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The Proprotein Convertases in the Murine Small Intestine

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THE PROPROTEIN CONVERTASES IN THE MURINE SMALL INTESTINE

By

Jeffrey Gagnon

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

The small intestine (SI) is a major endocrine organ with over 40 precursor hormones produced and proteolytically matured into active peptide hormones which signal throughout the body including the pancreas and CNS. One group of enzymes believed to be responsible for this maturation is the family of proprotein convertases (PCSKs). Using double immunofluorescent microscopy, the spatial localization of PCSK1, 2 and 3 in each region of the SI and colocalization with potential intestinal substrates was examined in mice. A unique regional expression pattern was observed for each of the PCSKs and several hormones examined exhibited high levels of colocalization. Next the gastrointestinal physiology of the PCSK2 knock out (KO) mouse was examined and correlated with the circulating levels of hormones known to mediate these functions. KO animals consume less food immediately after refeeding and have delayed intestinal transit. Several of the hormones responsible for feeding and intestinal motility were modulated in the PCSK KO animals. These studies suggest that the PCSK1 2 and 3 are present in the SI and required for normal functionality.

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Having started with this research group through high school co-op I wish to thank all the supervisors I have had over the 7 years at the OHRI. In particular, I thank my thesis supervisors Dr Michel Chrétien and Dr John Woulfe for their wisdom and guidance. I also thank Dr Janice Mayne and Dr Majambu Mbikay for their daily support and open ears. Direction was never in short supply.

Special thanks to all the research support staff including technicians Andrew Chen, Angela Raymond, Francine Sirois and administrative assistant JoAnn McDonald. Canadian Institutes of Health Research (CIHR) is acknowledged for their support of this research project through a grant to my supervisor Dr. Chrétien which supplied my graduate stipend.

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List of Abbreviations

PCSK: proprotein convertase subtilisin kexin type

EE: enteroendocrine

SI: small intestine

LPH: lipotrophic hormone

MSH: melanocyte stimulating hormone

CCK: cholecystokinin

GIP: glucose dependant insulinotropic polypeptide

SS: somatostatin

SP: substance P

GLP1: glucose dependant insulinotropic polypeptide

NPY: neuropeptide tyrosine

PYY: peptide tyrosine tyrosine

VIP: vasoactive intestinal polypeptide

GI: gastrointestinal

POMC: proopiomelanocortin

1. Introduction

1.1 The Proprotein Convertase Substilisin Kexin types (PCSKs)

1.1.1 Historical background

The concept that certain proteins such as enzymes are initially produced as inactive precursors (zymogens) before they mature to their active forms dates back to the 1950's with enzymatic work by Hans Neurath (86). The concept of limited proteolysis (as opposed to complete protein degradation) as the method of activating these zymogens was illustrated in the mid 1950's by showing that bacterial subtilisin could activate chymotrypsinogen (16).

The discovery that most peptide hormones are biosynthesized as larger inactive precursors which are modified into their mature active forms came a number of years later in 1967 from two separate groups. As a postdoctoral student at UC Berkeley, Michel Chrétien conducted chemical sequencing of β-and γ lipotrophic hormone (LPH) with sheep pituitary glands in which he found that within the peptide sequence of βLPH, another hormone, β melanocyte stimulating hormone (βMSH) was found (23). This was the chemical evidence, at the level of primary protein sequence that peptide hormones could be found within larger protein molecules. At the same time in the United States while working on the biosynthesis of insulin, Dr. Donald Steiner determined via pulse chase labeling that the smaller mature insulin was derived from a larger pro-insulin molecule (128). These two individuals conducted the seminal work indicating that mature peptide hormones could be derived from larger prohormones. However, the mechanisms

of how this was achieved would not be elucidated for many years. In the 1970's with the development of techniques such as cDNA cloning and DNA sequencing, it became clear that many mature hormones were arising from precursor forms (129) and that the cleavage of these precursors often occurred after paired basic amino acids (lysine or arginine) (22).

The elucidation of which endoproteases were responsible for these maturations came from knowledge in the yeast system. While working with yeast mating factors, Dr. Fuller in Michigan determined that the mating factor precursor was processed at a number of di-basic sites by an enzyme already known as kexin (40). Thanks to an expanding DNA database, he went on to compare the sequence of yeast kexin with other known proteins and found that kexin had almost 50% sequence similarity in the catalytic domain to another mammalian protein of unknown function known as furin (39). Furin (now known as PCSK3) then became a key candidate for the mammalian processing enzyme involved in the maturation of the growing list of prohormones. Due to its homology with bacterial substilisin and yeast kexin, the term proprotein convertase substilisin kexin-type (PCSK) was born (139).

With the advent of the polymerase chain reaction (PCR) in molecular biology, rapid studies searching cDNA libraries with probes designed around the catalytic domains of kexin and PCSK3 were carried out and 2 other proteases known as proprotein convertase 1 (PCSK1) and 2 (PCSK2) (117, 126) were discovered. These studies continued throughout the 1990's and even into 21st century with a current total of 9 PCSKs known to be present in humans with diverse functions and substrates (121).

1.1.2 PCSK domain structure and function

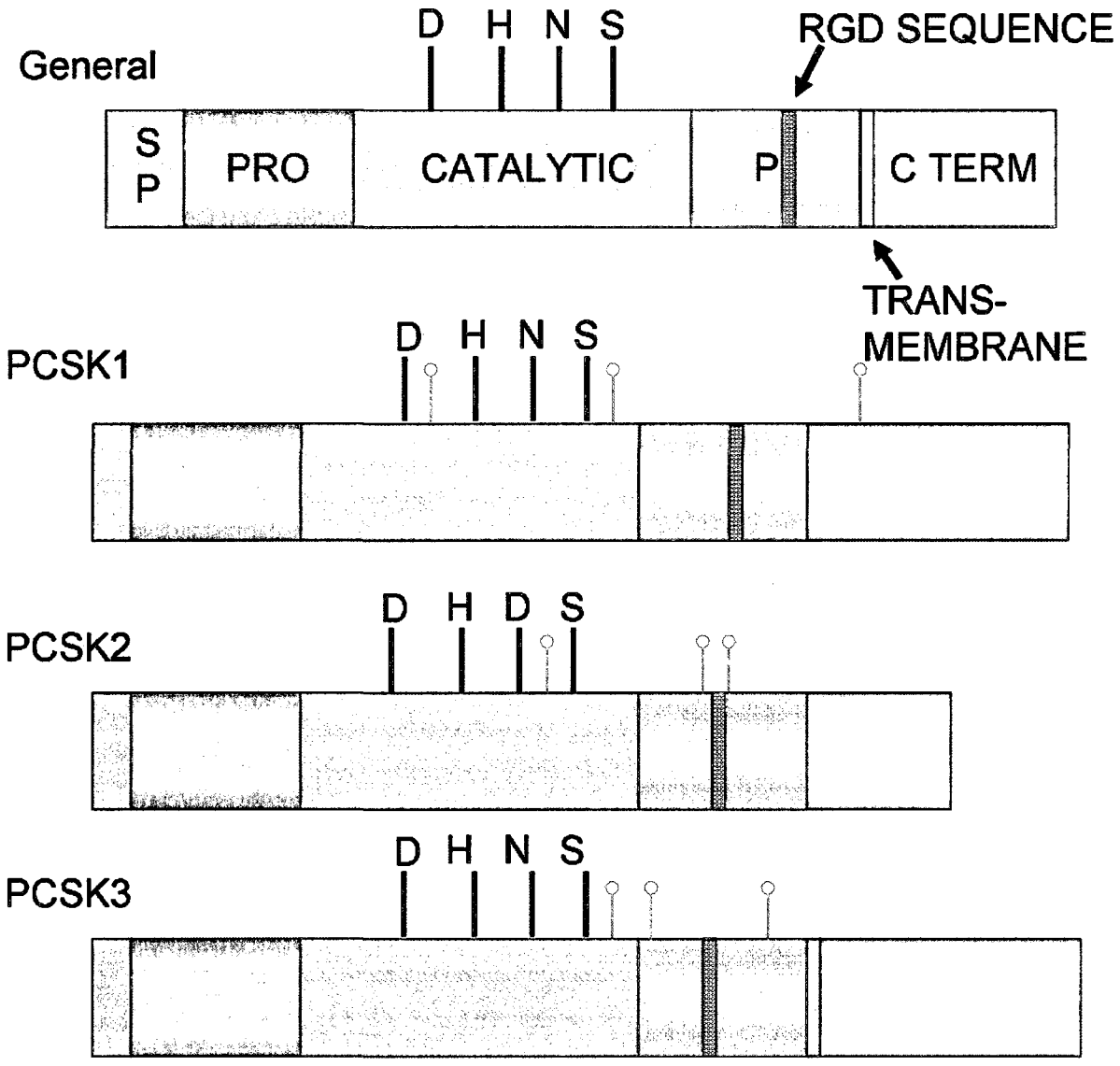
The PCSKs are a multi-domain protein family (Figure 1). As they are produced in the secretory pathway they contain a signal peptide domain at their N-terminus. The signal peptide is followed by a prodomain region which 1, initially is important in the successful folding of the growing polypeptide(12) and 2, acts as a intramolecular inhibitor that must be cleaved from the convertase for activity (12). This cleavage can occur co-translationally in the endoplasmic reticulum (ER) or can occur later in the trans-golgi network (TGN)(12). *In vitro*, it was later shown that certain peptides, based on the pro-domains of the several of the PCSKs, had the ability to act as a specific and potent inhibitors of the enzymes (154).

Immediately c-terminal to the pro-domain is the catalytic domain. This region has the common three residue "catalytic triad" found in other serine proteases, beginning with a histidine followed by an asparagine and then the catalytic serine (8).

The catalytic domain is N-terminal to a P domain. This region is important in the stability of the enzyme and what pH optimum it can be active within (155). Some studies have indicated that the arginine, glycine glutamine (RGD) sequence within this domain is required to traffic to secretory granules (65, 101).

