines. HuTu 80 is an immortal cell line taken from the duodenum of a small intestinal adenocarcinoma, and Fhs 74 Int is a primary cell line derived from normal fetal small intestine at 3-4 months gestation. The HuTu 80 line was shown to express *PYY* through non-quantitative RT-PCR analysis (Rozengurt et al 2006). Results of my experiments demonstrate that *PYY* and *NAGS* are expressed at similar, albeit low levels in both the HuTu 80 and Fhs 74 Int cells. As shown in Figure 4.3 the expression of *NAGS* and *PYY* in the HuTu80 (0.0004 pge) and Fhs 74 Int (0.0001 pge) cells was negligible compared to expression in normal human small intestine (0.070 pge *NAGS* and 0.030 pge *PYY*) and ileum (0.243 pge *NAGS* and 0.216 pge *PYY*). Expression of these genes could not be consistently induced above basal levels under any treatment condition suggesting these are not appropriate cell lines for investigating regulation of *PYY* and *NAGS*.

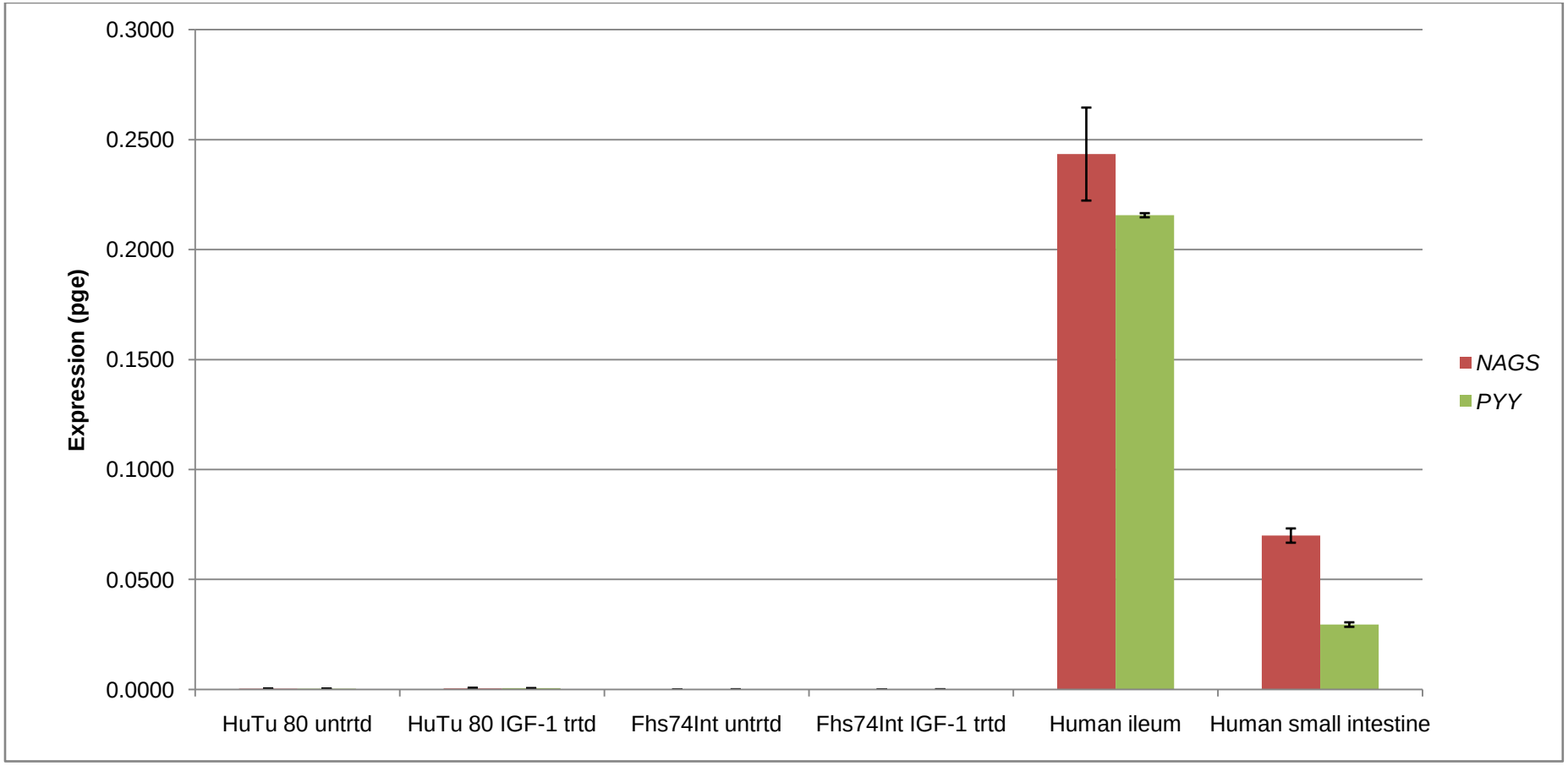


Figure 4.3: Expression of *NAGS* and *PYY* in HuTu 80 and Fhs 74 Int cell lines compared with human ileum and small intestine. Quantitative real-time RT-PCR was performed on each sample in triplicate. Plasmid cDNA was used to generate standard curves for each gene and the quantity of mRNA was calculated based on the standard curve. The expression (quantity) of transcript is given the unit designation pg of plasmid equivalents, or pge. The average quantity is graphed and error bars represent the standard deviation of triplicate measures.

Discussion

PYY and *NAGS* display distinct patterns of expression among the human tissues assayed. These genes are expressed concordantly in some tissues and discordantly in others. *PYY* and *NAGS* are expressed at similar levels to one another in the ileum and pancreas, with expression in the pancreas much lower than in the ileum. *PYY* was expressed highly in the colon where the expression of *NAGS* was low. Conversely, in the liver where *NAGS* was highly expressed, *PYY* was undetectable. Taken together, the expression data suggest that if *PYY* and *NAGS* are coordinately regulated it occurs in a tissue-specific manner in the ileum and pancreas.

The question then arises, why might *PYY* and *NAGS* be coordinately regulated in some tissues, but not in others? If *PYY* and *NAGS* were coordinately expressed in all tissues then it would be possible their correlated gene expression is simply a function of their location and arrangement. However, the possibility of tissue-specific coordinate regulation of *PYY* and *NAGS* implies there may be a functional link in tissues where they are coordinately expressed.

At first glance these genes have no apparent functional connection, but upon further examination a profound connection emerges. NAGS is necessary for the successful processing of waste nitrogen into urea. It is thought to regulate ureagenesis by varying the amount of NAG available for CPS1 which catalyzes the first and rate-determining step of the urea cycle. The primary source of waste nitrogen in humans is from our diet; the largest proportion coming from

digestion of dietary protein. One function of PYY is in appetite regulation inducing satiety in response to ingested nutrients. A greater and more sustained response has been observed upon ingestion of protein compared to fats and carbohydrates (Batterham et al 2006; Lomenick et al 2009). Both *NAGS* and *PYY* are upregulated in response to protein. The sustained increases of PYY in response to protein may suppress further nitrogen intake while excess nitrogen is processed through the urea cycle, of which *NAGS* is a potential regulator. Therefore, co-regulation of *PYY* and *NAGS* would provide a link between suppression of nitrogen intake and processing of waste nitrogen.

The differential regulation of this gene pair in tissues is likely governed by both the function of the gene products and the environmental context of the respective tissues. The small intestine is the principal site for protein absorption. The absorptive capacity for di- and tri- peptides is greater in the proximal small intestine, while in the distal small intestine (ileum) the absorptive capacity is greater for single amino acids (Johnson et al 2006). Thus, in the ileum, *PYY* and *NAGS* may be upregulated by amino acids which is consistent with the idea that co-regulation of this gene pair would contribute to the maintenance of nitrogen balance.

Upregulation of *PYY* and *NAGS* by amino acids may also explain why expression of this gene pair differs in the ileum and colon. By the time luminal contents enter the ileocecal junction absorption of protein is nearly complete (Johnson et al 2006). Thus, the concentration of amino acids is much lower in the colon. This change in the extracellular milieu from the ileum to the colon may

explain why there is decreased expression of *NAGS* in the colon. However, this does not explain the increased expression of *PYY* in the colon. Recall, that PYY serves multiple functions in addition to its role in appetite regulation. It functions to inhibit digestive enzyme secretion, gastric acid secretion, and gut motility. Furthermore, studies demonstrate a role for PYY in the maintenance of the mucosal epithelium of the gut. It is likely that the primary function(s) of PYY in the colon differs from that of the ileum and consequently the gene is alternatively regulated in these tissues. For example, PYY in the colon may function primarily in gastric motility and maintenance of gut epithelium. IGF-1 which has been shown to increase *PYY* promoter activity might be important in the regulation of *PYY* with regards to proliferation of the gut epithelium and may play a larger role in regulation of this gene in the colon.

The low expression of *NAGS* in the colon may simply be due to leaky transcription. *PYY* is highly expressed in the colon and because that region is actively transcribed, it is possible that some transcription of *NAGS* may also occur. While it is possible that expression of *NAGS* in the colon is the result of leaky transcription, I think the more plausible explanation is that colonic expression of *NAGS* serves as a sensor for ammonia load. The colon is the site of urea nitrogen salvage in which ammonia is produced from hydrolysis of urea by bacterial ureases. Additionally, there are multiple binding sites for the ubiquitous transcriptional regulator CCCTC-binding factor (CTCF) in the 5' flanking region shared by *PYY* and *NAGS*. CTCF can function as either a transcriptional repressor or activator, and importantly has been identified as the

vertebrate insulator protein (Bell, West, and Felsenfeld 1999; Kim et al 2007). Thus, CTCF may play an important role in the tissue-specific regulation of *NAGS* and *PYY*

Although, the expression pattern observed in the ileum is not inconsistent with the hypothesis of coordinate regulation of *PYY* and *NAGS*, the data are not definitive. To further investigate promoter function, cell lines were screened to identify one that endogenously expresses *PYY* and *NAGS*. While two small intestinal cell lines were identified that expressed *PYY* and *NAGS* at extremely low levels, I was unable to consistently induce expression above basal levels in either of these lines. One of these is derived from the duodenum where endogenous expression of *PYY* is low; therefore, it is possible these cell lines do not contain the full complement of regulatory factors necessary for enhanced expression of these genes. Furthermore, Dr. Andrew Leiter, who has been involved in research of PYY for greater than fifteen years also searched for a *PYY*-expressing line and was unable to identify one (personal communication). This suggests that current cell lines may not be a useful tool for investigating expression and regulation of *PYY* and *NAGS*. To address this issue, the experimental design was shifted to investigations using the mouse model organism and human populations. These investigations are detailed in Chapters V and VI of this dissertation.

While the close location of these genes to one another implies that chromatin structure might be favorable for transcription of both genes simultaneously, it is possible these genes are not coordinately regulated via

shared *cis*-regulatory elements. These genes may be co-regulated indirectly, that is, changes in expression in response to the same physiologic signal, but not through the same *cis*-regulatory element. *NAGS* and *PYY* could be upregulated concurrently through a common signaling pathway in response to protein. The mTOR pathway is an attractive candidate because it is upregulated in response to increases in amino acids. Consequently, even if *PYY* and *NAGS* are not coordinately regulated in *cis*, but indirectly through mTOR signaling via amino acids the proposed functional link in the maintenance of nitrogen balance would still hold. Therefore, further studies in whole animals are needed to firmly establish whether *PYY* and *NAGS* are indeed coordinately regulated in *cis* in a tissue-specific manner, and if co-regulation of this gene pair plays a role in nitrogen balance.

CHAPTER V

INVESTIGATING *IN VIVO* EXPRESSION OF *PYY* AND *NAGS* USING MOUSE AS A MODEL ORGANISM

Introduction

The experimental results described in Chapters III and IV established the following: *PYY* and *NAGS* are expressed concordantly in the ileum, but are not regulated by a bidirectional promoter in this tissue because the TSSs are separated by greater than 1000 bp. However, it is possible that *PYY* and *NAGS* are coordinately regulated via common regulatory elements located in their shared 5' flanking region.

A logical next step in testing the hypothesis that *PYY* and *NAGS* are coordinately regulated in *cis* would be to identify DNA sequence elements within the promoter region that are important for expression of both genes. Employing transient transfection assays with promoter deletion constructs is a commonly used method to pinpoint regions of the promoter that are critical for gene expression (Carey and Smale 2000). However, as discussed in Chapter IV, there are currently no cell lines which recapitulate endogenous expression of *PYY* and *NAGS*. Consequently, I was unable to examine promoter function using an *in vitro* cell culture system. This necessitated a move to an *in vivo* system for investigating regulation of *PYY* and *NAGS*.

There are limitations to studying regulation of *NAGS* and *PYY* in humans. The biggest obstacle is that expression of these two genes cannot be directly

measured under different treatment conditions. The focus of this study is on expression of *NAGS* and *PYY* in the ileum which cannot be measured without directly assaying tissue samples from human subjects which is not feasible. Evaluating the amount of NAGS and PYY protein could provide some clues; and while PYY is secreted and can be measured in plasma, NAGS is not secreted and cannot be assessed without directly assaying tissues. As a result, a model organism was employed to investigate regulation of *NAGS* and *PYY*.

The mouse model organism provides a good system for studying regulation of *NAGS* and *PYY in vivo*. First, like humans, mice process waste nitrogen through the urea cycle. Furthermore, while sequence conservation between *NAGS* of human and mouse varies across the gene, overall they share approximately 86% sequence homology. The conserved region, comprised of the 440 C-terminal amino acids that make up the catalytic domain of the protein, is 92% identical between human and mouse (Caldovic et al 2002a). The N-terminal region containing the putative mitochondrial targeting sequence shares 63% identity, while the variable domain shares only 35% identity (Caldovic et al 2002a).

Additionally, the genomes of mouse and human are similar with many regions of synteny. Indeed, in mouse the divergent arrangement of *Pyy* and *Nags* is conserved. These genes are located on mouse chromosome 11, and are separated by approximately 39 kb. Moreover, mice can be subjected to different feeding conditions, and tissues can be collected to examine expression of *Pyy* and *Nags*. For instance, *Pyy* and *Nags* are both upregulated in response

to dietary protein intake in mice and humans (Batterham et al 2006; Morris, Jr. 2002). I proposed in Chapter IV that increased concentration of amino acids in the ileum from digestion of dietary protein results in upregulation of *PYY* and *NAGS.* If expression of these genes is increased or decreased in response to alterations in dietary protein intake, then this would provide some evidence in support of my hypothesis of coordinate regulation. Thus, to determine if there is a coordinate response of *Pyy* and *Nags* to changes in dietary protein intake expression of these genes was measured in tissues of mice under a variety of feeding conditions.

Materials and Methods

Immunohistochemistry

A sample of ileum from a wild type male mouse at 8 weeks of age was obtained from Dr. Robert Coffey's lab (Vanderbilt University). This sample which was fixed and paraffin embedded in March 2009 was submitted to the Vanderbilt Immunohistochemistry (IHC) Core for sectioning and staining with PYY and NAGS antibodies. A rabbit polyclonal antibody to PYY was obtained from Abcam (ab22663). This antibody was previously used for IHC/IF applications (Ali-Rachedi et al 1984). Very few NAGS antibodies are commercially available, none of which have been tested for IHC applications. Three antibodies were selected and tested in these studies: 1) rabbit polyclonal to NAGS from Abcam (ab65536), 2) a goat polyclonal to a peptide mapping near the N-terminus of human NAGS from Santa Cruz Biotechnology (NAGS (Q12); sc-132384), and 3)

a rabbit polyclonal to NAGS from Aviva Systems Biology (ARP51183-T100). The immunogen sequence of NAGS (Q12) antibody from Santa Cruz Biotechnology is 100% homologous to mouse Nags and is thus predicted to react with the mouse homolog. The NAGS antibody from Aviva Systems Biology was raised against a synthetic peptide matching amino acids 433-482 in the C-terminal region of human NAGS. The 50 amino acid immunogen sequence is: PVLGGTPYLDKFVVSSSRQGQGSGQMLWECLRRD LQTLFWRSRVTNPINP. There is 100% sequence homology between the human-derived immunogen and mouse Nags, so this antibody is expected to cross react with mouse.