The final C-terminal domain of PCSKs has been shown to possess a variety of functions including trafficking. In PCSK 1, 2 and 5A the α-helical domain is required for trafficking to the appropriate vesicles (31). In the case of PCSK1 the C-terminal domain is actually cleaved off in a secondary cleavage step enabling the most active form of this enzyme (93). For the PCSK3, the c-terminal domain possesses an inter-membrane region

Figure 1. Schematic protein structure for PCSKs 1, 2 and 3. Different regions identified by color including; signal peptide (SP), pro domain (PRO), catalytic, P, transmembrane and C-terminal (C-TERM) domains. The indicated residues in the catalytic domain indicate conserved amino acids for catalytic activity. Blue bars indicate gylcosylation sites.


General
D H N S
RGD SEQUENCE
S P
PRO
CATALYTIC
P
C TERM
TRANS-
MEMBRANE
PCSK1
D H N S
PCSK2
D H D S
PCSK3
D H N S

that is unique to this convertase and allows it to be membrane-bound and cycle between the trans-golgi membrane (TGM), the plasma membrane and endosomes (14).

1.1.3 PCSK subcellular localization and tissue distribution

The distribution of each of the PCSKs has been examined initially with in situ hybridization and Northern blotting, and subsequently with Western blotting as antibodies became readily available. A summary of their tissue and sub-cellular localization is found in Table 1.

Several PCSKs, including PCSK1, 2, 4 and 5A, are in the regulated secretory pathway and as such are released into secretory granules (101). Their distribution, with the exception of PCSK5A is often endocrine tissue specific. PCSK1 and 2 are found throughout the endocrine system including neuroendocrine and enteroendocrine cell types (153, 156). They were originally discovered in the human insulinoma cell line (126). Later studies established that they are present in a variety of tissues including the pancreas (119), pituitary and other brain areas (10). These convertases are also present throughout GI tract (43, 138). Often these two PCSKs are found in the same tissues and even co-localize to the same cell so that they may both act on a similar precursor as is the case with proopiomelanocortin (POMC) in the pituitary (10). PCSK4 is exclusively expressed in the testis (62, 74) and is important for reproduction as males with an interrupted PCSK4 gene have impaired fertility (74). PCSK5 exists in two forms, PCSK5A and PCSK5B, that arise from alternative mRNA splicing (66). PCSK5A is the

Table 1. Tissue distribution and subcellular localization of the PCSKs. SG= secretory granules, TGN= trans golgi network and PM= plasma membrane.

PCSK	Alternate	Tissues	Subcellular
PCSK1	PC1/PC3	Neuro-& Enteroendocrine	SG
PCSK2	PC2	Neuro-& Enteroendocrine	SG
PCSK3	Furin/PACE/PC1	Ubiquitous	TGN, PM
PCSK4	PC4	Gonadal	TGN, PM
PCSK5A	PC5	Widespread	SG
PCSK5B	PC6	Widespread	TGN, PM
PCSK6	PACE4	Ubiquitous	TGN, PM
PCSK7	PC7/PC8	Ubiquitous	TGN, PM
PCSK8	Site 1 protease S1P/SKI	Ubiquitous	TGN, PM
PCSK9	NARC-1	Liver, Intestine	SECRETED

smaller isoform that is found in multiple tissues including the GI tract, the adrenal gland and the hippocampus (66).

The remaining PCSKs including PCSK3, 5B, 6, 7, 8 and 9 are found in the constitutive pathway and as such are often in found in many tissue types. PCSK3 is membrane bound and is actually detected in all major organ systems (27, 108). It is also expressed during development as seen with its ectoderm and mesoderm expression at embryonic day 7 (153). Its importance in development is perhaps best demonstrated with the PCSK3 knock out mouse model which displays developmental abnormalities and is embryonicaly lethal at E 11-12 (100). PCSK6 exists as several splice variants as well, and is shown to be constitutively transcribed (68). It is found in high levels in the anterior pituitary and in moderate levels throughout the brain (68). Additionally PCSK6 is known to colocalize with bone morphogenic proteins (BMP) (24) and has been implicated in the processing of BMP in tooth development (3). PCSK7 is the closest relative of yeast kexin and, like PCSK3 , 5B, 6 and 8, it contains a C-terminal transmembrane domain (120). Like PCSK3, it is highly expressed throughout the brain and other organ systems including the GI tract, spleen and pancreas (120). PCSK8, commonly known as site-1 protease, is found throughout the central nervous system during embryogenesis (133). It is widely expressed and plays important roles in cholesterol homeostasis (151). The most recent addition to this family is PCSK9. PCSK9 plays important roles in cholesterol balance and is highly expressed in the liver (103, 112) but is also found in peripheral tissues including colon (112).

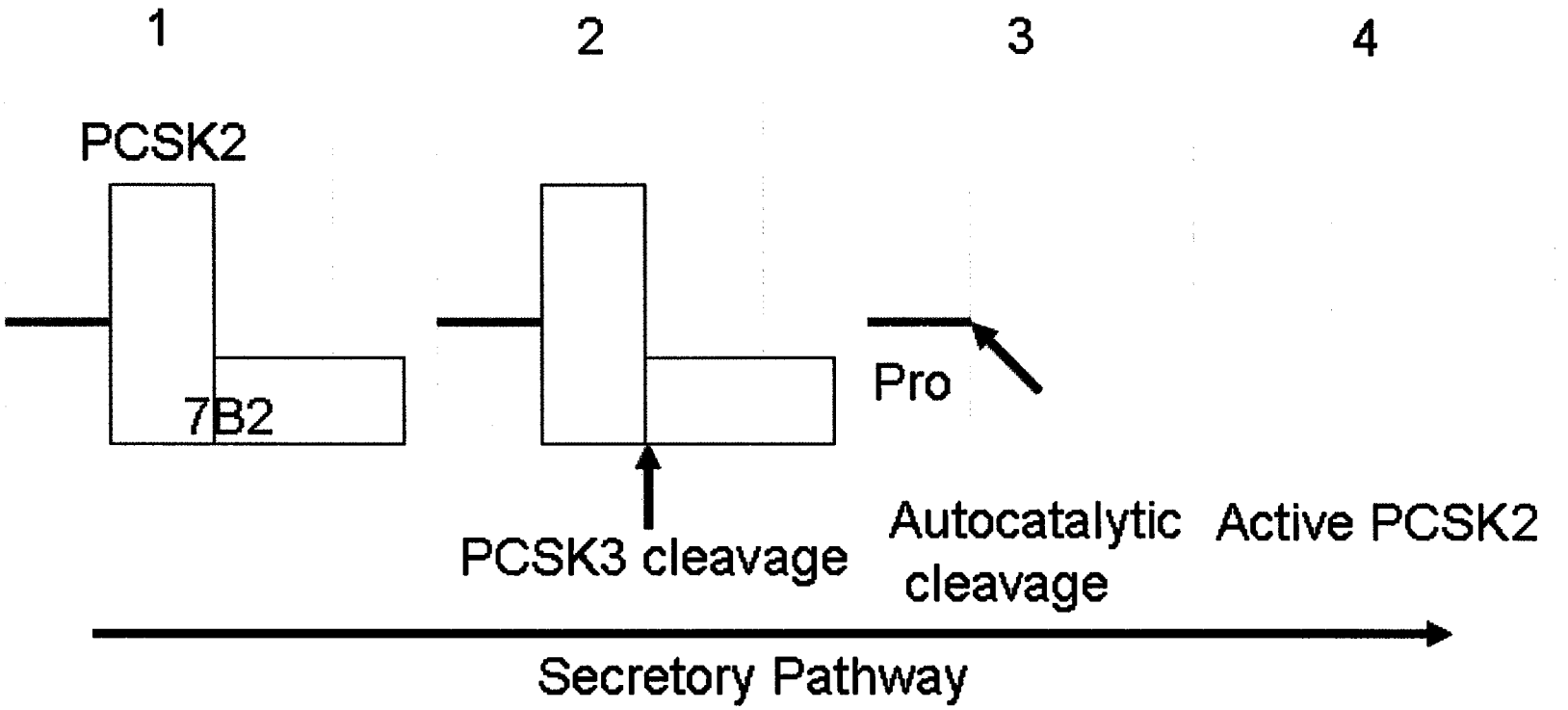
As indicated above and in Table 1, the nine different PCSKs are found in a variety of tissues. As PCSK1, 2 and 3 are expressed in the gastrointestinal (GI) tract, they will be the focus for the remainder of this Introduction.

1.1.4. Mechanisms of PCSK1, 2, and 3 activity

As previously indicated, the PCSKs are synthesized as inactive zymogens that undergo autocatalytic cleavage of their prodomains. This is a vital step in their activation cascade and occurs at different levels in the ER and Golgi apparatus. The prodomain of PCSK1 is efficiently processed in the ER during translation (12). PCSK3 is also shown to have its prodomain cleaved efficiently in the ER (83). PCSK2 on the other hand requires the endocrine chaperone 7B2 to help in its folding (157). 7B2 itself requires cleavage by PCSK3 to allow mature PCSK2 to be released (49) (Figure 2).

As discussed previously PCSK1 and PCSK2 are known to process substrates in the regulated secretory pathway and accordingly they contain a specific sorting sequence in their C-terminal domain which targets them to dense core secretory granules (31). Here, for activity, they require an acidic pH of 5.5 - 6 and millimolar calcium (156). The constitutively secreted PCSK3 differs in this respect as it cleaves substrates with a pH optimum of 5-8 (83) . It requires this pH variability as it is anchored by its transmembrane domain and must cycle between the membrane of the TGN, cell membrane and late endosomes (83). Additionally PCSK3 exists as a truncated secreted form (140) which retains enzymatic activity (90).

Figure 2. The maturation of PCSK2 by the interaction with chaperone protein 7B2 and PCSK3 adapted from **(73)**. Each panel (1-4) displays a different maturation event for PCSK2 along the secretory pathway. Panel 1 describes the interaction of PCSK2 and 7B2 during PCSK2 synthesis. Panel 2 describes the cleavage of 7B2 by PCSK3 which enables 7B2's release. In panel 3 as the pH lowers in the TGN, PCSK2 undergoes autocatalytic cleavage of its prodomain becoming its active form in panel 4. Below the events is the given pH of each indicated compartment.