Animal Care

Animals were group-housed in the pathogen free barrier facility at Vanderbilt University and maintained on a 12:12 hour light-dark cycle (light 0600h-1800h, dark 1800h-0600h) under controlled temperature (21 ± 2°C). Unless otherwise stated, all animals had *ad libitum* access to standard laboratory chow (Purina rodent diet 5001; Purina Mills, St Louis, MO) and water. Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Vanderbilt University.

Fast/re-feed study

The aim of this experiment was to compare expression of *Nags* and *Pyy* in tissues of mice that were fasted and then re-fed a low or high protein diet. Male

C57BL/6J mice were removed from group-housing and placed one mouse per cage. Mice were individually housed so that food intake could be monitored. After allowing the mice some time to acclimate to the new housing arrangement, they were fasted for 15 hours (1700h-0800h; n=10) and then given access to either a low protein (LP; n=5) or high protein diet (HP; n=5). The following Harlan Laboratories Teklad custom diets were used: 20% protein (TD.91352) and 40% protein (TD.90018). Macronutrient compositions of these isocaloric diets are given in Table 6.1. Body weight and food intake data were collected (Table 6.2). Initial food weight was recorded upon re-feeding and food weights were collected at 9 am, 10 am, 12 pm, and 2 pm. Intake was calculated for each time period by subtracting the new food weight from the previous food weight. The amount of protein consumed during each interval was calculated by multiplying percent protein in the diet by the amount of food consumed.

After six hours of *ad libitum* access to either the LP or HP diet, animals were euthanized using a terminal dose of isoflurane, and blood, liver, kidney, proximal intestine (duodenum), intermediate intestine (ileum), and distal intestine (colon) were collected. The intestine and colon samples were rinsed with phosphate-buffered saline (PBS) to remove any contents. All tissues were placed in RNALater (Ambion catalog # AM7020) and stored for a minimum of 24 hours before proceeding with RNA isolation.

Chronic LP or HP diets study

The aim of this study was to determine if there were differences in expression of *Pyy* and *Nags* in the ileum of mice chronically fed either a LP or HP diet. Male C57BL/6J mice between 13 and 16 weeks of age were switched from standard laboratory chow to either a low (20%; n=5) or high protein diet (40%; n=7). Diets were as described above (Table 6.1). Mice were kept on their respective diets for 3.5 weeks. Animals were euthanized using a terminal dose of isoflurane, and blood, liver, kidney, ileum (4 cm of the small intestine proximal to the cecum), and colon (2 cm just distal to the cecum) were collected. The ileum and colon were rinsed with PBS to remove any contents. All tissues were placed in RNALater (Ambion) and stored for a minimum of 24 hours before proceeding with RNA isolation.

Fasted/Fed study

The goal of this experiment was to determine if expression of *Pyy* and *Nags* changes in response to fasting. Male C57BL/6J mice, 8 weeks of age, were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were group-housed (4 mice to a cage). At 9 weeks of age experimental animals were fasted for 16 hours (1700h-0900h; n=8) while a control group was given free access to food (n=8). Animals were euthanized using a terminal dose of isoflurane, and blood, liver, kidney, ileum (4 cm small intestine proximal to cecum), and colon (2 cm just distal to the cecum) were collected. The ileum and colon were rinsed with PBS to remove fecal matter. All tissues were placed in

RNALater (Ambion) and stored for a minimum of 24 hours before proceeding with RNA isolation. Fasted mice were dissected first to minimize fasting time differences between these mice. All dissections (fasted and fed mice) were completed in approximately 1.5 hours.

Serum collection

Blood was allowed to coagulate on ice for approximately 2 hours after final dissection. It was then centrifuged at 2000 x g for 15 minutes; serum was collected and stored at -20°C.

Table 5.1: Composition of protein diets.
Nutrient composition of low and high protein diets used in mouse experiments. Diets are isocaloric; in low protein diet calories from carbohydrates are increased.

	Low protein	High protein
Protein	21.5%	42.6%
Carbohydrate	65.3%	44.3%
Fat	13.1%	13.2%
Kcal/g	3.8	3.8

RNA isolation

RNA was isolated from mouse tissues using TRIzol® reagent (Invitrogen cat #15596-018). The manufacturer's protocol was followed. Briefly, tissues were removed from RNALater and blotted with Kimwipes to remove excess RNALater. Tissues were weighed and placed in 1 mL of TRIzol® per 50-100 mg of tissue. An IKA Ultra Turrax T8 homogenizer was used to homogenize tissue

samples in the TRIzol®. The tissue homogenate was clarified by centrifugation at 12,000 x g for 10 minutes, and then clarified homogenates were transferred to fresh tubes. Chloroform was added and phases were separated by centrifugation. The aqueous phase (~400-600 µL) was transferred to a fresh tube. RNA was precipitated with 500 µL isopropyl alcohol and pellets were washed with 1000 µL of 75% ethanol. The RNA pellets were resuspended in nuclease-free water. After addition of nuclease-free water tubes were placed in a 55°C water bath for 10 minutes to aid in solubilization. Concentration and purity of RNA was assessed via spectrophotometric readings at 260, 280, and 230 nanometers.

Quantitative real-time RT-PCR

Primers and probes

Quantitative real time RT-PCR was used to determine the presence and amount of *Pyy* and *Nags* transcripts in mouse tissues. The following Applied Biosystems TaqMan gene expression assays were used: *Pyy* (Mm00520715_m1) and *Nags* (Mm00467530_m1). The mouse *Gapdh* endogenous control (VIC®/MGB probe, primer limited) TaqMan gene expression assay (ABI # 4352339E) was multiplexed with the *Pyy* and *Nags* reactions. Expression of *Hprt1* (Mm01318743_m1) was also determined.

First strand cDNA synthesis

First strand cDNA synthesis was performed using the SuperScript™ III First Strand Synthesis System (Invitrogen, catalog #18080-051). Following the manufacturer's instructions, 20 µL reactions were set up using oligo(dT) primer and 2 µg of total RNA. RNA/primer mixtures were prepared and incubated at 65°C and then placed on ice. Next, cDNA synthesis master mix was added to each tube and reactions were incubated at 50°C for 50 minutes and 85°C for 5 minutes. RNAse H was added to each tube and reactions were incubated at 37°C for 20 minutes.

Standard curve prep

For absolute quantitation of mRNA transcripts, standard curves were prepared using full-length cDNAs: *Pyy* (Open Biosystems clone ID 4218355), *Nags* (Open Biosystems clone ID 5100021), *Gapdh* (Open Biosystems clone ID 4159824) and Hprt1 (Open Biosystems clone ID 3500885). Standard curves consisted of ten-fold dilutions of full-length plasmid cDNA ranging from 5 fg to 500 pg.

To prepare plasmid cDNA for standard curves, 5 mL cultures of bacteria containing plasmid with full-length cDNA were grown overnight in a 37°C shaking incubator. Plasmid isolations were performed using the Qiagen plasmid miniprep kit. Plasmid DNA was eluted in nuclease-free water. DNA quality and concentration were assessed via spectrophotometric readings at 260, 280 and 230 nanometers. A 5 ng/µl dilution was prepared. This was then serially diluted 1:10 to a final concentration of 5×10^{-6} pg.

qRT-PCR set-up

All samples and standards were assayed in triplicate. Reactions were performed in a total volume of 10 µL and included: 5 µL TaqMan gene expression 2X master mix (Applied Biosystems cat #4369016), 0.5 µL TaqMan gene expression assay for the gene of interest, 0.5 µL TaqMan gene expression assay for endogenous control gene, and 4 µL of cDNA/nuclease free water mixtures. Standard curve cDNA/nuclease-free water mixes consisted of 1 µL of standard cDNA and 3 µL of nuclease-free water. The cDNA/nuclease-free water mixtures for the unknown samples consisted of 1.0 µL cDNA and 3.0 µL nuclease free water which corresponds to 100 µg RNA per reaction. The endogenous control gene assay was not included in the standard curve reactions, therefore, 0.5 µL of nuclease-free water was added to make up the volume.

qRT-PCR analyses were performed using Applied Biosystems Prism® 7900 HT Sequence Detection System and the accompanying software SDS 2.3 (Applied Biosystems, Inc., Foster City, CA). The following standard thermal cycler protocol was used: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The ramp rate for temperature change was 100%.

The SDS 2.3 analysis software generated an equation of the line for each set of standards. The highest concentration standard was dropped for each assay because it crossed the threshold before the set baseline. The r^2 was ≥ 0.99 for each standard curve. These curves were used to calculate the quantity

of *Pyy* and *Nags* in mouse tissues. The calculated quantities are equivalent to picograms (pg) of plasmid. Therefore, the expression (quantity) of transcript in the unknown samples is given the unit designation pg equivalents, or pge.

Results

Immunohistochemistry

IHC experiments were performed to determine if *Pyy* and *Nags* are expressed in the same cell type in the small intestine. Multiple cell types make up the small intestinal epithelium including: enterocytes, enteroendocrine cells and goblet cells. *Pyy* is expressed in the enteroendocrine L cells while expression of Nags has not been localized to a specific cell type. If the genes are expressed in the same cell type then coordinate regulation through shared *cis* elements would be possible. Alternatively, if they are not expressed in the same cell type then they might be coordinately regulated through the same signaling pathway such as the mTOR as was proposed in Chapter IV.

Results of IHC experiments were inconclusive due to the lack of a working Nags antibody. The mouse ileum sample used in these experiments was paraffin-embedded. Typically, better immunoreactivity is achieved with frozen specimens. Thus, it is possible one or more of the Nags antibodies would work better on frozen specimens since antigenicity is usually better with frozen versus paraffin-embedded tissue.

qRT-PCR

For accurate gene expression measurements, it is important to normalize expression of target genes with a reference gene that is not affected by the

factors or conditions being studied. Ideally, expression of the endogenous control should not vary across the samples being analyzed.

Gapdh is commonly used as an endogenous control gene. Expression of *Gapdh* was determined in the mouse tissues to be used as the endogenous control in these experiments. However, the expression of *Gapdh* was significantly lower in the fasted group compared with the fed group, 6.864 and 16.011 pge, respectively, suggesting this gene is differentially regulated under these conditions. A search in the literature revealed these data are consistent with a study by Yamada *et al* in which *Gapdh* mRNA was reduced in the small intestine of food-deprived rats compared with fed rats (Yamada et al 1997).

As previously mentioned, expression of an endogenous control gene should be invariant across the samples being analyzed. Since expression of *Gapdh* is reduced upon fasting it is not an appropriate endogenous control gene for studies in which mice are subjected to fasting.

I also determined expression of Hprt1 in the fasted versus fed study for use as an endogenous control. Hprt1 has been used as an endogenous control gene in a previous study comparing gene expression in tissues of fasted and fed mice (Batterham et al 2006). However, in the present study the expression of *Hprt1* was significantly lower in the fasted group compared with the fed group suggesting it may not be an ideal control for normalizing gene expression in these studies. Another search of the literature reveals that Tcea1 gene does not vary under fasting or caloric-restriction, thus this gene may serve as a better endogenous control for these studies (Dhahbi et al 2004; Selman et al 2006). As

a result, the non-normalized expression data for *Pyy* and *Nags* are presented and the results should be interpreted with caution until they can be normalized.

Fast/Re-feed study

Protein has been shown to illicit a greater and more sustained response in PYY secretion compared to fats and carbohydrates (Batterham et al 2006; Lomenick et al 2009). *NAGS* is also upregulated with increased dietary protein intake. In this experiment mice were fasted, and then allowed to re-feed. Upon re-feeding one group of mice (n=5) was given a low protein (LP) diet (20%) and the other group (n=5) was given a high protein (HP) diet (40%). The aim of this experiment was to determine if ileal expression of *Pyy* and *Nags* increases coordinately in mice given the HP diet compared with mice given the LP diet.

Body weight and food intake measures are presented in Table 6.2a. The mean weights were 22.7 and 22.1 grams in the LP and HP groups, respectively. The mice consumed variable amounts of food over the 6-hour re-feeding period. Thus, the amount of protein consumed by each mouse was highly variable within, and across the groups (Table 6.2b). Protein consumption for each mouse was calculated from food intake and the percent protein in the diet. One mouse (F/R 5) did not re-feed, and therefore, was not included in the analysis of LP versus HP diet. *Pyy* and *Nags* expression data for this mouse are included in Table 6.3.

The expression of *Pyy* and *Nags* across the tissues collected was similar to that observed in our screen of human tissues (Figure 6.1). *Nags* was highly

expressed in the liver, while *Pyy* expression was undetectable. *Pyy* was expressed at highest levels in the colon, while *Nags* was expressed at low levels in this tissue. Both *Nag* and *Pyy* were expressed at extremely low levels in the kidney. As expected both *Pyy* and *Nags* were expressed in the small intestinal samples.