Compartment	ER	TGN	Secretory Granules
pH	7.2	6.4	5.5

1.1.5 Substrates of PCSK1, 2 and 3

PCSK1, 2 and 3 have many known substrates. These enzymes process their substrates at the consensus sequence of Arg/Lys- $(X)_n$ –Lys/Arg where "X" is any amino acid other than cysteine and "n" can be 0, 2, 4 or 6 amino acids long (114). A list of the general consensus sites of each PCSK is provided in table 2. There are exceptions to these canonical sequences, as exemplified by PCSK8 (R/K-X-(L,I,V)-Z↓) and PCSK9 (V-F-A-Q↓S-I-P) cleavage sites (107).

Both PCSK1 and 2 are present in many endocrine tissues and have a large number of substrates (114). In the β-cells they are both involved in the maturation of pro-insulin where they cleave this substrate at 2 different positions (13, 125). In the α-cells of the pancreas, PCSK2 is involved in the processing of proglucagon to glucagon (30). In the pituitary, both of these convertases are involved in the processing of proopiomelanocortin (POMC). In this situation the differential expression of these 2 PCSKs dictates the differential distribution of the POMC processing, including α-MSH, ACTH and β-LPH (116). In the GI tract PCSK1 processes incretins like glucagon-like peptide 1 (GLP1)(148) from proglucagon and glucose dependent insulinotropic polypeptide (GIP) from pro GIP(137). PCSK2 is also involved in the processing of enteroendocrine prohormones. In the PCSK2 deficient mouse models, levels of both mature neuropeptide Y (NPY) and galanin were decreased compared to WT (82). Similarly, CCK-8 levels in the PCSK2 deficient brain were lower compared to WT. However other intermediate processing forms including CCK-58, -33 and -22 were elevated (95).

PCSK3, as previously mentioned, cleaves substrates in the constitutive secretory pathway. Some of these include growth factors like β nerve growth factor (37) and

Table 2. Established PCSK cleavage consensus sites. Arrows indicate cleavage point. Adapted from (107)

PCSK	Cleavage Motif
General PCSK1-7	K-R↓, R-R↓
Additional PCSK3, PCSK7	R-X-X-R↓
	X= any except C
Additional PCSK4	K-X-X-R↓
Additional PCSK5	H-X-X-R↓
PCSK8	R/K-X-(L,I,V)-Z↓
	Z=any except (P,C,E,V)
PCSK9	V-F-A-Q↓S-I-P (itself)

several tumor necrosis factor α releasing factors. For example, B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) (131) are required for development and are processed by PCSK3 which explains the developmental defects and embryonic lethality in PCSK3 KO mice (107). Aside from growth factors and cell surface receptors, PCSK3 is involved in processing prohormones including the proparathyroid hormone (PTH) (48), and in the circulation, the blood glycoprotein, Von Willebrand Factor (109) and the vasoconstrictor, endothelin (29). Elsewhere in the enteroendocrine system, PCSK3 processes the C-terminus of goblet cell secreted mucin 2, which is required for lubrication in the small intestine (150). It also is believed to cleave hydrolases in the intestine like prolactase (78). One unique set of substrates processed by PCSK3 are the bacterial and viral glycoproteins (gp) including the Ebola Zaire gp (142), the HIV gp160 (46) and the toxic anthrax protective agent (PA) (58).

1.1.6 PCSK deficiency and digestive abnormalities.

Some of the most important observations on the functional importance of convertases are the impact their deficiencies have on normal physiology. Much information has been derived through observation of human PCSK mutations and mouse models of PCSK deficiency.

1.1.6.1 PCSK1 deficiency

To date there are three documented cases of PCSK1 deficiency in humans (38, 53, 54). The first case was a female who is a compound heterozygote for mutations in her PCSK1 gene. She exhibited extreme childhood obesity along with impaired glucose homeostasis(54). The same authors documented a second case, again a compound heterozygote for novel PCSK1 mutations that had the obesity phenotype but presented clinically with extreme diarrhea and nutrient malabsorbtion (53). Re-examination of the first case revealed a similar small intestinal dysfunction. The last case, a six year old boy, had a novel missense mutation that produced auto-catalytically active PCSK1 lacking activity on other substrates. This patient did not exhibit the same glycemia phenotype as the others but still displayed marked diarrhea and nutrient malabsorbtion (38). The authors surmised that the small intestinal dysfunction is derived from the PCSK1 deficiency expressed in the enteroendocrine cells.

Mouse models of PCSK1 deficiency enable a detailed examination of the endocrine roles these enzymes have. Two different PCSK1 mouse models of deficiency have been developed. The first is a deletion of exon 1 as well as some upstream transcription elements resulting in a complete lack of PCSK1 expression. These animals lack mature growth hormone releasing hormone (GHRH) and this is manifest as dwarfism (158). A second mouse model that provides more information relative to the human cases is a single catalytic domain mutation. These mice, like the human examples, are obese and experience markedly hyperphagia (64).

1.1.6.2 PCSK2 deficiency

In mice, subcongenic breeding experiments with chromosomal regions known to associate with obesity identified linkage with the gene locus containing PCSK2. In this

study it was determined that lower levels of PCSK2 correlate with reduced adiposity index as well as reduced food consumption (20). The development of the PCSK2 KO mouse was carried out to investigate this enzyme's role in pancreatic islet physiology. Indeed there is altered morphology of the islets accompanied by elevated levels of proglucagon (41). However, the consequences of PCSK2 KO for the GI system were not investigated.

1.1.6.3 PCSK3 deficiency

The complete KO of PCSK3 is embryonic lethal. However, there have been several conditional KO animals developed including a liver specific KO (99) and a endothelial specific KO (personal communication Nabil Seidah). To date no intestinal specific KO for PCSK3 has been developed.

1.2 The Gastrointestinal System

1.2.1 General structure of the small intestine

The small intestine (SI) begins after the pyloric sphincter of the stomach and ends at the caecum before the large intestine (50). The basic microscopic unit of the SI is the crypt villus axis, the epithelium of which is made up of 4 distinct cell types differentiating from the stem cell population within the crypt. Paneth cells, which are responsible for the secretion of antimicrobial factors, differentiate and remain localized at the depth of the crypts in clusters. Other cells differentiate while migrating towards the villus tip including the enterocytes, which act as the nutrient absorbing brush border cells,

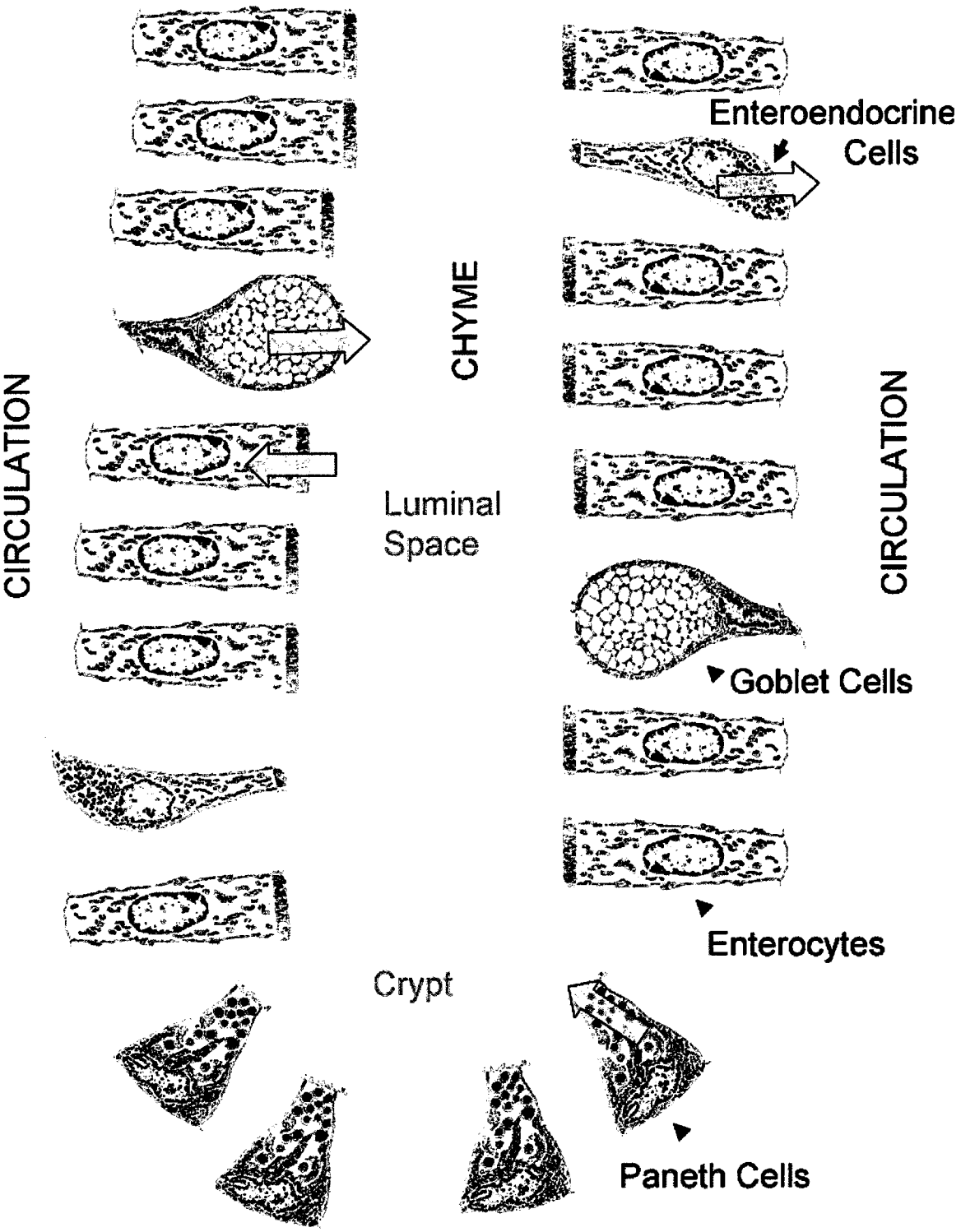
the goblet cells, which secrete mucin into the lumen of the gut for the purpose of lubrication, and the enteroendocrine (EE) cells which are the hormone secreting cells (Figure 3). These cells continue along the villus until they eventually senesce at the tips of villi and slough into the lumen in a dynamic process that takes 3-4 days in mice (2)

The SI is anatomically subdivided into three regions; the duodenum, the jejunum and the ileum. The duodenum is the first region to come into contact with the chyme entering from the stomach. This partially digested food is mixed with bile and digestive enzymes in this region (5). The jejunum has elongated villi which aid in its principle role of carbohydrate and protein absorption (25). The most distal region is the ileum and it possesses much shorter villi than the more proximal regions with larger pores that are tailored for lipid (55) and vitamin B12 absorption (122)

1.2.2 The small intestine as an endocrine organ

With its well understood roles of digestion and nutrient absorption the SI's role as an important endocrine organ are overshadowed. The cell types charged with this responsibility are the enteroendocrine (EE) cell types which themselves arise from the stem cell population at the base of the crypts (132). The EE cell population may be small in comparison to the enterocyte population however the sheer number of hormones produced (>100) as well as the number of EE cells together make the SI the largest endocrine organ in the body (1). Due to this large non-endocrine surrounding cell population the SI is also an excellent model of paracrine functionality. Structurally, the EE cells secrete their hormones towards the centers of the villi into the lamina propria

Figure 3. Cellular organization of the small intestinal crypt-villus. The four main intestinal cell types originating from the stem cell population are indicated. Direction of solute movement is indicated by yellow arrows. Adapted from (19)


CIRCULATION
CHYME
Luminal
Space
CIRCULATION
Enteroendocrine
Cells
Goblet Cells
Enterocytes
Crypt
Paneth Cells

which contains the circulatory system responsible for shuttling of both nutrients and hormones elsewhere in the body (17)

The first two hormones discovered in the early 1900s were in fact from the GI tract and included secretin and gastrin. With the advent of cDNA cloning, many more GI prohormones were discovered including cholecystokinin (CCK) (94), substance P (SP), somatostatin (SS), motilin, GIP, GLP1 and vasoactive intestinal polypeptide (VIP) (Figure 4). EE cell subtypes are defined on the basis of their peptide phenotype (94).

1.2.3 Endocrine signaling through the small intestine

To date several hormone signaling pathways in the SI have been established and these are important for maintaining homeostasis throughout the body.

One such example is the incretin effect. This effect was born from the observation that an enteral meal produced a more potent insulin release effect (insulinotropic) than a simple intravenous glucose challenge (32). The first hormone shown to have this effect was GIP (33) and the second was GLP-1 (141). These hormones are synthesized in the proximal regions (duodenum and jejunum) in response to food entering from the stomach (32). This system essentially acts as a primer to the pancreas when the need to store circulating glucose is imminent (Figure 5).

Another example of endocrine regulation in the GI tract arises from the recently described hormone ghrelin (59). This hormone is largely produced in the stomach and SI (26) and principally acts on the growth hormone secretagogue receptor which is involved

Figure 4. A timeline representation of discovered GI hormones. Pro-hormones with evidence of being processed by PCSKs in red and pro-hormones hypothesized based on sequence consensus in blue. Adapted from (94)

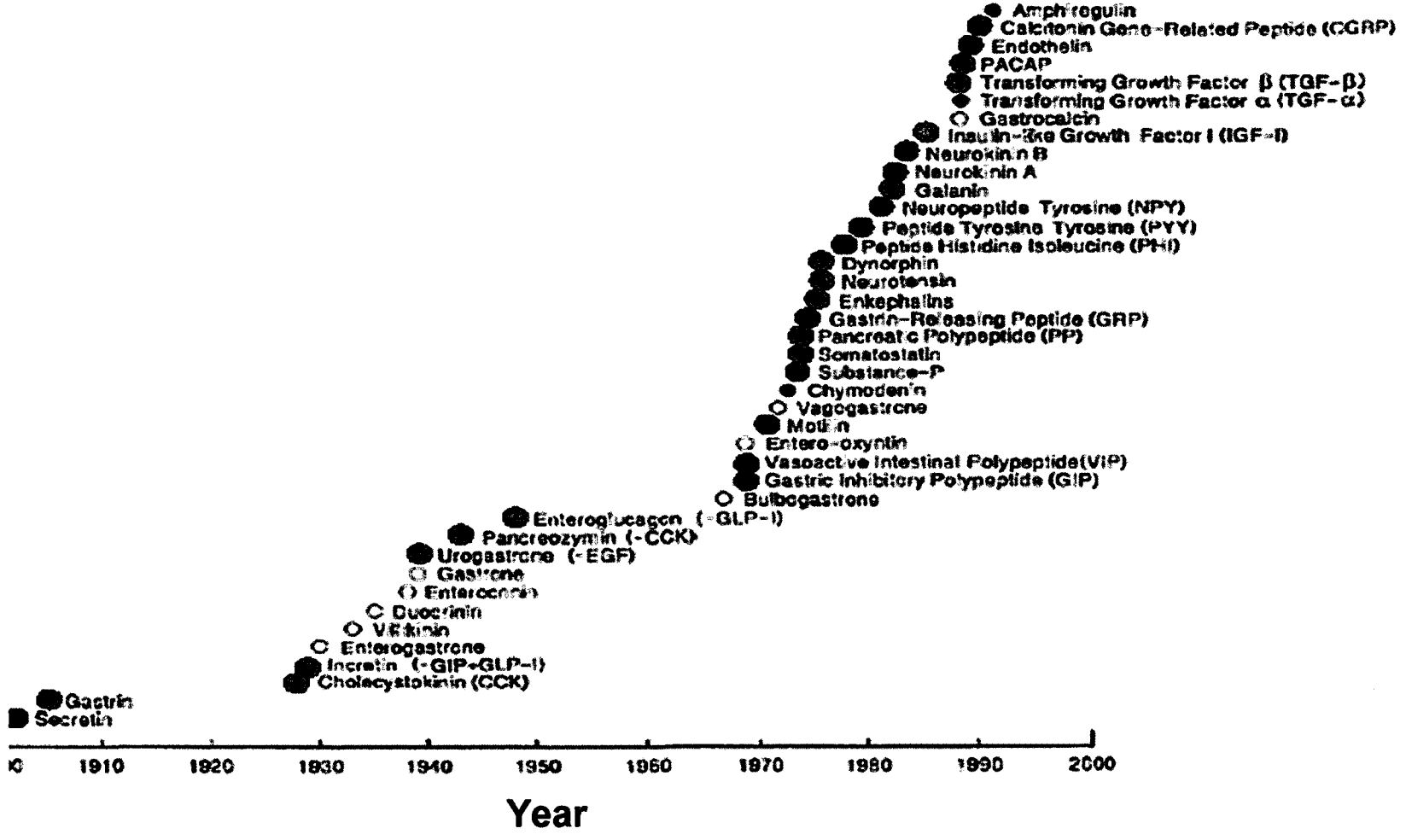
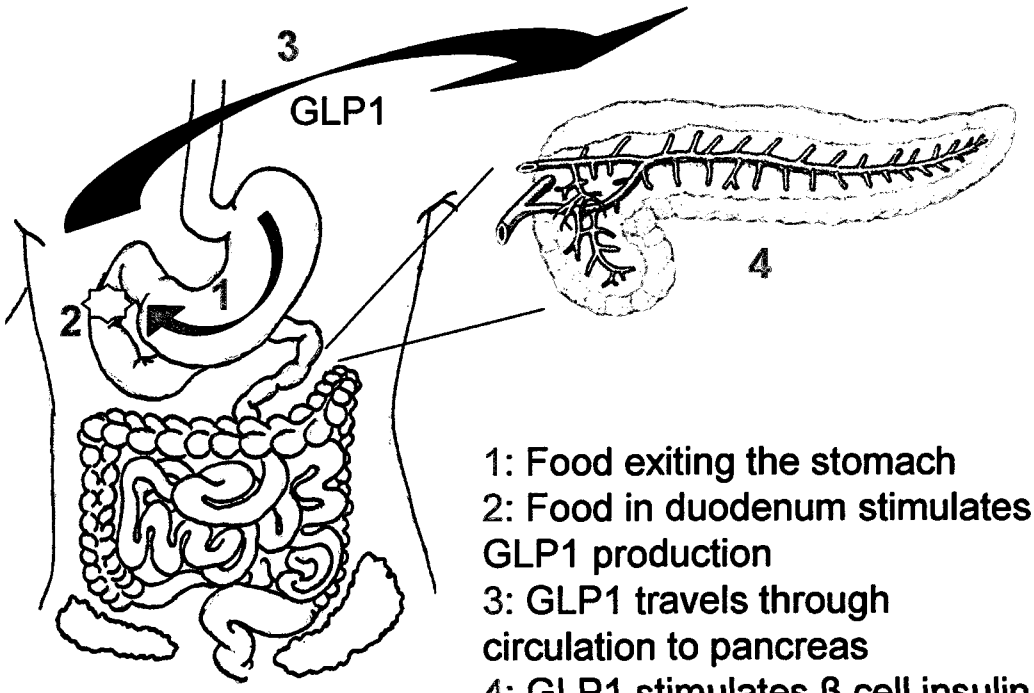

Amphiregulin
Calcitonin Gene-Related Peptide (CGRP)
Endothelin
PACAP
Transforming Growth Factor β (TGF-β)
Transforming Growth Factor α (TGF-α)
Gastrocalcin
Insulin-like Growth Factor I (IGF-I)
Neurokinin B
Neurokinin A
Galanin
Neuropeptide Tyrosine (NPY)
Peptide Tyrosine Tyrosine (PYY)
Peptide Histidine Isoleucine (PHI)
Dynorphin
Neurotensin
Enkephalins
Gastrin-Releasing Peptide (GRP)
Pancreatic Polypeptide (PP)
Somatostatin
Substance-P
Chymodenin
Vagogastrone
Motilin
Entero-oxyntin
Vasoactive Intestinal Polypeptide(VIP)
Gastric Inhibitory Polypeptide (GIP)
Bulbogastrone
Enteroglucagon (-GLP-I)
Pancreozymin (-CCK)
Urogastrone (-EGF)
Gastrone
Enterocrinin
Duocrinin
Villikinin
Enterogastrone
Incretin (-GIP+GLP-I)
Cholecystokinin (CCK)
Gastrin
Secretin
1910
1920
1930
1940
1950
1960
1970
1980
1990
2000
Year

Figure 5. The incretin effect of GLP 1 in the intestine and pancreas. Steps are indicated in numbers next to figure. Step 1 describes the exit of food from the stomach. Step 2 is the actual stimulation of GLP 1 production from the EE cells in the duodenum. In step 3 GLP 1 travels in circulation to the pancreas where it stimulates insulin production in the β cells (step 4).