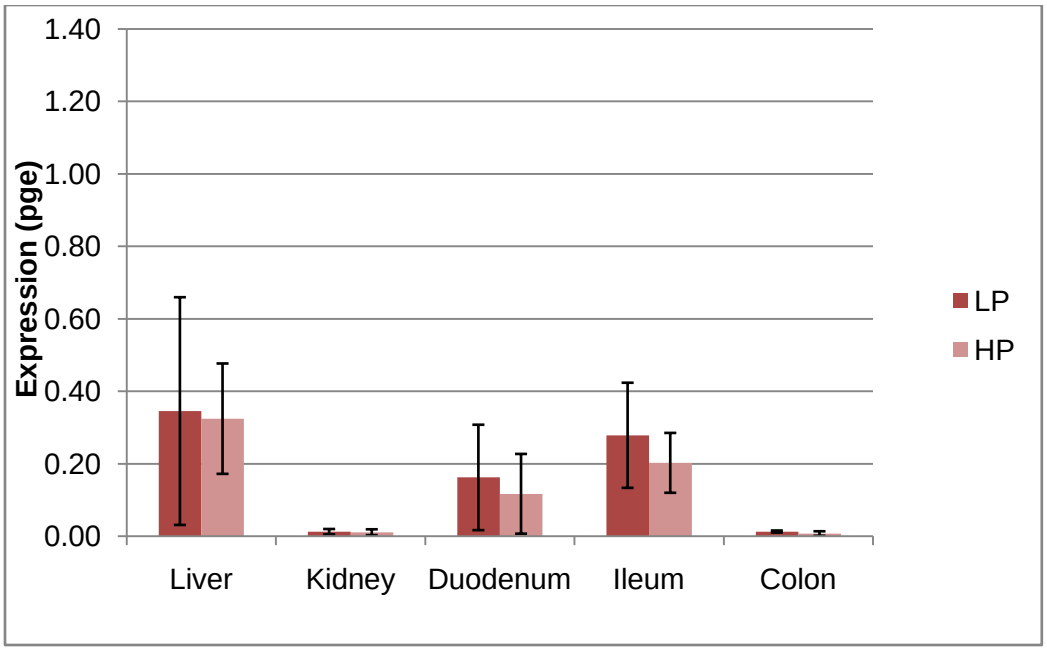
Table 5.2: Characteristics and food intake of mice in the fast/re-feed study

Animal ID	Weight (grams)	Diet	Food Consumption (grams)				
			8-9am	9-10am	10am-12pm	12-2pm	Total
F/R 1	22.7	LP	0.30	0.25	0.50	0.50	1.50
F/R 3	22.4	LP	0	0	0	0.40	0.40
F/R 5	24.8	LP	0	0	0	0	0
F/R 7	22.4	LP	0	0.4	0.20	0.60	1.20
F/R 9	21.4	LP	0.25	0.25	0.30	0.70	1.50
F/R 2	23.0	HP	0	0	0	0.70	0.70
F/R 4	19.7	HP	0.30	0.60	0	0.40	1.30
F/R 6	23.0	HP	0	0	0	0.90	0.90
F/R 8	22.0	HP	0.20	0.70	0.20	0.70	1..80
F/R 10	22.7	HP	0.35	0.55	0.40	0.90	2.20

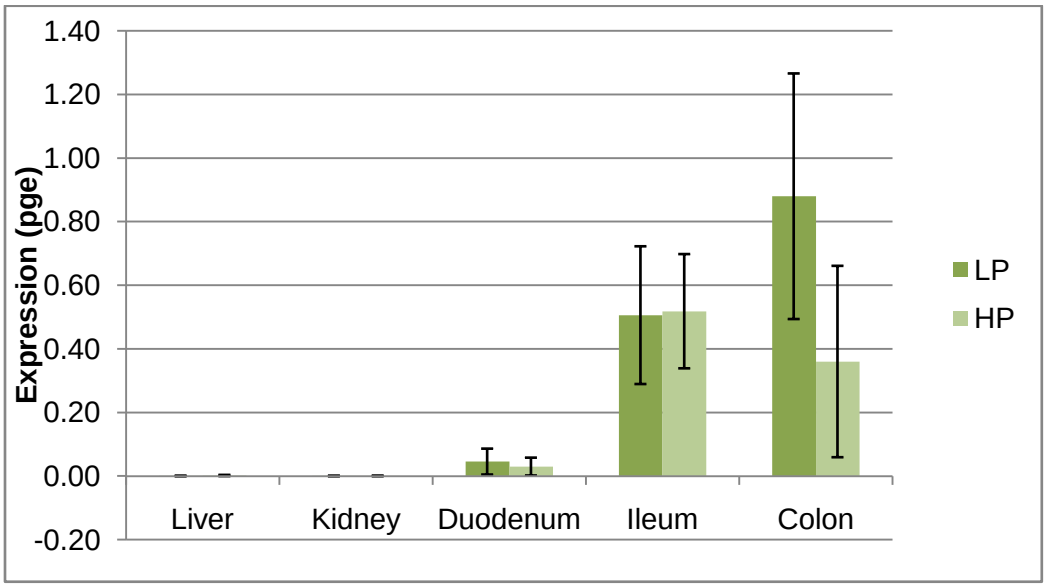
a) Body weight, diet and food consumption; LP = low protein, 20%, HP = high protein, 40%

Animal ID	Protein Consumption (grams)				
	8-9	9-10	10-12	12-2	Total
F/R 1	0.06	0.05	0.10	0.09	0.30
F/R 3	0.00	0.00	0.00	0.08	0.08
F/R 5	0.00	0.00	0.00	0.00	0.00
F/R 7	0.00	0.08	0.04	0.12	0.24
F/R 9	0.05	0.05	0.06	0.14	0.30
F/R 2	0.00	0.00	0.00	0.28	0.28
F/R 4	0.12	0.24	0.00	0.16	0.52
F/R 6	0.00	0.00	0.00	0.36	0.36
F/R 8	0.08	0.28	0.08	0.28	0.72
F/R 10	0.14	0.22	0.16	0.36	0.88

b) Protein consumed by each study animal at intervals over the re-feeding period



a) *Nags*



b) *Pyy*

Figure 5.1: Expression of *Nags* (a) and *Pyy* (b) in tissues of mice in the fast/re-feed study. The average expression is graphed and error bars represent the standard deviation. The expression (quantity) of transcript is given the unit designation pg of plasmid equivalents, or pge. LP = low protein (n =4), HP = high protein (n=5)

After establishing that expression patterns of *Pyy* and *Nags* in mice are consistent with that observed in human tissues (Chapter IV), I focused on the expression in the ileum, my primary tissue of interest (Figure 6.1). Overall, a comparison of ileal *Pyy* and *Nags* expression reveals a trend of higher expression of *Pyy* compared with *Nags*. The average *Nags* expression was 0.28 and 0.20 pge in the LP and HP groups, respectively, while *Pyy* expression was 0.51 and 0.52 pge in the LP and HP groups, respectively. This expression pattern was not observed in human ileum where *Pyy* and *Nags* were similarly expressed. It is possible the mice were fasted too long and had become catabolic, which upon re-feeding might result in an anabolic state in an effort to maintain lean body mass. Thus, more of the amino acids may be used for protein synthesis lessening the waste nitrogen load and consequently the need for urea synthesis. This may explain why *Nags* was expressed at lower levels than *Pyy* in the ileum of these mice.

Table 5.3: qRT-PCR average Ct and quantity values for fast/re-feed study arranged by tissue

a) Liver

Animal ID	*Pyy*		*Nags*		*Gapdh* (*Pyy*)	*Gapdh* (*Nags*)
	Ave Ct ± St dev	Ave Qty ± St dev	Ave Ct ± St dev	Ave Qty ± St dev	Ave Ct ± St dev	Ave Ct ± St dev
F/R 1	33.42 ± 0.18	<0.001 ± <0.001	18.38 ±0.12	0.703 ± 0.058	16.08 ± 0.12	16.09 ± 0.12
F/R 2	29.28 ±0.08	<0.001 ± <0.001	20.49 ±0.17	0.170 ± 0.019	17.76 ± 0.10	17.89 ± 0.23
F/R 3	34.78 ±0.48	<0.001 ± <0.001	25.56 ±0.29	0.006 ± 0.001	18.00 ± 0.25	17.80 ± 0.17
F/R 4	35.93 ±0.44	<0.001 ± <0.001	18.81 ±0.21	0.530 ± 0.072	17.60 ± 0.37	17.87 ± 0.07
F/R 5	32.18 ±0.41	<0.001 ± <0.001	26.02 ±0.14	0.004 ± <0.001	22.42 ± 0.10	22.35 ± 0.03
F/R 6	25.84 ±0.36	0.004 ± 0.001	19.17 ±0.13	0.413 ± 0.037	16.58 ± 0.03	16.75 ± 0.24
F/R 7	36.76 ±2.45	<0.001 ± <0.001	20.46 ±0.17	0.174 ± 0.021	18.86 ± 0.06	19.10 ± 0.23
F/R 8	29.35 ±0.13	<0.001 ± <0.001	20.34 ±0.09	0.188 ± 0.012	18.81 ± 0.13	18.80 ± 0.09
F/R 9	33.19 ±0.28	<0.001 ± <0.001	18.90 ±0.20	0.499 ± 0.068	17.54 ± 0.14	17.47 ± 0.12
F/R 10	26.69 ±0.38	0.002 ± <0.001	19.54 ±0.04	0.321 ± 0.009	17.94 ± 0.04	17.83 ± 0.07

Table 5.3: qRT-PCR average Ct and quantity values for fast/re-feed study arranged by tissue

b) Kidney

Animal ID	*Pyy*		*Nags*		*Gapdh* (*Pyy*)	*Gapdh* (*Nags*)
	Ave Ct ± St dev	Ave Qty ± St dev	Ave Ct ± St dev	Ave Qty ± St dev	Ave Ct ± St dev	Ave Ct ± St dev
F/R 1	36.20 ± 1.16	<0.001 ± <0.001	27.24 ± 0.61	0.002 ± 0.001	17.98 ± 0.08	17.84 ± 0.20
F/R 2	29.09 ± 0.41	<0.001 ± <0.001	23.60 ± 0.20	0.021 ± 0.003	16.72 ± 0.11	16.79 ± 0.06
F/R 3	31.80 ± 0.36	<0.001 ± <0.001	24.06 ± 0.09	0.015 ± 0.001	18.35 ± 0.31	18.23 ± 0.16
F/R 4	33.41 ± 0.24	<0.001 ± <0.001	24.50 ± 0.11	0.011 ± 0.001	18.18 ± 0.11	18.43 ± 0.16
F/R 5	29.98 ± 0.34	<0.001 ± <0.001	24.91 ± 0.06	0.009 ± <0.001	19.61 ± 0.04	19.42 ± 0.03
F/R 6	32.26 ± 0.32	<0.001 ± <0.001	27.69 ± 0.25	0.001± <0.001	19.17 ± 0.05	19.45 ± 0.06
F/R 7	33.26 ± 0.67	<0.001 ± <0.001	23.73 ± 0.13	0.019 ± 0.002	18.20 ± 0.10	18.10 ± 0.05
F/R 8	32.48 ± 0.60	<0.001 ± <0.001	24.67 ± 0.11	0.010 ± 0.001	18.65 ± 0.09	18.72 ± 0.09
F/R 9	34.28 ± 0.99	<0.001 ± <0.001	24.24 ± 0.12	0.014 ± 0.001	16.91 ± 0.11	17.03 ± 0.07
F/R 10	35.23 ± 1.64	<0.001 ± <0.001	undet	undet	26.57 ± 0.24	26.55 ± 0.03

undet = undetermined; indicates that the qRT-PCR reaction did not amplify

Table 5.3: qRT-PCR average Ct and quantity values for fast/re-feed study arranged by tissue

c) Duodenum

Animal ID	*Pyy*		*Nags*		*Gapdh* (*Pyy*)	*Gapdh* (*Nags*)
	Ave Ct ± St dev	**Ave Qty ± St dev**	**Ave Ct ± St dev**	**Ave Qty ± St dev**	**Ave Ct ± St dev**	**Ave Ct ± St dev**
F/R 1	36.58 ± 0.24	<0.001 ± <0.001	34.73 ± 0.64	<0.001 ± <0.001	18.48 ± 0.12	18.41 ± 0.07
F/R 2	21.88 ± 0.16	0.063 ± 0.007	20.23 ± 0.14	0.202 ± 0.020	17.68 ± 0.06	17.58 ± 0.12
F/R 3	21.266 ± 0.25	0.095 ± 0.015	19.77 ± 0.15	0.277 ± 0.028	18.05 ± 0.21	17.75 ± 0.14
F/R 4	33.97 ± 1.98	<0.001 ± <0.001	33.04 ± 0.32	<0.001 ± <0.001	23.96 ± 0.03	24.03 ± 0.07
F/R 5	undet	undet	35.02 ± 0.79	<0.001 ± <0.001	22.62 ± 0.10	22.48 ± 0.13
F/R 6	22.24 ± 0.19	0.049 ± 0.006	19.94 ± 0.08	0.246 ± 0.013	17.06 ± 0.15	16.99 ± 0.06
F/R 7	23.03 ± 0.20	0.029 ± 0.004	21.63 ± 0.11	0.079 ± 0.006	21.38 ± 0.02	21.27 ± 0.11
F/R 8	27.11 ± 0.32	0.002 ± <0.001	24.35 ± 0.10	0.013 ± 0.001	22.50 ± 0.11	22.55 ± 0.10
F/R 9	22.02 ± 0.15	0.057 ± 0.006	19.69 ± 0.17	0.293 ± 0.032	17.38 ± 0.05	17.33 ± 0.04
F/R 10	22.77 ± 0.07	0.034 ± 0.002	20.95 ± 0.01	0.124 ± 0.001	20.03 ± 0.10	19.98 ± 0.05

undet = undetermined; indicates that the qRT-PCR reaction did not amplify

Table 5.3: qRT-PCR average Ct and quantity values for fast/re-feed study arranged by tissue

d) Ileum

Animal ID	*Pyy*		*Nags*		*Gapdh* (*Pyy*)	*Gapdh* (*Nags*)
	Ave Ct ± St dev	**Ave Qty ± St dev**	**Ave Ct ± St dev**	**Ave Qty ± St dev**	**Ave Ct ± St dev**	**Ave Ct ± St dev**
F/R 1	19.08 ± 0.31	0.419 ± 0.092	20.06 ± 0.12	0.227 ± 0.019	17.05 ± 0.20	17.04 ± 0.18
F/R 2	18.40 ± 0.26	0.658 ± 0.112	19.93 ± 0.05	0.247 ± 0.008	17.07 ± 0.10	17.33 ± 0.03
F/R 3	18.13 ± 0.17	0.789 ± 0.095	19.04 ± 0.18	0.453 ± 0.055	17.29 ± 0.21	17.05 ± 0.12
F/R 4	18.97 ± 0.09	0.446 ± 0.026	20.74 ± 0.01	0.143 ± 0.001	18.95 ± 0.04	19.02 ± 0.06
F/R 5	-	-	-	-	-	-
F/R 6	18.44 ± 0.06	0.635 ± 0.027	19.79 ± 0.07	0.272 ± 0.012	16.76 ± 0.04	16.71 ± 0.15
F/R 7	19.68 ± 0.20	0.278 ± 0.038	21.12 ± 0.08	0.111 ± 0.006	18.88 ± 0.22	18.63 ± 0.05
F/R 8	18.49 ± 0.11	0.616 ± 0.048	19.84 ± 0.08	0.263 ± 0.015	17.16 ± 0.11	17.78 ± 0.02
F/R 9	18.69 ± 0.11	0.537 ± 0.039	19.54 ± 0.24	0.323 ± 0.050	17.59 ± 0.03	17.71 ± 0.14
F/R 10	19.92 ± 0.07	0.234 ± 0.010	21.48 ± 0.19	0.087 ± 0.011	20.78 ± 0.08	20.92 ± 0.15

(-) indicates no data due to missing tissue sample

Table 5.3: qRT-PCR average Ct and quantity values for fast/re-feed study arranged by tissue

e) Colon

Animal ID	*Pyy*		*Nags*		*Gapdh* (*Pyy*)	*Gapdh* (*Nags*)
	Ave Ct ± St dev	**Ave Qty ± St dev**	**Ave Ct ± St dev**	**Ave Qty ± St dev**	**Ave Ct ± St dev**	**Ave Ct ± St dev**
F/R 1	17.79 ± 0.10	0.983 ± 0.063	24.50 ± 0.03	0.011 ± <0.001	17.13 ± 0.09	16.91 ± 0.06
F/R 2	20.66 ± 0.36	0.145 ± 0.032	28.34 ± 0.71	0.001 ± <0.001	18.46 ± 0.39	18.43 ± 0.43
F/R 3	17.39 ± 0.03	1.29 ± 0.029	24.27 ± 0.11	0.013 ± 0.001	17.38 ± 0.24	17.29 ± 0.03
F/R 4	17.98 ± 0.09	0.869 ± 0.052	23.85 ± 0.12	0.018 ± 0.001	17.79 ± 0.03	17.89 ± 0.11
F/R 5	undet	undet	undet	undet	undet	21.72 ± 0.30
F/R 6	19.20 ± 0.06	0.380 ± 0.016	25.25 ± 0.07	0.007 ± <0.001	18.38 ± 0.05	18.28 ± 0.12
F/R 7	19.28 ± 0.10	0.361 ± 0.025	24.69 ± 0.10	0.010 ± 0.001	19.99 ± 0.15	19.82 ± 0.08
F/R 8	20.64 ± 0.18	0.145 ± 0.018	25.69 ± 0.06	0.005 ± <0.001	22.94 ± 0.07	23.09 ± 0.18
F/R 9	17.95 ± 0.05	0.884 ± 0.029	23.94 ± 0.07	0.017 ± 0.001	17.24 ± 0.10	17.36 ± 0.07
F/R 10	19.77 ± 0.22	0.261 ± 0.037	25.36 ± 0.11	0.006 ± <0.001	19.82 ± 0.05	19.92 ± 0.07

undet = undetermined; indicates that the qRT-PCR reaction did not amplify

Chronic LP or HP diets study

The mice in the fast/re-feed study were never exposed to the LP and HP diets prior to the study, and as a result did not re-feed well. Thus, the amount of protein consumed by each mouse was highly variable within and across the groups making it difficult to draw conclusions on how the higher protein diet affected expression of *Pyy* and *Nags*. Colonic and ileal *Pyy* mRNA levels had been previously shown to be increased in mice that were fed a HP diet for 16 weeks compared to mice fed a LP diet during the same period (Batterham et al 2006). Consequently, I modified the experimental design to determine if *Nags* mRNA levels would increase along with *Pyy* in the ileum of mice chronically fed the HP diet compared with mice fed the LP diet. Characteristics of study animals including body weight and age are presented in Table 6.4. Body weight was not significantly different between the two groups as determined by Wilcoxon rank-sum test ($p = 0.11$).