1: Food exiting the stomach
2: Food in duodenum stimulates GLP1 production
3: GLP1 travels through circulation to pancreas
4: GLP1 stimulates β cell insulin production

in appetite regulation (85). During a negative energy state before a meal, the level of ghrelin in circulation is high, once this meal comes into contact with the SI, the levels of ghrelin are suppressed (92) and satiety is induced.

1.3 Hypotheses

There is a wealth of research discussing the abundance of endocrine hormones in the GI tract. As many of these hormones are first produced as inactive proproteins and require proteolytic cleavage for their bioactivity, it is axiomatic that PCSKs should be present in the GI tract. There is evidence that this is the case. Indeed PCSK deficiency has consequences for GI function. The first hypothesis of this research project was PCSKs are expressed in a region-specific manner within the EE cells of the SI and to colocalize with prohormone substrates important for SI functions, including GIP, CCK, SS and SP. This was examined using double immunofluorescence.

In the context of the localization of PCSKs to SI EE cells, the second hypothesis was that a deficiency of PCSK2 would produce functional consequences in the GI tract physiology. Given the evidence for an important role for PCSK2 in the maturation of prohormones known to have GI functions, and considering the availability of a mouse model deficient in PCSK2 we chose to study this specifically. This was examined by measuring GI physiologies including hunger response and intestinal transit. We then correlated any observed physiological responses with the circulating levels of hormones required for feeding and gastric motility homeostasis.

1.4 Manuscript Layout

With the approval of the department and the thesis advisory committee the studies are presented in the form of manuscripts. The first manuscript was accepted by *Regulatory Peptides(43) 2008 Jul 22(Epub ahead of print)* and the second is in the final review phases for submission. The chapters in this thesis are exactly as they appear in their manuscript form with the exception of figure and reference numbers.

2 Chapters

2.1 Expression of PCSK1 (PC1/3), PCSK2 (PC2) and PCSK3 (furin) In Mouse Small Intestine

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Abstract

The family of serine proteases known as the proprotein convertases subtilisin/kexin type (PCSK) are responsible for the cleavage and maturation of many precursor hormones. Over its three successive regions, the duodenum, the jejunum and the ileum, the small intestine (SI) expresses over 40 peptide hormones necessary for normal intestinal physiology. Most of these hormones derive from proteolytic cleavage of their cognate inactive polypeptide precursors. Members of the PCSK family of proteases have been implicated in this process, although details of enzyme-substrate interactions are largely lacking. As a first step towards elucidating these interactions, we have analyzed by immunohistochemistry the regional distribution of PCSK1, PCSK2 and PCSK3 in mouse SI as well as their cellular co-localization with substance P (SP), cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide (GIP) and somatostatin (SS), 4 peptide hormones known to result from PCSK-mediated processing. Results indicate that PCSK1 is found in all three regions of the SI while PCSK2 and PCSK3 are primarily expressed in the upper two, the duodenum and the jejunum. In these proximal regions, PCSK1 was detectable in 100% of SP-positive (+) cells, 85% of CCK+ cells and 50% of GIP+ cells; PCSK2 was detectable in 40% of SS+ cells and 35% of SP+ cells; PCSK3 was detectable in 75% of GIP+ cells and 60% of SP+ cells. These histological data suggest that the 3 PCSKs may play differential and overlapping roles in prohormone processing in the three regions of the SI.

Keywords: PCSK, enteroendocrine, colocalization, small intestine, hormone

1. Introduction

Peptide hormones produced in endocrine cells are initially synthesized as prohormones and undergo post-translational modifications including cleavage by a group of serine proteases known as the proprotein convertases subtilisin kexin type (PCSK). The PCSK family contains 9 members that are collectively responsible for limited proteolysis of precursor proteins leading to the production of such bioactive molecules as growth factors, cytokines, hormones, cell surface proteins, receptors and transcription factors (113). PCSK 1-7 cleave their substrates following dibasic residues at the motif RX(R/K)(R/K)↓ (114) while PCSK8 (35) and PCSK9 (9) prefer hydrophobic and acidic residues, respectively. The roles of the mammalian PCSKs in neuroendocrine function have been studied extensively over the last two decades (106).

In a recent study the presence of PCSKs was examined in the human pancreas in detail (91). Their roles in enteroendocrine (EE) function are less well understood. Several lines of evidence indicate that these roles are important: (1) in humans, carriers of PCSK1 mutations have digestive abnormalities including diarrhea and nutrient mal-absorption (51, 52), a phenotype was also observed in PCSK1-null mice (158); (2) PCSK2-null mice exhibit delayed growth (21, 42) and strain-specific PCSK2-deficiency to delayed growth and resistance to obesity (21, 42). PCSK3 (furin)-null mice die in utero and, to date, no information on its role in gut physiology is known; however, since this enzyme is widely expressed in the gut (44, 61, 96), it warrants investigation.

The small intestine (SI) is anatomically subdivided into three regions; the duodenum, the jejunum and the ileum. The basic microscopic unit of the SI is the crypt

villus axis, the epithelium of which is made up of 4 distinct cell types differentiating from the stem cell population within the crypt. Paneth cells, which are responsible for the secretion of antimicrobial factors, differentiate and remain localized at the depth of the crypts in clusters. Other cells differentiate while migrating towards the villus tip including the enterocytes, which act as the nutrient absorbing brush border cells, the goblet cells, which secrete mucin into the lumen of the gut for the purpose of lubrication, and the enteroendocrine (EE) cells which are the hormone secreting cells. These cells continue down the villus until they eventually senesce at the tips of villi and pass into the lumen in a dynamic process that takes 3-4 days in mice (2).

The processing of peptide hormones by PCSKs in the brain has shed light on potential processing roles PCSKs in the GI tract as several of these hormones are found in both endocrine systems (28). Some of these include cholecystokinin (CCK, a regulator of the satiety response) (97), substance P (SP, a potent inducer of contractility) (123), and somatostatin (SS, a modulator of the effects and levels of various intestinal hormones) (105). In addition to these, there is some preliminary evidence that intestinal hormones like glucose dependent insulinotropic polypeptide or GIP may be processed by PCSKs (136).

To begin to gain insights into the roles of PCSKs in EE system and in intestinal functions, we have studied the distribution of PCSK1, PCSK2 and PCSK3 in EE cells of the duodenum, jejunum and ileum as well as their co-localization with CCK, GIP, SS and SP in these SI regions.

2. Materials and Methods

2.1. Preparation of intestinal sections

Mice were used under a protocol approved by the Animal Care Committee of the Ottawa Health Research Institute. Small intestines were surgically removed from 9-week old male and female CD1 mice and placed on ice. The duodenum, jejunum and ileum were cut transversely into 3-cm sections located immediately after the pyloric sphincter, 40% down the intestine (3-4cm after the ligament of treitz) and immediately before the caecum, respectively. These three pieces were flushed with PBS buffer pH 7.4 to remove waste, then cut into 1-cm pieces and fixed in 10% buffered formalin (Fisher Scientific, Mississauga Canada) overnight at room temperature (RT). The fixed tissue was processed by dehydrating in graded alcohols (70%, 80%, 90%, 100%, 100%) cleared in toluene (3x 2 hours) and infused with paraffin. The tissues were then embedded in paraffin blocks so that a transverse section of the intestine could be obtained. This orientation is ideal for visualizing the entire crypt-villus axis.

2.2. Antibodies

Primary and secondary antibodies used and their appropriate dilutions are displayed in Table 3.

Table 3. Antibodies used for immunohistochemistry. Antibodies directed against the indicated antigen were raised in rabbit (Rb), goat (Gt) and mouse (M). Abcam (Cambridge, MA) Zymed (San Fransisco, CA) Santa Cruz Buiotechnology (Santa Cruz, CA) Biomeda (Foster city, CA) Invitrogen (Burlington, Canada)

	Antibody information		
Antibody	Dilution	Cat Number	Company
Rb-PCSK1	1/200	ab3532	Abcam
Rb-PCSK2	1/200	ab3533	Abcam
Rb-PCSK3	1/100	36-1800	Zymed
Gt-Chromogrannin A	1/600	sc-1488	Santa Cruz
Gt-GIP	1/200	sc-23554	Santa Cruz
Gt-Subtance P	1/200	sc-9758	Santa Cruz
M-Somatostatin	1/500	v1169	Biomeda
Gt-Cholecystokinin	1/50	sc-21617	Santa Cruz
anti Rb-594	1/150	A11012	Invitrogen
anti Gt-488	1/150	A11055	Invitrogen
anti M-488	1/150	A21200	Invitrogen

2.3. Immunohistochemistry

Tissue blocks were cut at 4-μm and sections mounted onto positive treated superfrost plus slides (VWR, Mississauga Canada). Sections were allowed to adhere overnight at 37C. Tissue sections were deparaffinized using toluene and rehydrated in graded alcohols then running tap water. All antibodies used in the experiments required microwave antigen retrieval. The slides were placed in 0.01M citrate buffer (pH 5.6) and microwaved for 2x5 minutes with a 5-minute room temperature (RT) cool down between microwaving. Sections were incubated in Tris buffered buffered saline (TBS) at pH 7.4 for 5 minutes at RT then blocked in TBS + 20% normal horse serum (NHS) (Vector laboratories, Burlington Canada) for 15 minutes on shaker at RT. Immediately after blocking, sections were incubated in primary antibodies at the noted dilutions (Table 3) in TBS + 20% NHS ON at 4°C with shaking. After 3x5-minute washes with TBS, the sections were incubated for 45 minutes at RT with the indicated secondary antibodies in the TBS + 20% NHS. Sections were then washed 3 times in TBS, coated with vectashield mounting media (Vector Laboratories, Burlington Canada), coverslipped, and finally sealed with nail polish.

2.4 Controls

For controls, each of the primary antibodies was preincubated with its commercially available complementary peptide (PCSK1 ab5011, Abcam; PCKS2 ab5012, Abcam; GIP sc-23554 p, Santa Cruz; CCK. sc-21617 p, Santa Cruz; SP sc-9758 p, Santa Cruz;

Chr A, sc-1488 P, Santa Cruz)), prior to application. Incubation with pre-absorbed primary antibodies resulted in no staining. Additional negative controls were performed by incubation of sections lacking primary antisera.

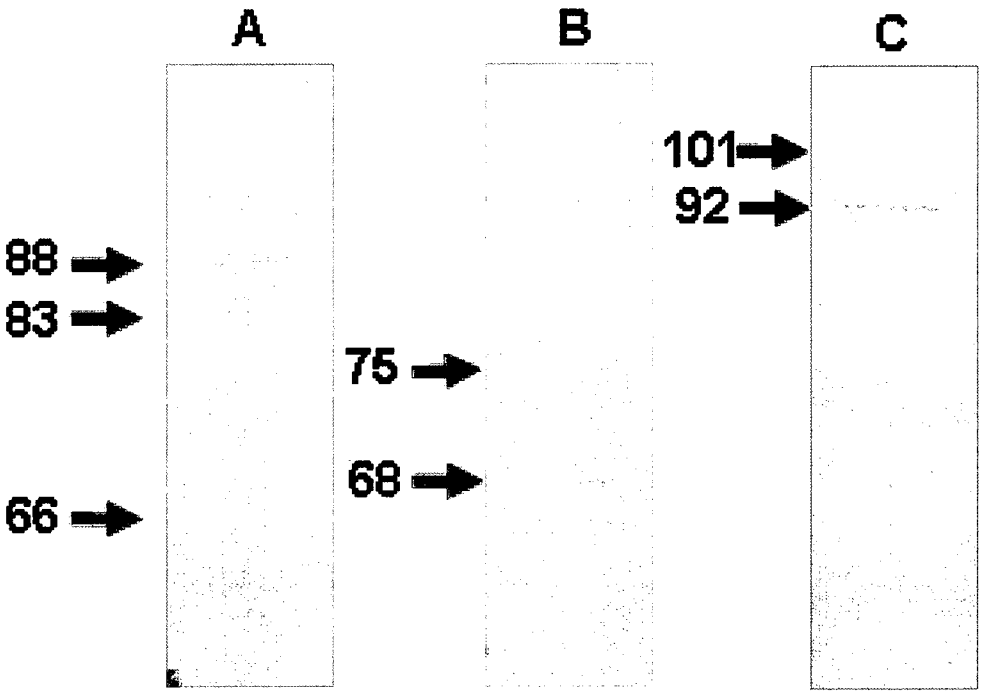
In addition to the immunochemistry controls each of the PCSK antibodies was tested in immunoblots to verify the detection of correct mass products. For these experiments tissue lysates were used from mouse brain as crude intestinal extracts contain a much lower proportion of EE cells and hence are below the threshold for Western blot detection of PCSKs. The results from these controls are shown in supplemental figure 1 (figure 6).

2.5 Immunoblotting

Tissues were lysed in 1X RIPA (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% (v/v) NP-40, 0.5% deoxycholate (w/v), 0.1% (w/v) sodium dodecyl sulfate (SDS), PIC) by mechanical homogenization at 4°C. Samples were then incubated on ice for 10 minutes then sonicated for 2 minutes at 50% amplitude. Samples were then centrifuged at 13,000 x g for 10 min and total tissue lysates (TTL) collected. Protein concentrations were determined by BioRad Protein Assay.

50ug of TTL were fractionated through a 7% Nu-Page Tris-Acetate gel (Invitrogen) and proteins immunoblotted (IB) following standard protocol. The primary anti-IB PCSK1, PCSK2 and PCSK3 were used at 1/1000 and the secondary antibody (goat anti rabbit horseradish perozidase) at 1/5000 dilutions. Immunoblots were revealed by chemiluminescence (Western Lightening Plus; Perkin-Elmer) on X-OMAT film (Kodak). The apparent PCSK molecular mass was determined using Syngene's

Figure 6. Western blot analysis on crude mouse brain extract. Strip A is PCSK1, B, PCSK2 and C PCSK3. Masses are in Kd and are representative of the pro and mature forms of these PCSKs.


A
B
C
101
92
88
83
75
68
66

Chemigenius 2XE imager and GeneTool software in comparison to See Blue Plus 2 mass standard (invitrogen).

2.6 Quantification of immunostaining

Sections were visualized on a Zeiss Axioplan 2 microscope and double labeled overlays were produced through multidimensional acquisition software (Zeiss Axiovision 4.5). Counting was restricted to the villi as these EE cells would represent terminally differentiated as opposed to a mixture of differentiated and undifferentiated cells of the crypts(110). Assessment of the regional distribution in the duodenum, jejunum, and ileum for each of the 3 PCSKs examined was accomplished by double labelled immunofluorescence with Chromogranin A (Chr A), a general enteroendocrine (EE) marker. The use of this marker served as a baseline for comparing different PCSK amounts in given region relative to Chr A+ cells. The count of PCSK positive (+) cells was calculated per 100 Chr A+ cells in a given section.

For the co-localization experiments, tissue sections of each of the three regions of the SI taken from three different 9-week old female mice were double-labelled for individual hormone and individual PCSK. Data is expressed in histograms of percentage of PCSK-positive cells among hormone-positive cells ± standard errors of means (SEM).

3. Results

3.1. PCSK1, 2 and 3 are differentially expressed in the duodenum, jejunum and ileum

Previous work suggests that Chr A is a protein that is produced by essentially all hormone-producing cells (89). As such, it is a reliable marker for the identification of endocrine cells. In this study, we have used Chr A to identify EE cells in the duodenum, the jejunum and the ileum and to determine for each region the proportion of EE cells that contain a particular PCSK. The results are shown in Figure 7. In total, over the entire length of the SI, PCSK1+ EE cells were more frequent than PCSK2+ and PCSK3+ EE cells. The frequency was highest in the jejunum and ileum and slightly lower in the duodenum. The amount of PCSK2+ EE cells was similar to that of PCSK1+ EE cells in the duodenum but gradually decreased over the length of the SI to fewer then 25 + cells per 100 Chr A cells in the ileum. PCSK3 + cells were the least frequent in all three regions of the SI with fewer than 25 positive cells per 100 Chr A+ cells (Figure 7).

3.2. Co-localization of CCK, GIP, SP and SS with PCSK1, 2 and 3

Intestinal sections were double-labelled for each one of the hormones and each of the PCSKs. The degree of co-localization is represented by histograms of the percentage of hormone-positive cells that contain the PCSK and by micrographs of the most intense double-labelling shown beneath in Figs. 8-11. In the duodenum and the jejunum, among CCK+ cells, >80% were PCSK1+ , <25% PCSK2+ and 0% were PCSK3+ (Fig. 8, A-D).

Figure 7. Panel A: The range of PCSK-positive cells per 100 Chr A+ cells counted. Counts were performed in all three regions of the SI and for each PCSK separately with n =3. + = 0-25 cells, ++ = 25-50 cells, +++ =50-100 cells, ++++ >100 cells. Panel B Representative figures of PCSK/ Chr A double staining in duodenum. 488nm image I, IV and VII are Chr A , and 594nm image II, V and VIII are PCSK1, 2 and 3 respectively. The last column (III, VI and IX) are overlays. Bars =50 μm