Table 5.4: Characteristics of mice on chronic diets study

Animal ID	Weight (grams)	Age (weeks)	Diet
CD 1	26.2	13	LP
CD 2	27.3	13	LP
CD 3	-	13	LP
CD 4	24.7	13	LP
CD 5	24.8	13	LP
CD 6	26.9	13	HP
CD 7	27.1	13	HP
CD 8	27.4	13	HP
CD 9	27.2	13	HP
CD 10	32.4	16	HP
CD 11	27.3	16	HP
CD 12	25.9	16	HP

LP = low protein, 20%, HP = high protein, 40%

(-) indicates missing data

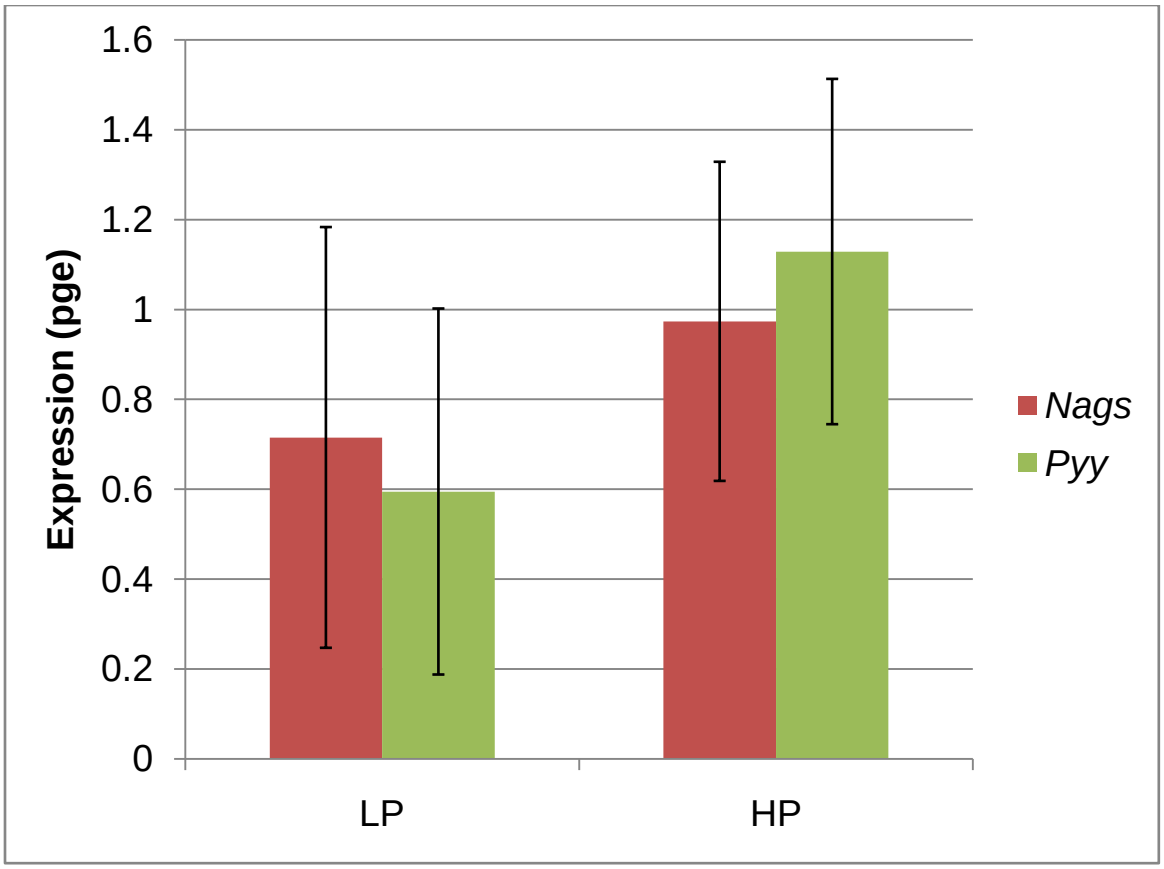


Figure 5.2: Ileal expression of *Nags* and *Pyy* in mice from chronic LP and HP diets study. Mice were chronically fed either a low protein (LP, 20%) or a high protein (HP, 40%) diet and ileal expression of *Nags* and *Pyy* was determined using qRT-PCR. The average expression is graphed and error bars represent the standard deviation. The expression (quantity) of transcript is given the unit designation pg of plasmid equivalents, or pge. LP = low protein (n = 5), HP = high protein (n = 7)

The average ileal expression of the non-normalized expression of *Nags* (0.715 pge) and *Pyy* (0.595 pge) in the LP group was not significantly different, as determined by a Wilcoxon rank-sum test. However, in the HP group the ileal expression of *Pyy* (1.129 pge) was significantly higher ($p = 0.04$) than *Nags* (0.973 pge). This result was driven by the greater increase in expression of *Pyy* compared to *Nags* in response to the HP diet.

Since these mice were not fasted, but were given *ad libitum* access to either the LP or HP diet, *Gapdh* may be an appropriate endogenous control gene for this experiment. Indeed, *Gapdh* levels in the ileum are not significantly different in the LP and HP fed mice. I normalized expression of *Pyy* and *Nags* to *Gapdh*; these data are graphed in figure 6.3. The normalized data show the same trend as that observed in the non-normalized data. *Pyy* expression increases significantly ($p = 0.004$) in mice fed HP diet compared to those fed LP diet. *Nags* expression was not significantly different between these groups. These data suggest that *Pyy* and *Nags* may not be coordinately regulated in response to chronic feeding of a high protein diet.

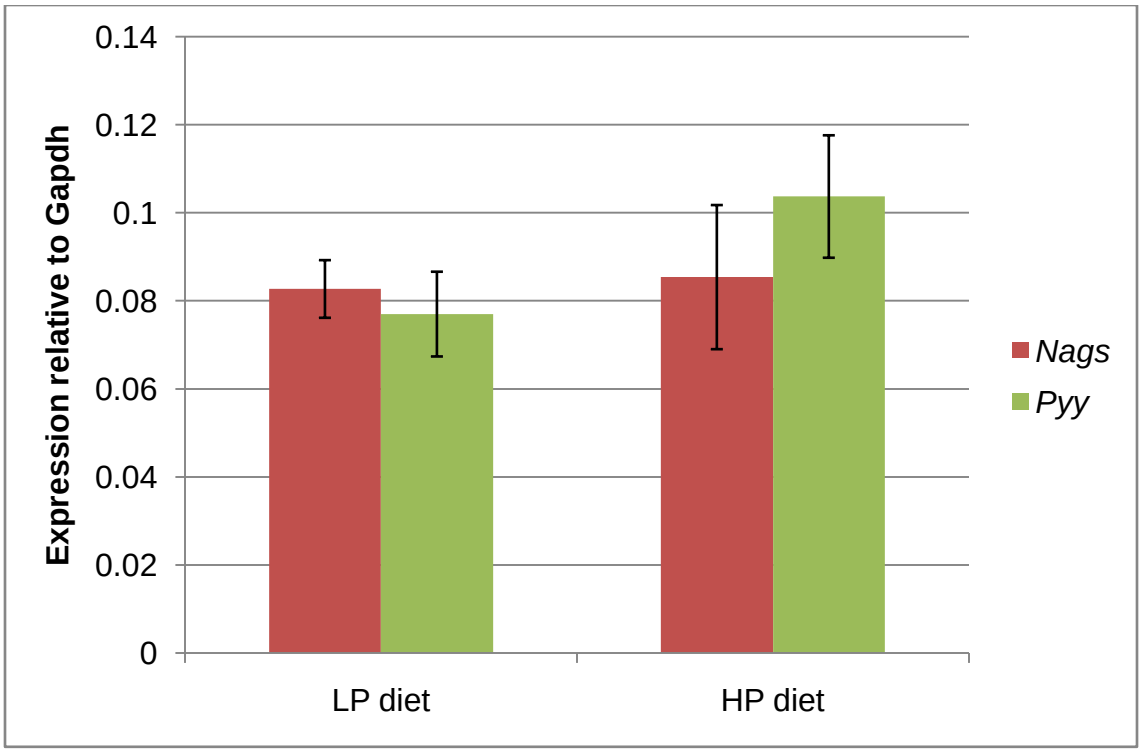


Figure 5.3: Normalized ileal expression *Nags* and *Pyy* in mice from chronic diets study. Mice were chronically fed either a low protein (LP, 20%) or a high protein (HP, 40%) diet and ileal expression of *Nags* and *Pyy* was determined using qRT-PCR. The average expression relative to Gapdh is graphed and error bars represent the standard deviation. The expression (quantity) of transcript is given the unit designation pg of plasmid equivalents, or pge. LP = low protein (n = 5), HP = high protein (n = 7)

Fasted/fed study

The aim of the fasted versus fed study was to examine expression of *Pyy* and *Nags* in mice that were fasted compared with mice given *ad libitum* access to their normal chow. This study design allows for examination of expression of these genes under acute conditions. Pyy is a satiety factor; therefore, the expectation is it would be decreased in the fasted mice. If *Pyy* and *Nags* are coordinately regulated then it would follow that *Nags* expression would also be decreased in the fasted mice. Characteristics of the mice are given in Table 6.5.

Pyy and *Nags* are expressed is at similar levels within each group (Figure 6.8). In the fasted group, the average expression of *Pyy* and *Nags* was the same: 1.106 pge. In the fed group the average non-normalized expression for *Nags* was 1.630 pge while *Pyy* expression was 1.547 pge. The expression of *Nags* is not statistically different from that of *Pyy* in either the fasted or the fed group. Expression of both genes is decreased in the fasted group compared with the fed group. As determined by a Wilcoxon rank-sum test, *Nags* expression is significantly decreased ($p = 0.02$) and while not significant the decrease in *Pyy* expression in the fasted group is nearing significance ($p = 0.08$). These data are consistent with the hypothesis that *Pyy* and *Nags* are coordinately regulated.

Table 5.5: Characteristics of mice in the fasted/fed study

Animal ID	Weight (grams)	Group
FF 1	21.3	Fasted
FF 2	21.6	Fasted
FF 3	20.5	Fasted
FF 4	21.3	Fasted
FF 5	22.4	Fasted
FF 6	23.0	Fasted
FF 7	22.8	Fasted
FF 8	21.7	Fasted
FF 9	23.7	Fed
FF 10	25.6	Fed
FF 11	23.8	Fed
FF 12	25.2	Fed
FF 13	25.5	Fed
FF 14	27.0	Fed
FF 15	26.1	Fed
FF 16	22.8	Fed

Table 5.6: qRT-PCR average Ct and quantity values for ileal *Pyy* and *Nags* expression in fasted (1-8) and fed (9-16) mice

	Pyy		*Nags*	
Animal ID	**Ave Ct ± St dev**	**Ave Qty ± St dev**	**Ave Ct ± St dev**	**Ave Qty ± St dev**
FF 1	19.12 ± 0.07	1.664 ± 0.074	20.61 ± 0.10	1.533 ± 0.096
FF 2	20.62 ± 0.15	0.625 ± 0.060	22.56 ± 0.11	0.435 ± 0.031
FF 3	19.84 ± 0.09	1.039 ± 0.063	21.08 ± 0.07	1.132 ± 0.048
FF 4	20.09 ± 0.07	0.883 ± 0.039	21.41 ± 0.07	0.912 ± 0.040
FF 5	19.45 ± 0.04	1.338 ± 0.037	20.65 ± 0.07	1.494 ± 0.067
FF 6	19.90 ± 0.03	0.994 ± 0.020	21.04 ± 0.15	1.161 ± 0.112
FF 7	22.70 ± 4.08	0.499 ± 0.426	21.64 ± 0.16	0.788 ± 0.084
FF 8	19.05 ± 0.49	1.804 ± 0.618	20.75 ± 0.09	1.395 ± 0.078
FF 9	19.19 ± 0.13	1.593 ± 0.137	20.25 ± 0.09	1.933 ± 0.108
FF 10	18.61 ± 0.04	2.328 ± 0.058	19.99 ± 0.09	2.288 ± 0.128
FF 11	19.65 ± 0.25	1.185 ± 0.203	20.88 ± 0.10	1.286 ± 0.083
FF 12	19.06 ± 0.04	1.729 ± 0.045	20.58 ± 0.03	1.563 ± 0.035
FF 13	19.40 ± 0.03	1.386 ± 0.026	20.80 ± 0.02	1.357 ± 0.019
FF 14	18.86 ± 0.12	1.974 ± 0.152	20.21 ± 0.12	1.979 ± 0.150
FF 15	19.61 ± 0.12	1.210 ± 0.093	20.68 ± 0.08	1.466 ± 0.078
FF 16	19.94 ± 0.04	0.972 ± 0.026	21.09 ± 0.53	1.168 ± 0.424

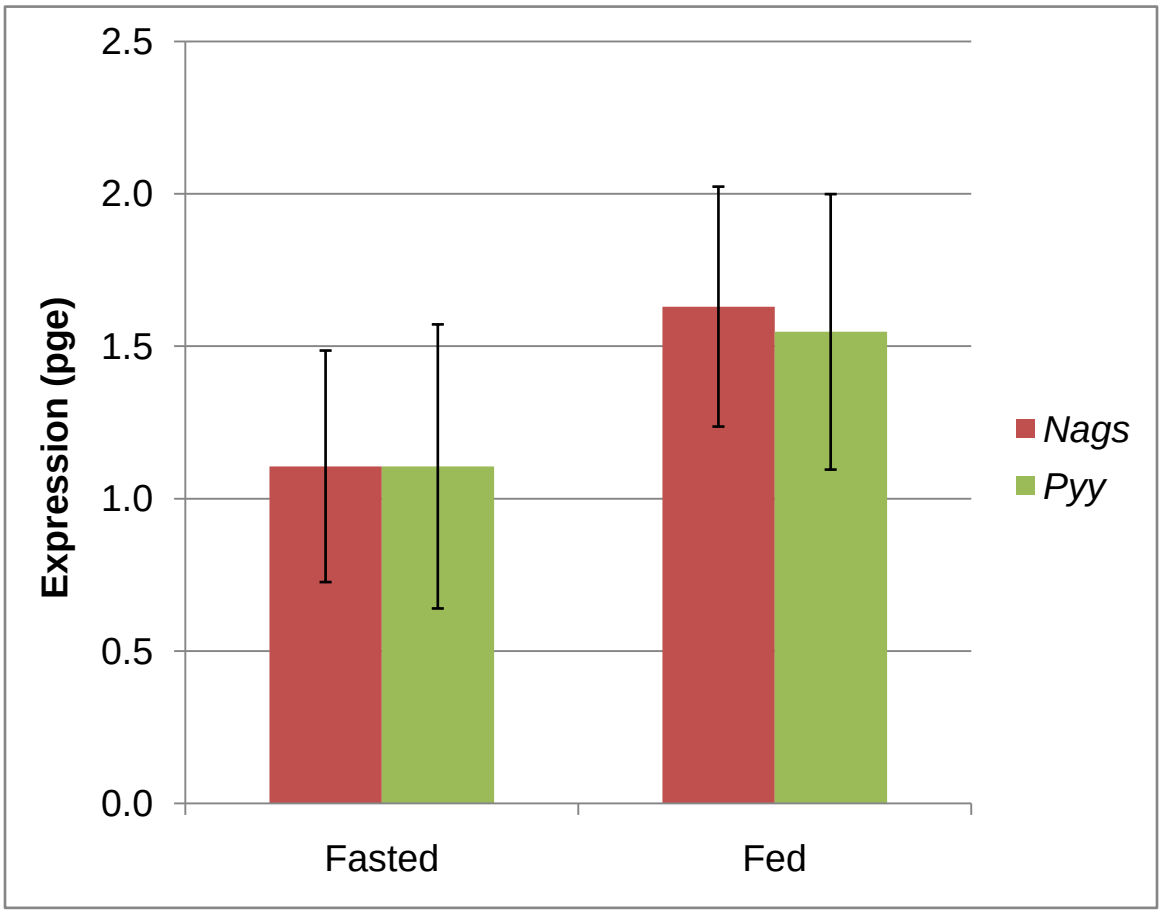


Figure 5.4: Ileal expression of *Nags* and *Pyy* in fasted and fed mice. Quantitative real time RT-PCR was used to determine expression of *Nags* and *Pyy* in mice that were fasted (n=8) or fed (n=8). The average expression is graphed and error bars represent the standard deviation. The expression (quantity) of transcript is given the unit designation pg of plasmid equivalents, or pge.