A

Region:	PCSK1	PCSK2	PCSK3
Duodenum	+++	+++	+
Jejunum	++++	++	+
Ileum	++++	+	+

B

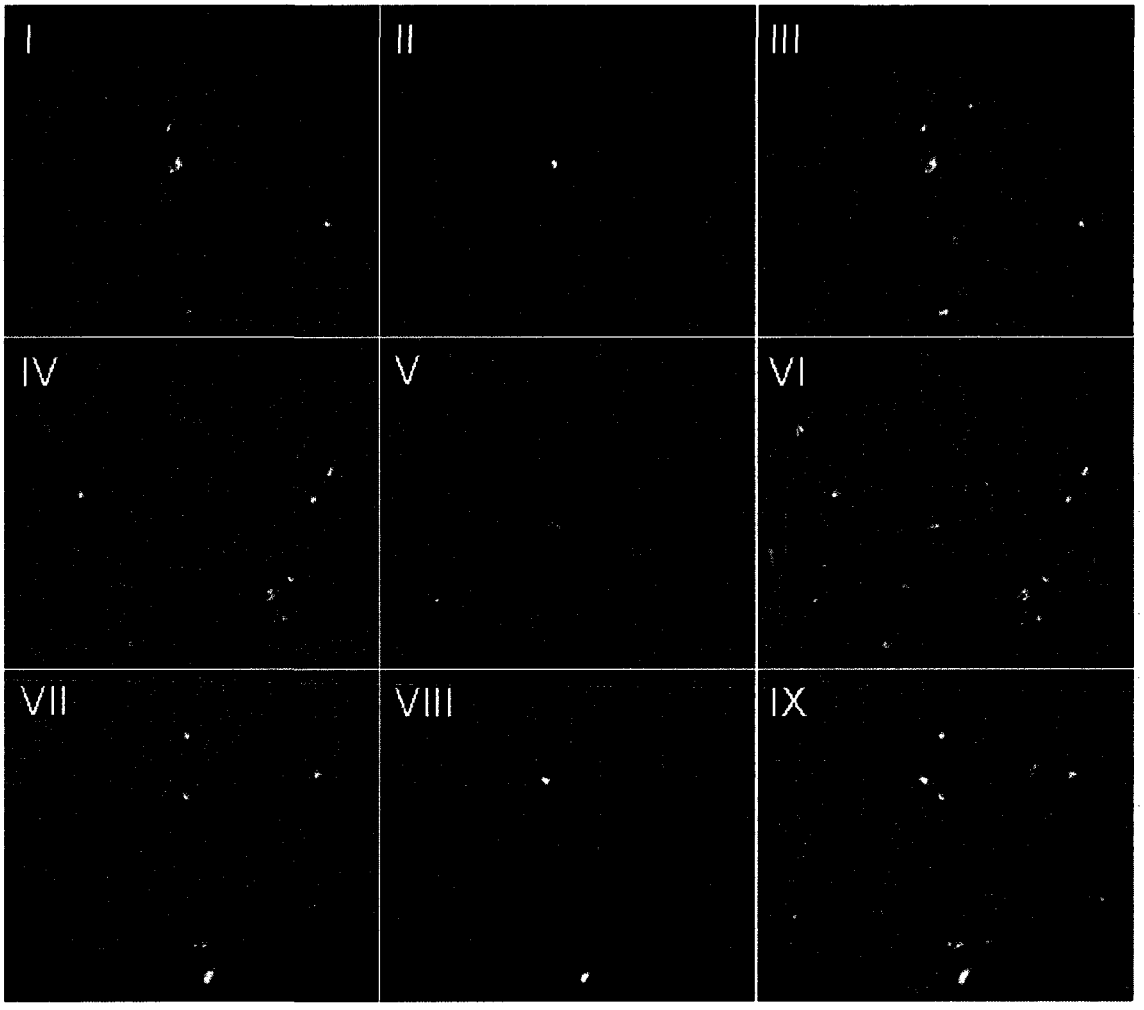
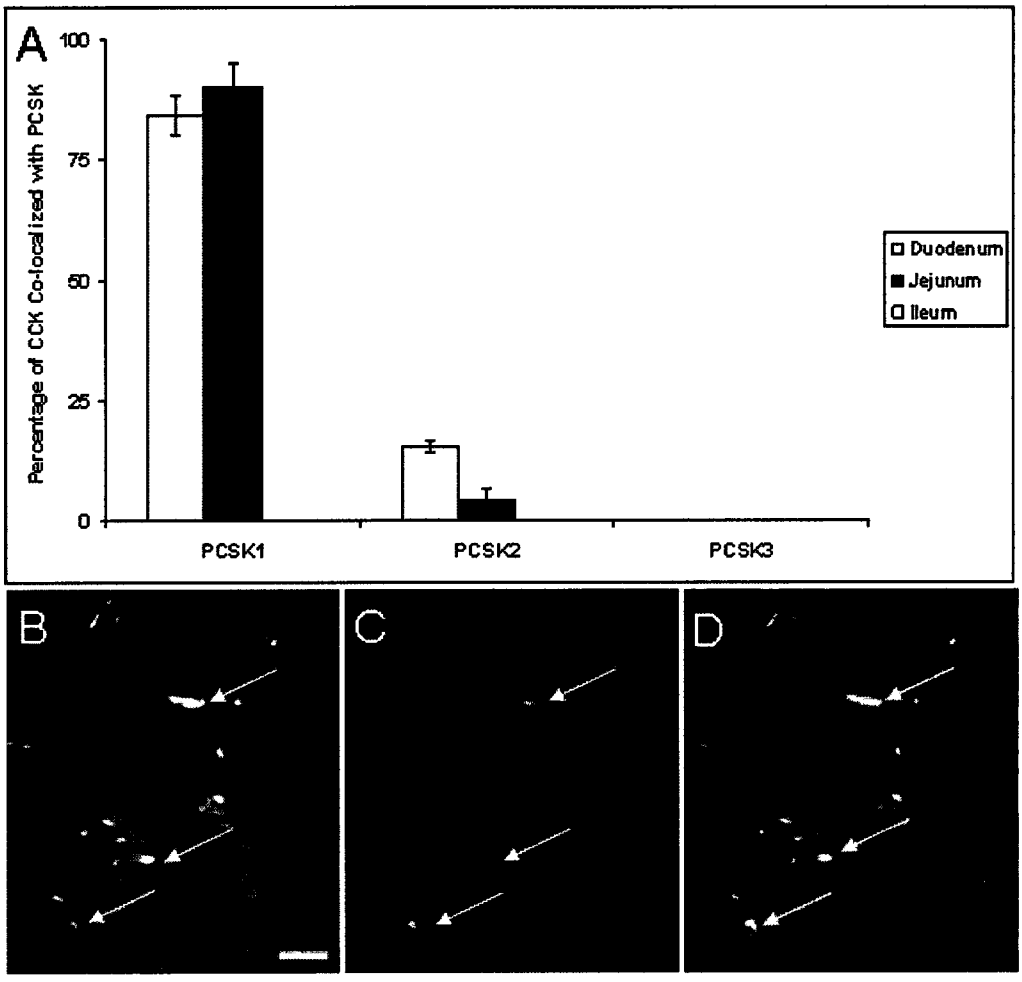


Figure 8 Cholecystokinin co-localizes with PCSK1 and 2 in the duodenum. The histogram above indicates the percentage of CCK cells (y-axis) co-localized with indicated PCSK (x-axis) for duodenum, jejunum and ileum (A). The micrograph below illustrates co-localizing cell(s) in mouse duodenum. In green (B) is CCK with red (C) PCSK1 and an overlay with DAPI counterstain (D). White arrows indicate single labelled cells and yellow arrows indicate double labelled cells in overlay (D). Bars = 50 μm.


A
100
75
50
25
0
Percentage of CCK Co-localized with PCSK
PCSK1
PCSK2
PCSK3
Duodenum
Jejunum
Ileum
B
C
D

Among GIP+ cells in these regions, ~70% were PCSK3+ (Fig. 9, A, E-G), and 50% were PCSK1+ (Figure 9, A, B-D). Over the length of the SI, nearly all SP+ cells were PCSK1+; in the duodenum and the jejunum, 30% of them were PCSK2+ and ~50% PCSK3+ (Figure 10). In the duodenum, 40% of SS+ cells were PCSK2+ (Figure 11, A-D); in the duodenum and the jejunum, 20% of them were PCSK3+ (Figure 11, A).

Figure 9. GIP co-localizes with PCSK1 and 2. The histogram above indicates the percentage of GIP cells (y-axis) co-localized with indicated PCSK (x-axis) for duodenum, jejunum and ileum (A). The micrograph below illustrates co-localizing cell(s) in mouse duodenum. In green (B and E) is GIP with red (C) PCSK1 or (F) PCSK3 and an overlay with DAPI counterstain (D and G). White arrows indicate single labelled cells and yellow arrows indicate double labelled cells in overlay . Bars = 50μm.

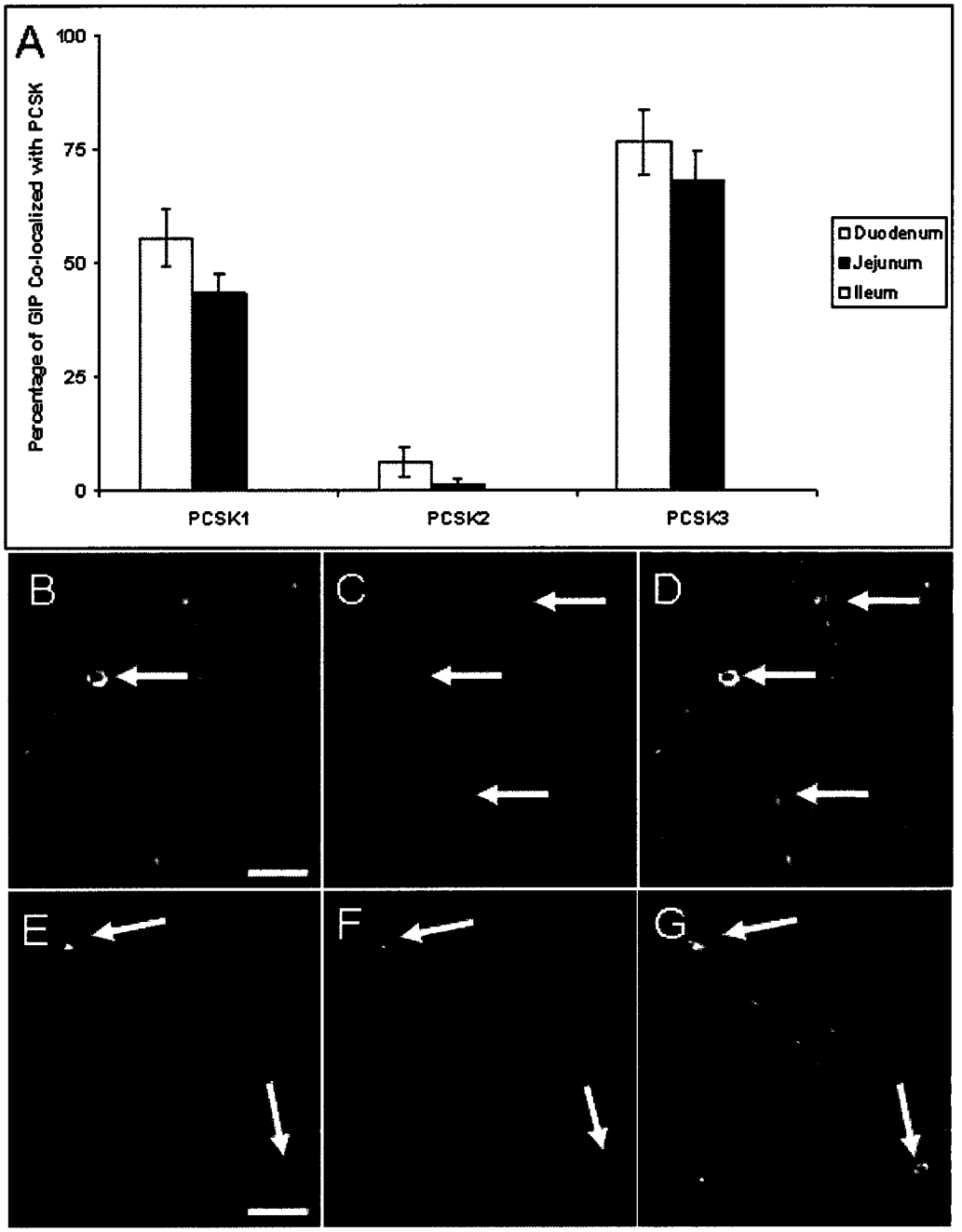

A
Percentage of GIP Co-localized with PCSK
100
75
50
25
0
PCSK1
PCSK2
PCSK3
Duodenum
Jejunum
Ileum
B
C
D
E
F
G