Discussion

The experiments described in this chapter examined expression of *Pyy* and *Nags* in tissues of mice under different dietary conditions to determine if there was coordinate change in gene expression in response to these dietary conditions. In the fast/re-feed study, multiple tissues were examined and results reveal a tissue-specific expression pattern of *Nags* and *Pyy* similar to that observed in human tissue expression studies (Chapter IV). This implies that these genes may be regulated similarly in human and mouse, confirming that mouse is an appropriate model organism for investigating regulation of *Nags* and *Pyy*.

Data from the fast/re-feed experiment is difficult to interpret, primarily due to the variable amounts of food and consequently protein consumed by the mice. Although on average the protein consumption for mice on the HP diet was higher than for those on the LP diet, some mice in the LP and HP groups actually consumed similar amounts of protein. This makes it difficult to determine what impact the percent of protein in the diet might have on expression of *Pyy* and *Nags*.

Furthermore, the eating style of the mice upon re-feeding varied with some eating fairly consistently throughout while others waited until the last 2 hours to eat. Switching the mice from normal chow to the new diets without previous acclimation to the diet likely contributed to their reticence to eat after the prolonged fast. The Pyy response to ingested nutrients is an acute one (shown, in human, to peak at approximately 1 hour after eating). The variability observed

in the frequency and timing of re-feeding may have impacted the expression profiles of *Pyy* and *Nags*, independent of the amount of protein consumed. Thus, it is not possible to draw meaningful conclusions from this study about the effect of these diets on expression of *Nags* and *Pyy*.

In the chronic diets study, *Nags* expression in the ileum was not significantly different between mice on the LP and HP diets. However, mice fed the HP diet had significantly higher ileal *Pyy* expression than those on the LP diet. This is consistent with a previous report demonstrating higher fasting and fed ileal *Pyy* levels in mice fed a high protein diet for 16 weeks as compared to control mice fed a normal protein diet for the same period (Batterham et al 2006). Taken together, the significant increase in expression of *Pyy*, but not *Nags*, suggests that under these conditions, *Pyy* and *Nags* may not be coordinately regulated.

In the final *in vivo* experiment, expression of *Pyy* and *Nags* was examined in a group of fasted mice compared with a control group of mice fed *ad libitum*. In the fasted/fed experiment *Pyy* and *Nags* are expressed at similar levels within each group. The average ratio of expression *Pyy*/*Nags* was 1.0 and 0.95 in the fasted and fed groups, respectively, indicating these genes are expressed at approximately equal levels in both the fasted and fed mice. *Nags* expression is significantly lower in the fasted group and *Pyy* expression is trending in that direction. This is consistent with the hypothesis that *Pyy* and *Nags* are coordinately regulated.

Overall, results from the *in vivo* experiments are inconclusive. From these data it cannot be determined if *Pyy* and *Nags* are coordinately regulated. The fasted/fed study results are consistent with my hypothesis of coordinate regulation, but the data are not normalized and results must be interpreted with caution. The data from the chronic diets study suggest *Pyy* and *Nags* may not be coordinately regulated. It is possible that under the chronic feeding conditions *Pyy* is functioning in the capacity of a long-term regulator of energy homeostasis. It may well be that co-regulation of *Pyy* and *Nags* would function in the short-term regulation of energy homeostasis in determining the size and timing of single meals, thereby providing a mechanism to prevent overconsumption of dietary protein. Although not conclusive, these studies lay the ground work for future *in vivo* experiments investigating the regulation of *Pyy* and *Nags*. Ultimately further studies are needed to understand the relationship between *Pyy*, *Nags*, and dietary protein intake.

CHAPTER VI

PEPTIDE YY LEVELS ARE INCREASED IN PATIENTS WITH INBORN ERRORS OF AMINO ACID METABOLISM

Introduction

Inborn errors of metabolism (IEM) are genetic disorders in which defects in enzymes or transport proteins cause a block in a metabolic pathway and a subsequent accumulation of toxic substrates (Gupta 2007; Weiner 2009). IEM encompass disorders of mitochondrial and peroxisomal function, lysosomal storage, and carbohydrate, organic acid, fatty acid, and amino acid metabolism. Phenylkenoturia (PKU) (MIM# 261600) is the most prevalent inborn error of amino acid metabolism, occurring in approximately 1 in 14,000 to 1 in 20,000 live births (Rezvani 2007). The majority of PKU cases are caused by a deficiency of phenylalanine hydroxylase (PAH) (EC 1.14.16.1), the enzyme that converts phenylalanine to tyrosine. This enzyme deficiency results in an increased concentration of phenylalanine which, if left untreated, leads to significant cognitive impairment (Rezvani 2007).

Urea cycle disorders (UCDs) are another, and potentially more serious, inborn error of amino acid metabolism. Collectively, these disorders have an estimated prevalence of 1 in 30,000 live births (Rezvani 2007). The urea cycle is a biochemical process of ammonia detoxification in which excess nitrogen, in the form of ammonia, is converted to urea and excreted as waste (Krebs and

Henseleit 1932; Rezvani 2007). Five enzymes make up the urea cycle: carbamoyl-phosphate synthase 1 (CPS1) (EC 6.3.5.5), ornithine carbamoyltransferase (OTC) (EC 2.1.33), argininosuccinate synthase 1 (ASS1) (EC 6.3.4.5), argininosuccinate lyase (ASL) (EC 4.3.2.1), and arginase 1 (ARG1) (EC 3.5.3.1). An additional enzyme, N-acetylglutamate synthase (NAGS) (EC 2.3.1.1), is critical for urea cycle function, providing CPS1 with its obligatory co-factor, N-acetylglutamate (NAG). Deficiencies in these enzymes result in hyperammonemia leading to central nervous system dysfunction and, if left untreated, death (Rezvani 2007).

Nutrition is the foundation of both the acute and chronic management of patients with inborn errors of amino acid metabolism (Mofidi and Kronn 2009). There are a number of challenges associated with nutritional management of PKU and UCD patients including strict dietary restrictions and patient compliance. Dietary protein is a major source of phenylalanine and nitrogen; therefore its intake is strictly controlled in these patients. However, protein is critical for normal growth and development; a sufficient amount must be supplied to meet the minimal growth requirement while limiting intake of toxic substrates (Lee et al 2005; Mofidi and Kronn 2009). Natural sources of protein are severely constrained for these patients and protein requirements are filled via medical foods comprised of single amino acids rather than polypeptides (MacDonald et al 1994; Mofidi and Kronn 2009; Singh 2007). Patients are often supplemented with additional calories from non-protein sources in an effort to promote

anabolism and prevent catabolism which is critical for maintaining metabolic control (Lee et al 2005; Mofidi and Kronn 2009; Singh et al 2005).

Feeding problems, including lack of appetite, are common in both PKU and UCD patients (MacDonald et al 1994; Rezvani 2007; Summar and Tuchman 2001; Weiner 2009). Poor appetite can result in reduced caloric intake leading to catabolism and a subsequent buildup of toxic substrates. The anorexia observed in UCD patients has traditionally been attributed to elevated ammonia levels, while in PKU patients, a lack of variety and palatability of foods offered are suggested causes (MacDonald et al 1994). Alternatively, altered secretion patterns of appetite regulating hormones may play a role in the lack of appetite in these patients. Indeed, one study found a significant reduction of the orexigenic hormone ghrelin in poorly controlled PKU patients compared to both diet-controlled PKU patients and normal controls (Schulpis et al 2004). A similar study found a significant increase of leptin in poorly controlled PKU patients compared to both diet-controlled PKU patients and normal controls (Schulpis, Papakonstantinou, and Tzamouranis 2000).

Given previous reports that appetite regulating hormones, such as ghrelin, may affect appetite in patients with IEM, we hypothesized that the neuroendocrine hormone peptide tyrosine tyrosine (PYY) may also be altered in these patients compared to other groups. Secreted by the endocrine L cells of the distal small intestine and colon, PYY induces satiety, inhibits gastric acid secretion, delays gastric emptying and slows gut motility (Korner and Leibel 2003; Leiter et al 1987). Therefore, elevated PYY levels may contribute to the

lack of appetite and other gastrointestinal problems common in PKU and UCD patients. To explore this possible relationship further, we measured and describe here PYY levels in both PKU and UCD patients and compare both patient groups with healthy controls.

Materials and Methods

Study Populations

De-identified surplus plasma samples from urea cycle disorder (UCD; n=66) and phenylketonuria (PKU; n=61) patients undergoing clinical testing were collected from the Vanderbilt Pathology Lab. UCD diagnoses included: CPS1 (MIM# 237300), OTC (MIM# 311250), ASS1 (MIM# 215700), and ASL (MIM# 207900) deficiencies. Both study groups included samples from infants, children, and adults. A subset of the patients had multiple plasma samples available for study; the most recent sample was used in the analysis. Samples from patients less than one year of age were excluded from the analysis because plasma PYY levels are increased in infants compared with children and adults (Adrian et al 1986; Siahanidou et al 2005). The control group consisted of fasted, normal weight and obese children ages 7-11 years. The control subjects were from a previously published prospective study examining PYY levels in prepubertal children (Lomenick et al 2009). Surplus fasting plasma samples from these subjects were retrieved for analysis in the present study.

Relevant demographic and clinical patient data was gathered for this study, including age, sex, race/ethnicity, body mass index (BMI), and glutamine

and arginine levels. Blood ammonia concentration was also collected for the UCD patients. This study was approved by the Institutional Review Board (IRB) at Vanderbilt University Medical Center (IRB# 081080).

Plasma PYY determination

Total PYY was measured for all samples by ELISA (Millipore, EZHPYYT66K). This assay measures human PYY_{1-36} and PYY_{3-36}; it does not cross-react with neuropeptide Y or pancreatic polypeptide. Samples were measured in duplicate. If the difference between duplicate results of a sample was greater than 15% coefficient of variation (CV), the sample was assayed again in duplicate. If the % CV of the measurements was within acceptable limits the data was included for analysis. Because surplus plasma samples were collected for this study, a proportion of the samples did not have an adequate volume available to perform the assay multiple times. Therefore, if the % CV was greater than 15 for duplicate results of a sample and the sample could not be assayed again, it was excluded from the analysis.

Statistical Analysis

Statistical analyses were performed using STATA 10.1. Data are summarized as median and interquartile range (IQR). Sex differences between groups were determined by Fisher's exact test. Age, BMI and plasma PYY concentrations were not normally distributed; therefore a Kruskal-Wallis test was

used to test for differences among groups. Pair-wise comparisons of BMI and PYY levels between groups were performed using a Wilcoxon rank-sum test.

To test for differences in PYY levels by UCD diagnosis, the patients were stratified by type of UCD diagnosis: ornithine transcarbamylase deficient (OTCD) patients (n=28) and all other UCD patients - CPS1 (n=2), ASS1 (n=8) and ASL (n=4). A Kruskal-Wallis test was used to test for differences in PYY levels among controls, OTCD patients, and patients with all other types of UCDs. A Wilcoxon rank-sum test was used to perform pair-wise comparisons between groups. Results were considered significant at $p \leq 0.05$.

To identify variables significantly associated with PYY levels, we performed linear regressions where log-transformed PYY was the dependent variable and age, sex, and BMI were independent variables. Linear regressions were performed for each group (controls, PKU patients and UCD patients).

Results

Study population characteristics are given in Table 5.1. Overall, sex differed across study groups ($p = 5x10^{-4}$) as the control group was mostly male (82%) compared with the UCD and PKU groups (38% and 43% male, respectively). The median age among the three study groups was not significantly different. As expected based on ascertainment (see Methods and Materials), median BMI was significantly different across the study groups (p=0.04), a result driven by the higher median BMI observed among controls compared with the other study groups.

Table 6.1: Study Population Characteristics

	Controls (n=28)	UCD patients (n=42)	PKU patients (n=36)	p-value
% Male	82	38	43	$5x10^{-4*}$
Median Age years (IQR)	9.7 (2.1)	9.5 (13)	7 (9)	0.08**
Median BMI kg/m^2(IQR)	22.9 (10.1)	18.8 (6.7)	17.9 (5.6)	0.04**

Abbreviations: Body mass index (BMI), Interquartile range (IQR).
*Fisher's exact test
**Kruskal-Wallis test

Median plasma PYY levels were 82 pg/mL, 146 pg/mL, and 202 pg/mL for the controls, UCD patients, and PKU patients, respectively. Across the study groups, median plasma PYY levels were significantly different (Figure 5.1; $p = 1x10^{-4}$). Pair-wise comparisons revealed that median plasma PYY levels were significantly higher in both UCD patients ($p = 3.5x10^{-5}$) and PKU patients ($p = 5x10^{-9}$) compared to controls. There was no significant difference between median PYY levels in UCD and PKU patients ($p>0.05$).

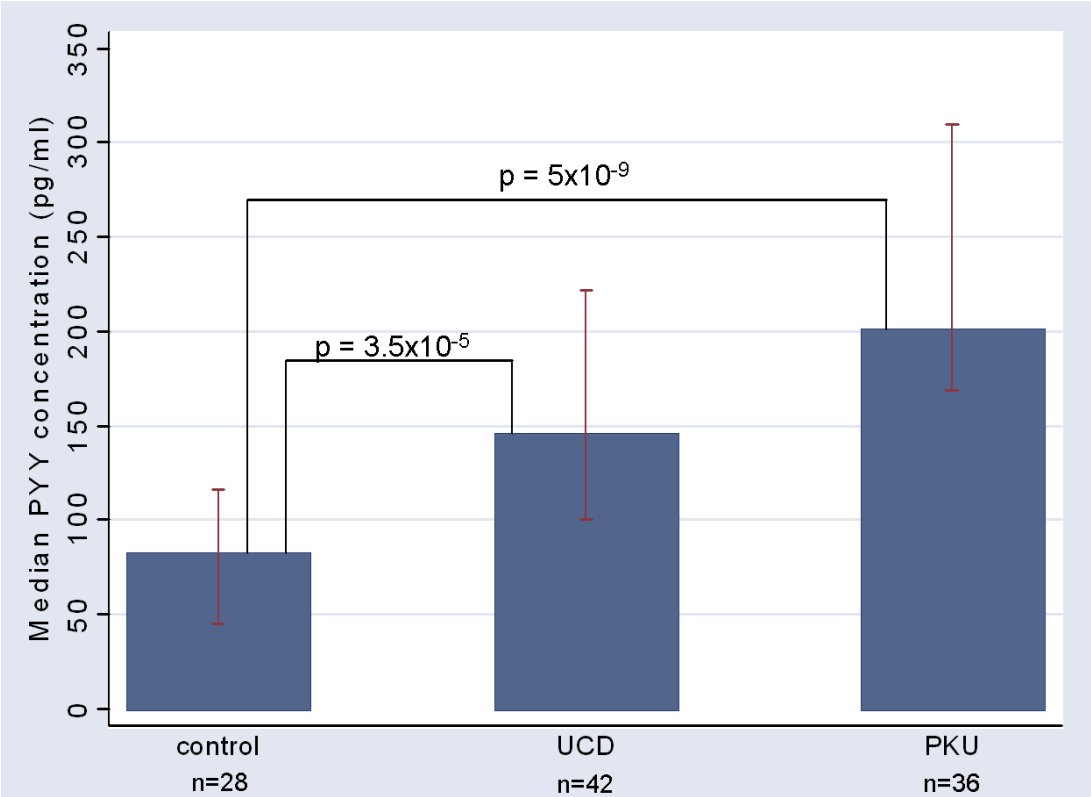


Figure 6.1: Median plasma PYY concentration in UCD and PKU patients and controls Median plasma PYY concentration (pg/mL) are reported; the red bars represent the interquartile range (IQR) for each study group (control IQR = 70.6, UCD IQR = 122.0, PKU IQR = 139.8). A Kruskal-Wallis test was performed to determine if median PYY levels differ across the three groups ($p=1x10^{-4}$), and a Wilcoxon rank-sum test was performed for pair-wise comparisons. PYY = peptide tyrosine tyrosine, UCD = urea cycle disorder, PKU=phenylketonuria, IQR = interquartile range

Linear regression identified variables significantly associated with PYY levels in each group (Table 5.2). In the control group BMI was significantly associated with increased PYY levels ($\beta = 0.47$; $p = 0.02$). Age was significantly associated with decreased PYY levels in the PKU group ($\beta = -0.03$; $p = 2x10^{-3}$), while diagnosis ($\beta = 0.319$; $p = 5x10^{-4}$) was significantly associated with PYY levels in the UCD group.

Table 6.2: Demographic and epidemiologic factors associated with PYY levels in

a) Controls

Independent variable	β-coefficient	95% CI	p-value
Sex (n=28)	-0.50	-1.170 0.166	0.14
Race (n=28)	-0.29	-0.761 0.181	0.22
Age (n=28)	-0.04	-0.236 0.164	0.71
BMI (n=28)	0.05	0.010 0.833	**0.02**

b) PKU patients

Independent variable	β-coefficient	95% CI	p-value
Sex (n=35)	0.16	-0.137 0.458	0.28
Race (n=31)	-0.18	-0.833 0.465	0.57
Age (n=35)	-0.03	-0.053 -0.013	$2x10^{-3}$
BMI (n=28)	-0.01	0.058 0.038	0.68

c) UCD patients

Independent variable	β-coefficient	95% CI	p-value
Sex(n=42)	0.05	-0.393 0.484	0.84
Race (n=39)	-0.14	-0.409 0.130	0.30
Age (n=42)	-0.01	-0.032 0.004	0.13
BMI (n=36)	-0.01	-0.043 0.025	0.59
Diagnosis (n=42)	0.32	0.149 0.489	$1x10^{-3}$

Linear regression of log-transformed PYY levels for Controls (a), PKU patients (b), and UCD patients (c)
UCD diagnosis includes: CPS1, OTC, ASS1, and ASL deficiencies
BMI = Body mass index

Ornithine transcarbamylase deficiency (OCTD) is the most common UCD (Nassogne et al 2005; Rezvani 2007), and differs from other UCDs in that it is an

X-linked, partially dominant disorder with highly variable clinical phenotypes (McCullough et al 2000; Nassogne et al 2005; Tuchman et al 1998). Given that the type of UCD diagnosis was significantly associated with PYY levels (Table 5.2), we stratified by type of UCD to test for differences in PYY levels in OTCD patients compared with all other UCD patients. The median PYY level (128 pg/mL) in the OTCD group was significantly higher compared to controls (Figure 5.2; $p = 1.6x10^{-3}$), and it was significantly lower compared to all other UCD diagnoses (Figure 5.2; $p = 9x10^{-3}$). As expected, the median PYY level in the all other UCDs patient group (235 pg/mL) was significantly increased compared to controls (Figure 5.2; $p = 1x10^{-4}$).

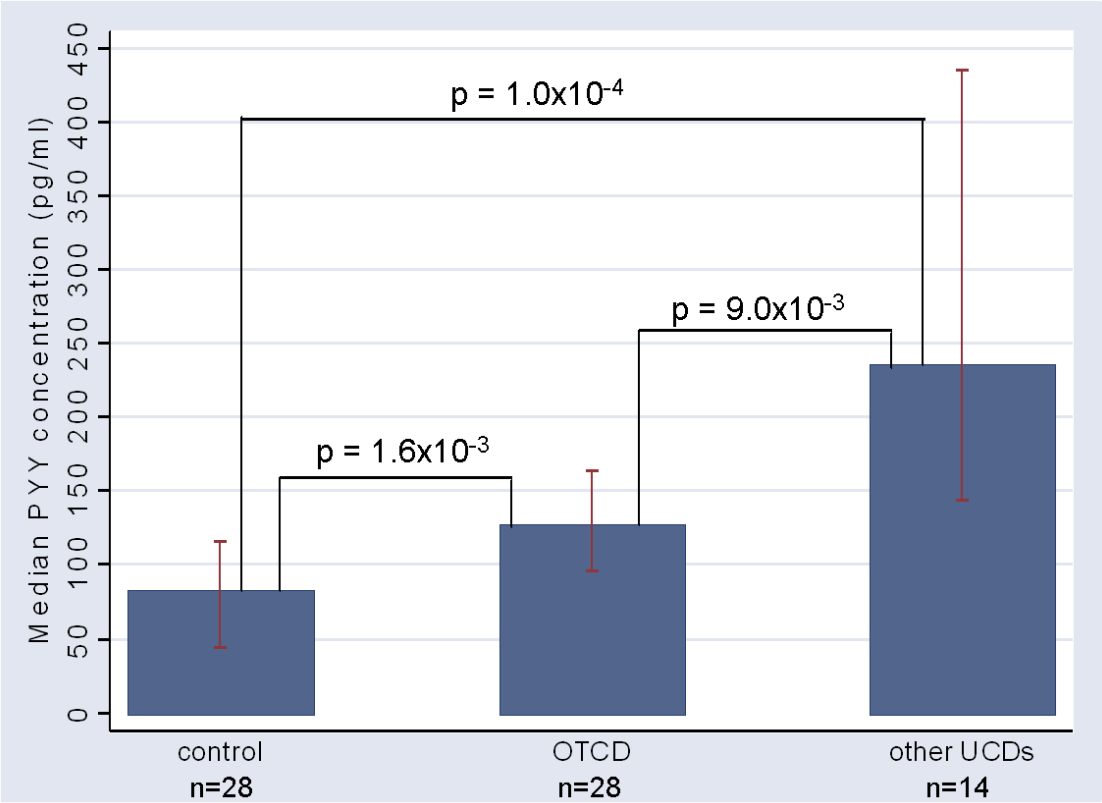


Figure 6.2: Median plasma PYY concentration in OTCD, all other UCD diagnoses and controls Median plasma PYY concentration (pg/mL) are reported, and the red bars show the IQR for each study group (control IQR = 70.6, OTCD IQR =68.1, other UCDs IQR =292.0). A Kruskal-Wallis test was performed to determine if median PYY levels differ across the three groups ($p=1x10^{-4}$), and a Wilcoxon rank-sum test was performed for pair-wise comparisons. PYY = peptide tyrosine tyrosine, OTCD = ornithine transcarbamylase deficiency, other UCDs = other urea cycle disorders (including CPS1, ASS1 & ASL deficiencies), IQR = interquartile range

Discussion

Nutritional management is a mainstay of treatment in PKU and UCD patients (Mofidi and Kronn 2009). Lack of appetite in these patients contributes to poor metabolic control, leading to episodes of metabolic decompensation (Lee et al 2005; Singh et al 2005). The etiology of anorexia in PKU and UCD patients is unknown although there is evidence that altered secretion patterns of appetite

regulating hormones may play a role (Schulpis et al 2004; Schulpis, Papakonstantinou, and Tzamouranis 2000). To extend these preliminary findings, we determined the concentration of the neuroendocrine hormone PYY in the plasma of PKU and UCD patients and a control group from a previously published study (Lomenick et al 2009). PYY levels were significantly increased in both patient groups compared to controls, which supports our hypothesis that the anorexigenic hormone PYY may contribute to loss of appetite among PKU and UCD patients.

It is unclear why PYY levels are increased in PKU and UCD patients, but one possible explanation relates to control of nitrogen intake and co-regulation of *PYY* and *NAGS*. The genes for *PYY* and *NAGS* are divergently transcribed, an arrangement consistent with coordinate regulation (Adachi and Lieber 2002; Beck and Warren 1988; Trinklein et al 2004; Yang and Elnitski 2007). *PYY* and *NAGS* are both upregulated in response to protein; PYY induces satiety, while NAGS provides the co-factor for the first enzyme of the urea cycle (Batterham et al 2006; Colombo, Pfister, and Cervantes 1990; Lomenick et al 2009; Morris, Jr. 2002). Co-regulation of *PYY* and *NAGS* is a potential mechanism linking suppression of nitrogen intake to processing of waste nitrogen. In UCD and PKU patients this may result in prolonged satiation manifesting as a lack of appetite. Additional studies are needed to confirm the proposed mechanism of coordinate regulation of *PYY* and *NAGS* and the potential role it plays in maintenance of nitrogen balance.

Among UCD patients, diagnosis is associated with PYY levels. Patients with OTCD have significantly lower PYY levels compared with patients of all other UCD diagnoses; however OTCD patient PYY levels are still significantly higher compared with controls. One possible explanation for these findings may relate to the clinical variability of OTCD and the predominance of carrier females in the study population. Urea cycle function is less compromised in OTCD carrier females than in patients with other UCD diagnoses (Maestri et al 1998). OTCD carrier females will be able to clear waste nitrogen faster than patients with other UCDs, but not as quickly as normal controls. Therefore, if *PYY* and *NAGS* are coordinately regulated as a means to suppress nitrogen intake while waste nitrogen is processed through the urea cycle, one would expect PYY levels to be lower in the OTCD group compared to the all other UCDs group and higher compared to controls.

Increased body mass index (BMI) was significantly associated with increased PYY levels in the control group but not in the PKU or UCD groups. The reasons for this are unclear, however, the relationship between BMI and PYY is inconsistent in the literature with some studies showing a negative correlation (Batterham et al 2003a; Le Roux et al 2006), some a positive correlation (Lomenick, Clasey, and Anderson 2008), and still others no correlation at all (Kim et al 2005). The control group, which was ascertained for a previously published study, includes both normal weight and obese children (Lomenick et al 2009). In comparison, there were few patients within the PKU (n=1) and UCD (n=8) groups that were overweight. Small sample size and a

relative lack of overweight patients in the PKU and UCD groups may contribute to our inability to detect an association of BMI with PYY levels in these groups.

Increased age was significantly associated with decreased PYY levels in the PKU group but not in the UCD or control groups. The age range of the study groups was variable with the control group ranging from 7-11 years, PKU patients from 1-26 years and UCD patients from 1-51 years. The more homogenous age of the controls is the most likely explanation for our inability to detect an association among this group. The lack of a significant association with PYY and age in the UCD group may be due to heterogeneity of this group or simply a lack of power. Although not significant, the age effect among UCD patients in this study ($\beta = -0.014$) trended in the same direction as the significant effect among PKU patients ($\beta = -0.03$). It is possible an age effect would be detected in a larger study of UCD patients.

Interestingly, two of the infants excluded from analysis at the onset of this study had extraordinarily high PYY concentrations (4629 pg/mL and 6133 pg/mL). Both of these samples were newborns with an UCD. The PYY concentrations in these patients are two to three times higher than values reported for healthy pre-term and term neonates (Adrian et al 1986). To our knowledge there are no previous reports of PYY levels of this magnitude. The effect of such extreme PYY levels is not yet known and requires further study.

There are several limitations of this study. First, due to the retrospective nature of this study, we were unable to directly test loss of appetite with PYY levels. We are assuming that all PKU and UCD patients have a loss of appetite,

and in turn expect higher PYY levels compared with controls. It is possible not all patients tested had a loss of appetite and this could explain the large variability in PYY levels observed in the PKU and UCD patient groups. Another limitation of our study is that fasting times for PKU and UCD patients were unknown. Minimally, these patients were fasted for two hours prior to sample collection whereas control subjects were fasted overnight.

In conclusion, we demonstrate that PYY levels are elevated among PKU and UCD patients compared with controls. Factors associated with PYY levels, depending on the study group, include BMI, age, and UCD diagnosis, which is consistent with previous literature and observations. These data support the hypothesis that increased PYY levels may be associated with loss of appetite and other gastrointestinal symptoms in PKU and UCD patients. However, larger, prospective studies are needed to gain a better understanding of how appetite regulating hormones such as PYY impact the nutritional management of PKU and UCD patients.

CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Human *PYY* and *NAGS* are divergently transcribed and consequently share a 5' flanking region. Therefore, the possibility exists for coordinate regulation via shared regulatory elements in the intergenic space. Despite the interest in *PYY* as a potential therapeutic target for obesity, the regulation of this gene has not been investigated. Studies on the regulation of *NAGS* are also limited.

Multiple experimental approaches were employed to investigate the relationship between *PYY* and *NAGS*. Based solely on the data presented here, it is possible my hypothesis is incorrect and *PYY* and *NAGS* are not coordinately regulated via shared *cis* elements. It is, in fact, possible that *PYY* and *NAGS* are completely independently regulated. Despite my best efforts, I have no definitive data demonstrating these genes are coordinately regulated. However, when all of the results from my investigation are taken together with data published in the literature, coordinate regulation of these two genes is still an attractive model. The location and arrangement of these genes, their concordant expression in the ileum, the increased plasma PYY levels in UCD patients, the trend toward upregulation of both genes in fed mice compared with fasted mice, and the fact

that both are upregulated in response to ingested protein offer support to the proposed model.

Multiple TSSs were identified for *PYY* and *NAGS* in different tissues indicating these genes are regulated by alternative promoters in a tissue specific manner. The results discussed in Chapter IV demonstrate that *PYY* and *NAGS* display distinct expression patterns across human tissues. These genes are discordantly expressed in the majority of human tissues with the exception of the ileum and pancreas where they are coordinately expressed. Taken together, these data suggest if *PYY* and *NAGS* are coordinately regulated it is most likely in a tissue-specific fashion.

Next, if *PYY* and *NAGS* are co-regulated, then, in patients with disrupted nitrogen metabolism, the expectation is that *PYY*, like *NAGS*, would be upregulated. Results of our studies reveal that plasma PYY levels are increased in patients with urea cycle disorders. Interestingly, PYY levels are also increased in other populations which have a diminished capacity for urea synthesis, for example patients with liver cirrhosis and the elderly (Bianchi et al 1991; Fabbri et al 1993; Fabbri et al 1994; Marchesini et al 1990). This may explain, in part, the lack of appetite in these populations. Taken together this supports the global hypothesis of a functional relationship between PYY, NAGS, and nitrogen balance.

The findings from studies using mouse as a model organism provide additional clues to understanding the relationship between PYY and NAGS. We demonstrate that ileal *Pyy* and *Nags* expression is similar within individual mice

chronically fed a low protein or high protein diet and in those on normal chow that were fasted or fed. Although not conclusive, experimental results point to higher expression of both *Pyy* and *Nags* in fed mice compared to fasted mice. This suggests that under these conditions *Pyy* and *Nags* are coordinately regulated.

Overall, our results are consistent with the hypothesis of coordinate regulation of *PYY* and *NAGS*. The mechanism of coordinate regulation could occur through shared *cis* regulatory elements or in a more classic sense, that is, changes in gene expression occurring in response to the same hormone or stimulant. Upon initial examination this divergently transcribed gene pair appears to have non-homologous functions. The work presented here points to a potential functional link in the maintenance of nitrogen balance. Co-regulation of *PYY* and *NAGS* may be a protective mechanism against overconsumption of dietary protein, which is a major contributor to the waste nitrogen pool. Overconsumption of protein could lead to ammonia toxicity if the capacity of the urea cycle is exceeded. Thus, coordinate regulation of these genes may play a part in nitrogen balance by linking the control of nitrogen intake to the processing of waste nitrogen. Furthermore, coordinate regulation of this gene pair may provide some explanation for why protein is the most satiating macronutrient.

Future Directions

The work presented here establishes a foundation for future investigations into the relationship between *PYY* and *NAGS*. To extend the findings presented here, it will be important to identify *cis* elements that are essential for regulation

of *PYY* and *NAGS*, and determine if any of these elements are necessary for regulation of both genes. The lack of an available cell line appropriate for assaying promoter function points toward continued *in vivo* examination of regulatory function using model organisms.

The mechanisms of nitrogen balance are largely unresolved. Additional studies examining PYY levels in human populations would be useful in confirming the functional relationship between PYY and NAGS and its effects on nitrogen balance. The data presented in Chapter VI demonstrating significantly higher plasma PYY concentrations in patients with inborn errors of metabolism are compelling, however, a prospective study using appropriate controls and including hunger/satiety ratings is necessary to determine what role, if any, PYY plays in the lack of appetite common in UCD patients. It would be informative to examine plasma PYY levels in conjunction with blood urea nitrogen and urine urea in UCD patients and other populations with diminished capacity for urea synthesis such as the elderly and patients with liver cirrhosis to determine if there is a relationship between nitrogen clearance and PYY levels.

Finally, it would be interesting to examine the relationship of *PYY* and *NAGS* in an evolutionary context. Koyanagi *et al* suggest that the evolution of the bidirectional arrangement of genes may be linked with the evolution of their coordinated function (Koyanagi et al 2005). For example, the *SERPINI1* and *PDCD10* genes are found in both vertebrates and invertebrates, but the bidirectional arrangement of this gene pair was found only in mammals and in none of other animal species studied (Koyanagi et al 2005). A preliminary

examination of gene arrangement indicates that the location and arrangement of *PYY* and *NAGS* is not conserved in fish, birds, or reptiles. Fish excrete ammonia directly through their gills, while in birds and reptiles it is excreted as uric acid. This preliminary data suggests that co-regulation of *PYY* and *NAGS* may be unique to ureotelic organisms. Ultimately, further studies are needed to elucidate the mechanisms by which this gene pair is regulated and to clarify the functional and evolutionary relationship of *PYY* and *NAGS*.

REFERENCES

1. Adachi, N., Lieber, M. R. (2002) Bidirectional gene organization: a common architectural feature of the human genome. Cell 109, 807-809.

2. Adrian, T. E., Ferri, G. L., Bacarese-Hamilton, A. J., Fuessl, H. S., Polak, J. M., Bloom, S. R. (1985) Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology 89, 1070-1077.

3. Adrian, T. E., Smith, H. A., Calvert, S. A., Aynsley-Green, A., Bloom, S. R. (1986) Elevated plasma peptide YY in human neonates and infants. Pediatr.Res. 20, 1225-1227.

4. Ahituv, N., Kavaslar, N., Schackwitz, W., Ustaszewska, A., Collier, J. M., Hebert, S., Doelle, H., Dent, R., Pennacchio, L. A., McPherson, R. (2006) A PYY Q62P variant linked to human obesity. Hum.Mol.Genet. 15, 387-391.

5. Ali-Rachedi, A., Varndell, I. M., Adrian, T. E., Gapp, D. A., Van, N. S., Bloom, S. R., Polak, J. M. (1984) Peptide YY (PYY) immunoreactivity is co-stored with glucagon-related immunoreactants in endocrine cells of the gut and pancreas. Histochemistry 80, 487-491.

6. Asakawa, A., Inui, A., Ueno, N., Fujimiya, M., Fujino, M. A., Kasuga, M. (1999) Mouse pancreatic polypeptide modulates food intake, while not influencing anxiety in mice. Peptides 20, 1445-1448.

7. Asakawa, A., Inui, A., Yuzuriha, H., Ueno, N., Katsuura, G., Fujimiya, M., Fujino, M. A., Niijima, A., Meguid, M. M., Kasuga, M. (2003) Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. Gastroenterology 124, 1325-1336.

8. Batshaw, M. L., Berry, G. T. (1991) Use of citrulline as a diagnostic marker in the prospective treatment of urea cycle disorders. J.Pediatr. 118, 914-917.

9. Batterham, R. L., Cohen, M. A., Ellis, S. M., Le Roux, C. W., Withers, D. J., Frost, G. S., Ghatei, M. A., Bloom, S. R. (2003a) Inhibition of food intake in obese subjects by peptide YY3-36. N.Engl.J.Med. 349, 941-948.

10. Batterham, R. L., Cowley, M. A., Small, C. J., Herzog, H., Cohen, M. A., Dakin, C. L., Wren, A. M., Brynes, A. E., Low, M. J., Ghatei, M. A., Cone,

R. D., Bloom, S. R. (2002) Gut hormone PYY(3-36) physiologically inhibits food intake. Nature 418, 650-654.

11. Batterham, R. L., Heffron, H., Kapoor, S., Chivers, J. E., Chandarana, K., Herzog, H., Le Roux, C. W., Thomas, E. L., Bell, J. D., Withers, D. J. (2006) Critical role for peptide YY in protein-mediated satiation and body-weight regulation. Cell Metab 4, 223-233.

12. Batterham, R. L., Le Roux, C. W., Cohen, M. A., Park, A. J., Ellis, S. M., Patterson, M., Frost, G. S., Ghatei, M. A., Bloom, S. R. (2003b) Pancreatic polypeptide reduces appetite and food intake in humans. J.Clin.Endocrinol.Metab 88, 3989-3992.

13. Beck, C. F., Warren, R. A. (1988) Divergent promoters, a common form of gene organization. Microbiol.Rev. 52, 318-326.

14. Bell, A. C., West, A. G., Felsenfeld, G. (1999) The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. Cell 98, 387-396.

15. Bianchi, G., Marchesini, G., Vilstrup, H., Fabbri, A., De Mitri, M. S., Zoli, M., Pisi, E. (1991) Hepatic amino-nitrogen clearance to urea-nitrogen in control subjects and in patients with cirrhosis: a simplified method. Hepatology 13, 460-466.

16. Boey, D., Heilbronn, L., Sainsbury, A., Laybutt, R., Kriketos, A., Herzog, H., Campbell, L. V. (2006a) Low serum PYY is linked to insulin resistance in first-degree relatives of subjects with type 2 diabetes. Neuropeptides 40, 317-324.

17. Boey, D., Lin, S., Karl, T., Baldock, P., Lee, N., Enriquez, R., Couzens, M., Slack, K., Dallmann, R., Sainsbury, A., Herzog, H. (2006b) Peptide YY ablation in mice leads to the development of hyperinsulinaemia and obesity. Diabetologia 49, 1360-1370.

18. Brusilow, S. W. (1985) Disorders of the urea cycle. Hosp.Pract.(Off Ed) 20, 65-72.

19. Burbelo, P. D., Martin, G. R., Yamada, Y. (1988) Alpha 1(IV) and alpha 2(IV) collagen genes are regulated by a bidirectional promoter and a shared enhancer. Proc.Natl.Acad.Sci.U.S.A 85, 9679-9682.

20. Caldovic, L., Morizono, H., Gracia, P. M., Gallegos, R., Yu, X., Shi, D., Malamy, M. H., Allewell, N. M., Tuchman, M. (2002a) Cloning and expression of the human N-acetylglutamate synthase gene. Biochem.Biophys.Res.Commun. 299, 581-586.

21. Caldovic, L., Morizono, H., Yu, X., Thompson, M., Shi, D., Gallegos, R., Allewell, N. M., Malamy, M. H., Tuchman, M. (2002b) Identification, cloning and expression of the mouse N-acetylglutamate synthase gene. Biochem.J. 364, 825-831.

22. Carey, M., Smale, S. (2000) Transcriptional Regulation in Eukaryotes Concepts, Strategies and Techniques, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

23. Cohen, N. S., Cheung, C. W., Raijman, L. (1987) Channeling of extramitochondrial ornithine to matrix ornithine transcarbamylase. J.Biol.Chem. 262, 203-208.

24. Colombo, J. P., Pfister, U., Cervantes, H. (1990) The regulation of N-acetylglutamate synthetase in rat liver by protein intake. Biochem.Biophys.Res.Commun. 172, 1239-1245.

25. Dhahbi, J. M., Kim, H. J., Mote, P. L., Beaver, R. J., Spindler, S. R. (2004) Temporal linkage between the phenotypic and genomic responses to caloric restriction. Proc.Natl.Acad.Sci.U.S.A 101, 5524-5529.

26. Ekblad, E., Sundler, F. (2002) Distribution of pancreatic polypeptide and peptide YY. Peptides 23, 251-261.

27. El-Salhy, M., Suhr, O., Danielsson, A. (2002) Peptide YY in gastrointestinal disorders. Peptides 23, 397-402.

28. Fabbri, A., Marchesini, G., Bianchi, G., Bugianesi, E., Bortoluzzi, L., Zoli, M., Pisi, E. (1993) Unresponsiveness of hepatic nitrogen metabolism to glucagon infusion in patients with cirrhosis: dependence on liver cell failure. Hepatology 18, 28-35.

29. Fabbri, A., Marchesini, G., Bianchi, G., Bugianesi, E., Zoli, M., Pisi, E. (1994) Kinetics of hepatic amino-nitrogen conversion in ageing man. Liver 14, 288-294.

30. Fong, C. T. (1995) Principles of inborn errors of metabolism: an exercise. Pediatr.Rev. 16, 390-395.

31. Glavas, M. M., Grayson, B. E., Allen, S. E., Copp, D. R., Smith, M. S., Cowley, M. A., Grove, K. L. (2008) Characterization of brainstem peptide YY (PYY) neurons. J.Comp Neurol. 506, 194-210.

32. Gropper, S. S., Smith, J. L., Groff, J. L. (2009) Advanced Nutrion and Human Metabolism, Fifth ed. Wadsworth, Belmont, CA.

33. Grossman, L. I., Seelan, R. S., Jaradat, S. A. (1998) Transcriptional regulation of mammalian cytochrome c oxidase genes. Electrophoresis 19, 1254-1259.

34. Guo, Y., Ma, L., Enriori, P. J., Koska, J., Franks, P. W., Brookshire, T., Cowley, M. A., Salbe, A. D., Delparigi, A., Tataranni, P. A. (2006) Physiological evidence for the involvement of peptide YY in the regulation of energy homeostasis in humans. Obesity.(Silver.Spring) 14, 1562-1570.

35. Gupta, A. (2007) To Err is Genetics: Diagnosis and Management of Inborn Errors of Metabolism (IEM). The Anthropologist Special 3, 415-423.

36. Haberle, J., Schmidt, E., Pauli, S., Kreuder, J. G., Plecko, B., Galler, A., Wermuth, B., Harms, E., Koch, H. G. (2003) Mutation analysis in patients with N-acetylglutamate synthase deficiency. Hum.Mutat. 21, 593-597.

37. Halton, T. L., Hu, F. B. (2004) The effects of high protein diets on thermogenesis, satiety and weight loss: a critical review. J.Am.Coll.Nutr. 23, 373-385.

38. Heikkila, P., Soininen, R., Tryggvason, K. (1993) Directional regulatory activity of cis-acting elements in the bidirectional alpha 1(IV) and alpha 2(IV) collagen gene promoter. J.Biol.Chem. 268, 24677-24682.

39. Herr, D. R., Harris, G. L. (2004) Close head-to-head juxtaposition of genes favors their coordinate regulation in Drosophila melanogaster. FEBS Lett. 572, 147-153.

40. Hort, Y., Baker, E., Sutherland, G. R., Shine, J., Herzog, H. (1995) Gene duplication of the human peptide YY gene (PYY) generated the pancreatic polypeptide gene (PPY) on chromosome 17q21.1. Genomics 26, 77-83.

41. Ikeda, S., Ayabe, H., Mori, K., Seki, Y., Seki, S. (2002) Identification of the functional elements in the bidirectional promoter of the mouse O-sialoglycoprotein endopeptidase and APEX nuclease genes. Biochem.Biophys.Res.Commun. 296, 785-791.

42. International Human Genome Sequencing Consortium (2004) Finishing the euchromatic sequence of the human genome. Nature 431, 931-945.

43. Johnson, L. R., Barret, K. E., Ghishan, F. K., Merchant, J. L., Said, H. M., Wood, J. D. (2006) Physiology of the Gastrointestinal Tract, Volume 2 Fourth ed. Elsevier Academic Press, London.

44. Karra, E., Batterham, R. L. (2010) The role of gut hormones in the regulation of body weight and energy homeostasis. Mol.Cell Endocrinol. 316, 120-128.

45. Kensche, P. R., Oti, M., Dutilh, B. E., Huynen, M. A. (2008) Conservation of divergent transcription in fungi. Trends Genet. 24, 207-211.

46. Kim, B. J., Carlson, O. D., Jang, H. J., Elahi, D., Berry, C., Egan, J. M. (2005) Peptide YY is secreted after oral glucose administration in a gender-specific manner. J.Clin.Endocrinol.Metab 90, 6665-6671.

47. Kim, T. H., Abdullaev, Z. K., Smith, A. D., Ching, K. A., Loukinov, D. I., Green, R. D., Zhang, M. Q., Lobanenkov, V. V., Ren, B. (2007) Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. Cell 128, 1231-1245.

48. Knutson, B. A., Drennan, M., Liu, X., Broyles, S. S. (2009) Bidirectional transcriptional promoters in the vaccinia virus genome. Virology 385, 198-203.

49. Kohri, K., Nata, K., Yonekura, H., Nagai, A., Konno, K., Okamoto, H. (1993) Cloning and structural determination of human peptide YY cDNA and gene. Biochim.Biophys.Acta 1173, 345-349.

50. Korner, J., Leibel, R. L. (2003) To eat or not to eat - how the gut talks to the brain. N.Engl.J.Med. 349, 926-928.

51. Koyanagi, K. O., Hagiwara, M., Itoh, T., Gojobori, T., Imanishi, T. (2005) Comparative genomics of bidirectional gene pairs and its implications for the evolution of a transcriptional regulation system. Gene 353, 169-176.

52. Krasinski, S. D., Wheeler, M. B., Kopin, A. S., Leiter, A. B. (1990) Pancreatic polypeptide and peptide YY gene expression. Ann.N.Y.Acad.Sci. 611, 73-85.

53. Krebs, H., Henseleit, K. (1932) Studies on urea formation in the animal organism. Hoppe-Seylers Z.Physiol.Chem. 210, 33-36.

54. Latner, J. D., Schwartz, M. (1999) The effects of a high-carbohydrate, high-protein or balanced lunch upon later food intake and hunger ratings. Appetite 33, 119-128.

55. Le Roux, C. W., Batterham, R. L., Aylwin, S. J., Patterson, M., Borg, C. M., Wynne, K. J., Kent, A., Vincent, R. P., Gardiner, J., Ghatei, M. A., Bloom, S. R. (2006) Attenuated peptide YY release in obese subjects is associated with reduced satiety. Endocrinology 147, 3-8.

56. Lee, B., Singh, R. H., Rhead, W. J., Sniderman, K. L., Smith, W., Summar, M. L. (2005) Considerations in the difficult-to-manage urea cycle disorder patient. Crit Care Clin. 21, S19-S25.

57. Lee, S. G., Song, K. (2000) Identification and characterization of a bidirectional promoter from the intergenic region between the human DDX13 and RD genes. Mol.Cells 10, 47-53.

58. Leiter, A. B., Toder, A., Wolfe, H. J., Taylor, I. L., Cooperman, S., Mandel, G., Goodman, R. H. (1987) Peptide YY. Structure of the precursor and expression in exocrine pancreas. J.Biol.Chem. 262, 12984-12988.

59. Lejeune, M. P., Westerterp, K. R., Adam, T. C., Luscombe-Marsh, N. D., Westerterp-Plantenga, M. S. (2006) Ghrelin and glucagon-like peptide 1 concentrations, 24-h satiety, and energy and substrate metabolism during a high-protein diet and measured in a respiration chamber. Am.J.Clin.Nutr. 83, 89-94.

60. Lin, J. M., Collins, P. J., Trinklein, N. D., Fu, Y., Xi, H., Myers, R. M., Weng, Z. (2007) Transcription factor binding and modified histones in human bidirectional promoters. Genome Res. 17, 818-827.

61. Lomenick, J. P., Clasey, J. L., Anderson, J. W. (2008) Meal-related changes in ghrelin, peptide YY, and appetite in normal weight and overweight children. Obesity.(Silver.Spring) 16, 547-552.

62. Lomenick, J. P., Melguizo, M. S., Mitchell, S. L., Summar, M. L., Anderson, J. W. (2009) Effects of meals high in carbohydrate, protein, and fat on ghrelin and peptide YY secretion in prepubertal children. J.Clin.Endocrinol.Metab 94, 4463-4471.

63. Ma, L., Tataranni, P. A., Hanson, R. L., Infante, A. M., Kobes, S., Bogardus, C., Baier, L. J. (2005) Variations in peptide YY and Y2 receptor genes are associated with severe obesity in Pima Indian men. Diabetes 54, 1598-1602.

64. MacDonald, A., Rylance, G. W., Asplin, D. A., Hall, K., Harris, G., Booth, I. W. (1994) Feeding problems in young PKU children. Acta Paediatr.Suppl 407, 73-74.

65. Maestri, N. E., Lord, C., Glynn, M., Bale, A., Brusilow, S. W. (1998) The phenotype of ostensibly healthy women who are carriers for ornithine transcarbamylase deficiency. Medicine (Baltimore) 77, 389-397.

66. Maljaars, P. W., Peters, H. P., Mela, D. J., Masclee, A. A. (2008) Ileal brake: a sensible food target for appetite control. A review. Physiol Behav. 95, 271-281.

67. Marchesini, G., Bianchi, G. P., Fabbri, A., Lolli, R., Bugianesi, E., Zoli, M., Pisi, E. (1990) Synthesis of urea after a protein-rich meal in normal man in relation to ageing. Age Ageing 19, 4-10.

68. McCullough, B. A., Yudkoff, M., Batshaw, M. L., Wilson, J. M., Raper, S. E., Tuchman, M. (2000) Genotype spectrum of ornithine transcarbamylase deficiency: correlation with the clinical and biochemical phenotype. Am.J.Med.Genet. 93, 313-319.

69. Mian, A., Lee, B. (2002) Urea-cycle disorders as a paradigm for inborn errors of hepatocyte metabolism. Trends Mol.Med. 8, 583-589.

70. Minth, C. D., Andrews, P. C., Dixon, J. E. (1986) Characterization, sequence, and expression of the cloned human neuropeptide Y gene. J.Biol.Chem. 261, 11974-11979.

71. Misra, M., Miller, K. K., Tsai, P., Gallagher, K., Lin, A., Lee, N., Herzog, D. B., Klibanski, A. (2006) Elevated peptide YY levels in adolescent girls with anorexia nervosa. J.Clin.Endocrinol.Metab 91, 1027-1033.

72. Mofidi, S., Kronn, D. (2009) Emergency Management of Inherited Metabolic Disorders. Topics in Clinical Nutrition 24, 374-384.

73. Morris, S. M., Jr. (2002) Regulation of enzymes of the urea cycle and arginine metabolism. Annu.Rev.Nutr. 22, 87-105.

74. Morris, S. M., Jr., Kepka-Lenhart, D. (2002) Hormonal induction of hepatic mitochondrial ornithine/citrulline transporter mRNA. Biochem.Biophys.Res.Commun. 294, 749-752.

75. Morris, S. M., Jr., Moncman, C. L., Rand, K. D., Dizikes, G. J., Cederbaum, S. D., O'Brien, W. E. (1987) Regulation of mRNA levels for five urea cycle enzymes in rat liver by diet, cyclic AMP, and glucocorticoids. Arch.Biochem.Biophys. 256, 343-353.

76. Nassogne, M. C., Heron, B., Touati, G., Rabier, D., Saudubray, J. M. (2005) Urea cycle defects: management and outcome. J.Inherit.Metab Dis. 28, 407-414.

77. Paddon-Jones, D., Westman, E., Mattes, R. D., Wolfe, R. R., Astrup, A., Westerterp-Plantenga, M. (2008) Protein, weight management, and satiety. Am.J.Clin.Nutr. 87, 1558S-1561S.

78. Pedrazzini, T., Pralong, F., Grouzmann, E. (2003) Neuropeptide Y: the universal soldier. Cell Mol.Life Sci. 60, 350-377.

79. Pfluger, P. T., Kampe, J., Castaneda, T. R., Vahl, T., D'Alessio, D. A., Kruthaupt, T., Benoit, S. C., Cuntz, U., Rochlitz, H. J., Moehlig, M., Pfeiffer, A. F., Koebnick, C., Weickert, M. O., Otto, B., Spranger, J., Tschop, M. H. (2007) Effect of human body weight changes on circulating levels of peptide YY and peptide YY3-36. J.Clin.Endocrinol.Metab 92, 583-588.

80. Pray, L. (2008) Eukaryotic Genome Complexity. Nature Education 1.

81. Rezvani, I. (2007) Defects in Metabolism of Amino Acids, in Nelson Textbook of Pediatrics 18th ed. Saunders Elsevier, Philadelphia.

82. Rolls, B. J., Hetherington, M., Burley, V. J. (1988) The specificity of satiety: the influence of foods of different macronutrient content on the development of satiety. Physiol Behav. 43, 145-153.

83. Rozengurt, N., Wu, S. V., Chen, M. C., Huang, C., Sternini, C., Rozengurt, E. (2006) Colocalization of the alpha-subunit of gustducin with PYY and GLP-1 in L cells of human colon. Am.J.Physiol Gastrointest.Liver Physiol 291, G792-G802.

84. Schulpis, K. H., Papakonstantinou, E. D., Tzamouranis, J. (2000) Plasma leptin concentrations in phenylketonuric patients. Horm.Res. 53, 32-35.

85. Schulpis, K. H., Papassotiriou, I., Vounatsou, M., Karikas, G. A., Tsakiris, S., Chrousos, G. P. (2004) Morning preprandial plasma ghrelin and catecholamine concentrations in patients with phenylketonuria and normal controls: evidence for catecholamine-mediated ghrelin regulation. J.Clin.Endocrinol.Metab 89, 3983-3987.

86. Selman, C., Kerrison, N. D., Cooray, A., Piper, M. D., Lingard, S. J., Barton, R. H., Schuster, E. F., Blanc, E., Gems, D., Nicholson, J. K., Thornton, J. M., Partridge, L., Withers, D. J. (2006) Coordinated multitissue transcriptional and plasma metabonomic profiles following acute caloric restriction in mice. Physiol Genomics 27, 187-200.

87. Sheikh, S. P., Holst, J. J., Orskov, C., Ekman, R., Schwartz, T. W. (1989) Release of PYY from pig intestinal mucosa; luminal and neural regulation. Regul.Pept. 26, 253-266.

88. Shih, P. A., Wang, L., Chiron, S., Wen, G., Nievergelt, C., Mahata, M., Khandrika, S., Rao, F., Fung, M. M., Mahata, S. K., Hamilton, B. A., O'Connor, D. T. (2009) Peptide YY (PYY) gene polymorphisms in the 3'-untranslated and proximal promoter regions regulate cellular gene expression and PYY secretion and metabolic syndrome traits in vivo. J.Clin.Endocrinol.Metab 94, 4557-4566.

89. Siahanidou, T., Mandyla, H., Vounatsou, M., Anagnostakis, D., Papassotiriou, I., Chrousos, G. P. (2005) Circulating peptide YY concentrations are higher in preterm than full-term infants and correlate negatively with body weight and positively with serum ghrelin concentrations. Clin.Chem. 51, 2131-2137.

90. Singh, R. H. (2007) Nutritional management of patients with urea cycle disorders. J.Inherit.Metab Dis. 30, 880-887.

91. Singh, R. H., Rhead, W. J., Smith, W., Lee, B., Sniderman, K. L., Summar, M. (2005) Nutritional management of urea cycle disorders. Crit Care Clin. 21, S27-S35.

92. Soderberg, C., Pieribone, V. A., Dahlstrand, J., Brodin, L., Larhammar, D. (1994) Neuropeptide role of both peptide YY and neuropeptide Y in vertebrates suggested by abundant expression of their mRNAs in a cyclostome brain. J.Neurosci.Res. 37, 633-640.

93. Stein, L. D. (2004) Human genome: end of the beginning. Nature 431, 915-916.

94. Strausberg, R. L., Feingold, E. A., Grouse, L. H., Derge, J. G., Klausner, R. D., Collins, F. S., Wagner, L., Shenmen, C. M., Schuler, G. D., Altschul, S. F., Zeeberg, B., Buetow, K. H., Schaefer, C. F., Bhat, N. K., Hopkins, R. F., Jordan, H., Moore, T., Max, S. I., Wang, J., Hsieh, F., Diatchenko, L., Marusina, K., Farmer, A. A., Rubin, G. M., Hong, L., Stapleton, M., Soares, M. B., Bonaldo, M. F., Casavant, T. L., Scheetz, T. E., Brownstein, M. J., Usdin, T. B., Toshiyuki, S., Carninci, P., Prange, C., Raha, S. S., Loquellano, N. A., Peters, G. J., Abramson, R. D., Mullahy, S. J., Bosak, S. A., McEwan, P. J., McKernan, K. J., Malek, J. A., Gunaratne, P. H., Richards, S., Worley, K. C., Hale, S., Garcia, A. M., Gay, L. J., Hulyk, S. W., Villalon, D. K., Muzny, D. M., Sodergren, E. J., Lu, X., Gibbs, R. A., Fahey, J., Helton, E., Ketteman, M., Madan, A., Rodrigues, S., Sanchez, A., Whiting, M., Madan, A., Young, A. C., Shevchenko, Y., Bouffard, G. G., Blakesley, R. W., Touchman, J. W., Green, E. D., Dickson, M. C., Rodriguez, A. C., Grimwood, J., Schmutz, J., Myers, R. M., Butterfield, Y. S., Krzywinski, M. I., Skalska, U., Smailus, D. E., Schnerch, A., Schein, J. E., Jones, S. J., Marra, M. A. (2002) Generation

and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc.Natl.Acad.Sci.U.S.A 99, 16899-16903.

95. Summar, M., Tuchman, M. (2001) Proceedings of a consensus conference for the management of patients with urea cycle disorders. J.Pediatr. 138, S6-10.

96. Takiguchi, M., Matsubasa, T., Amaya, Y., Mori, M. (1989) Evolutionary aspects of urea cycle enzyme genes. Bioessays 10, 163-166.

97. Takiguchi, M., Mori, M. (1995) Transcriptional regulation of genes for ornithine cycle enzymes. Biochem.J. 312 (Pt 3), 649-659.

98. Trinklein, N. D., Aldred, S. F., Hartman, S. J., Schroeder, D. I., Otillar, R. P., Myers, R. M. (2004) An abundance of bidirectional promoters in the human genome. Genome Res. 14, 62-66.

99. Tuchman, M., Morizono, H., Rajagopal, B. S., Plante, R. J., Allewell, N. M. (1998) The biochemical and molecular spectrum of ornithine transcarbamylase deficiency. J.Inherit.Metab Dis. 21 Suppl 1, 40-58.

100. Ueda, R., Iketaki, H., Nagata, K., Kimura, S., Gonzalez, F. J., Kusano, K., Yoshimura, T., Yamazoe, Y. (2006) A common regulatory region functions bidirectionally in transcriptional activation of the human CYP1A1 and CYP1A2 genes. Mol.Pharmacol. 69, 1924-1930.

101. Ueno, H., Yamaguchi, H., Mizuta, M., Nakazato, M. (2008) The role of PYY in feeding regulation. Regul.Pept. 145, 12-16.

102. Wang, G., Leiter, A. B., Englander, E. W., Greeley, G. H., Jr. (2004) Insulin-like growth factor I increases rat peptide YY promoter activity through Sp1 binding sites. Endocrinology 145, 659-666.

103. Waterlow, J. C. (1999) The mysteries of nitrogen balance. Nutr.Res.Rev. 12, 25-54.

104. Weiner, D. L. (2009) Pediatrics, Inborn Errors of Metabolism. eMedicine.

105. Westerterp-Plantenga, M. S., Rolland, V., Wilson, S. A., Westerterp, K. R. (1999) Satiety related to 24 h diet-induced thermogenesis during high protein/carbohydrate vs high fat diets measured in a respiration chamber. Eur.J.Clin.Nutr. 53, 495-502.

106. Yamada, H., Chen, D., Monstein, H. J., Hakanson, R. (1997) Effects of fasting on the expression of gastrin, cholecystokinin, and somatostatin genes and of various housekeeping genes in the pancreas and upper digestive tract of rats. Biochem.Biophys.Res.Commun. 231, 835-838.

107. Yang, M. Q., Elnitski, L. L. (2007) A computational study of bidirectional promoters in the human genome. Lect.Notes Comput.Sci. 4463, 361-371.

108. Zanotto, E., Shah, Z. H., Jacobs, H. T. (2007) The bidirectional promoter of two genes for the mitochondrial translational apparatus in mouse is regulated by an array of CCAAT boxes interacting with the transcription factor NF-Y. Nucleic Acids Res. 35, 664-677.

109. Zhang, T., Uchida, T., Gomez, G., Lluis, F., Thompson, J. C., Greeley, G. H., Jr. (1993) Neural regulation of peptide YY secretion. Regul.Pept. 48, 321-328.