Figure 10. Substance P co-localizes with PCSK1, 2 and 3. The histogram above indicates the percentage of SP cells (y-axis) co-localized with indicated PCSK (x-axis) for duodenum, jejunum and ileum (A). The micrograph below illustrates co-localizing cell(s) in mouse duodenum. In green (B and E) is SP with red (C) PCSK1 or (F) PCSK3 and an overlay with DAPI counter stain (Dand G). White arrows indicate single labelled cells and yellow arrows indicate double labelled cells in overlay. Bars = 50μm.

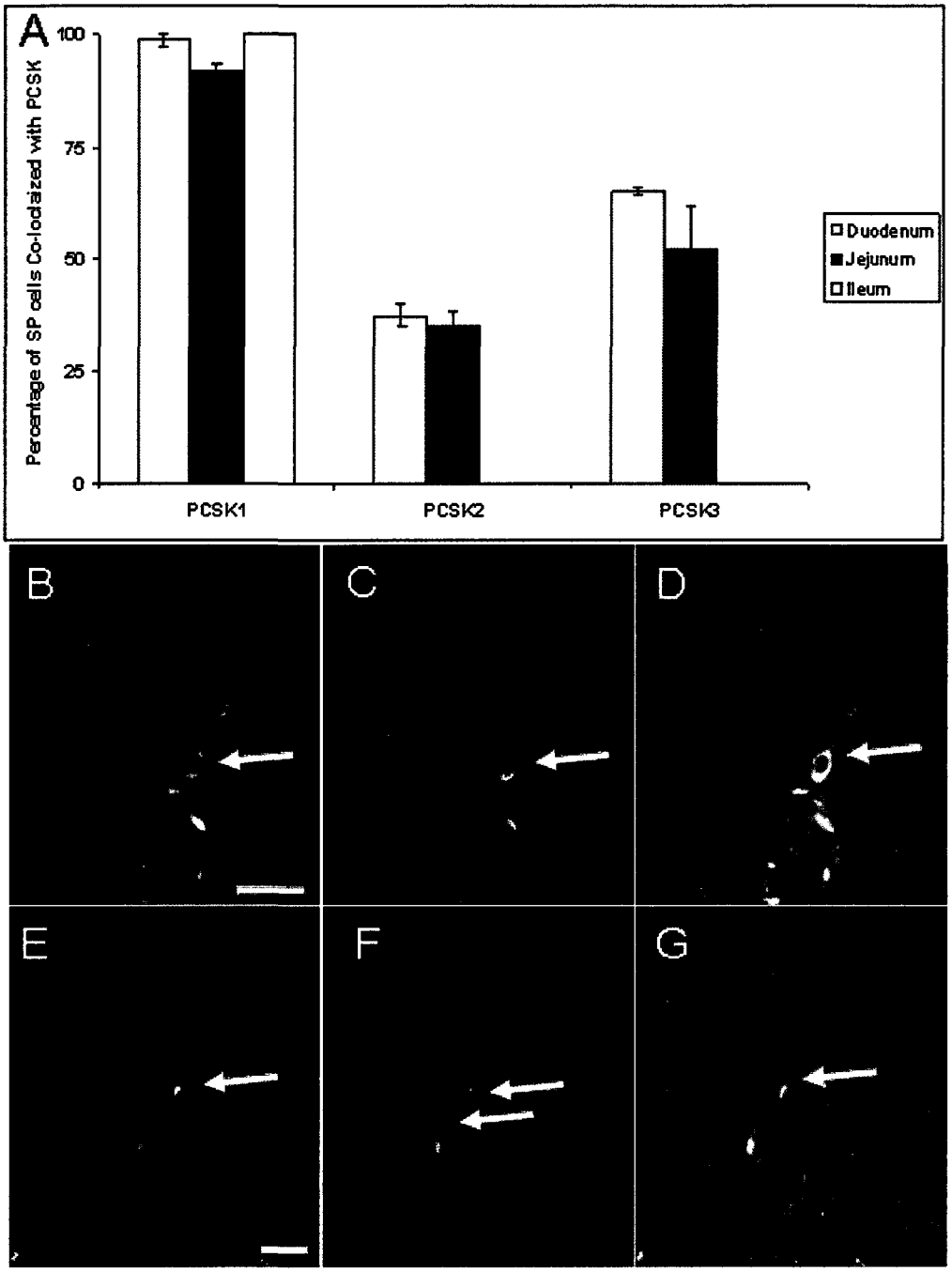
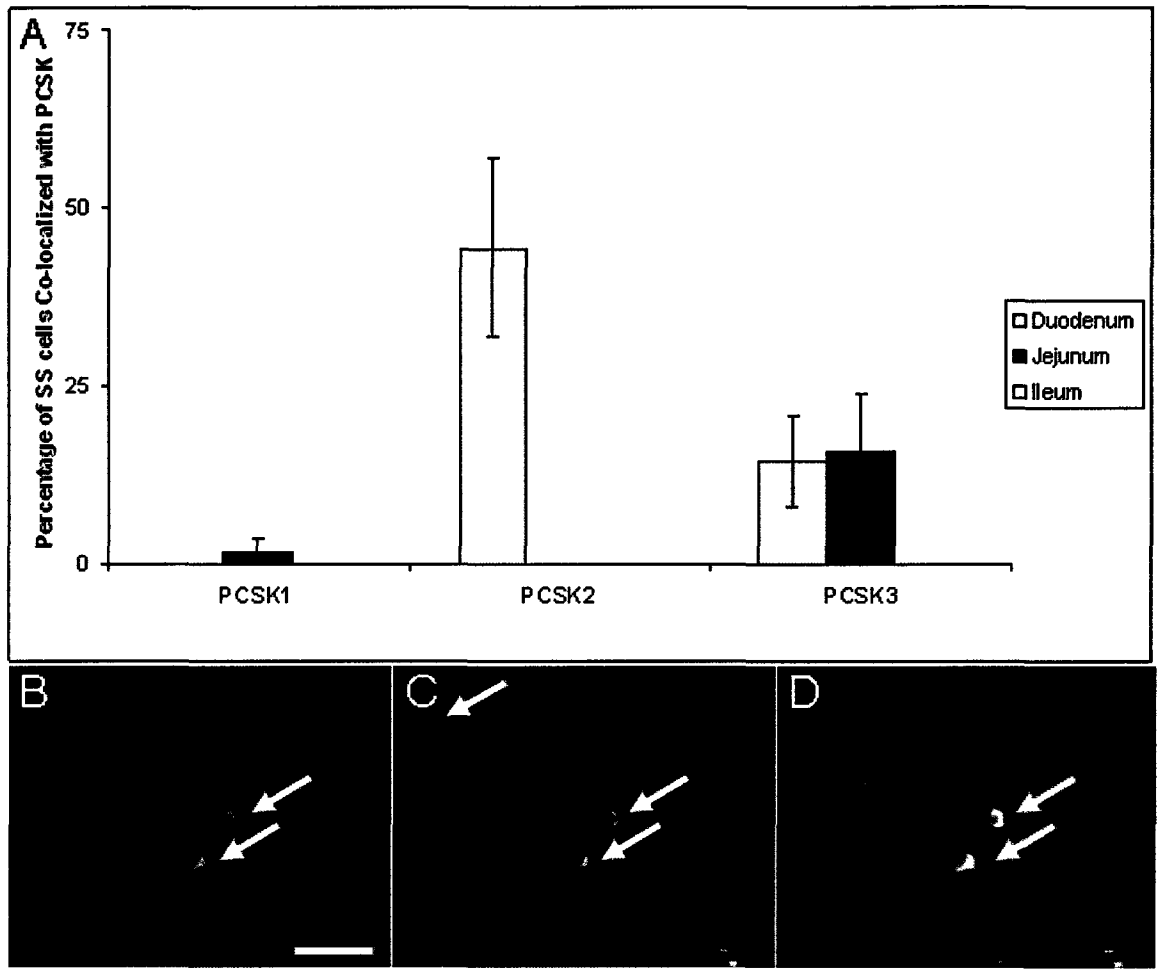

A
Percentage of SP cells Co-localized with PCSK
100
75
50
25
0
PCSK1
PCSK2
PCSK3
Duodenum
Jejunum
Ileum
B
C
D
E
F
G

Figure 11. Somatostatin co-localizes with PCSK2 and 3. The histogram above indicates the percentage of SS cells (y-axis) co-localized with indicated PCSK (x-axis) for duodenum, jejunum and ileum (A). The micrograph below illustrates co-localizing cell(s) in mouse duodenum. In green (B) is SS with red (C) PCSK2 and an overlay with DAPI counter stain (D). White arrows indicate single labelled cells and yellow arrows indicate double labelled cells in overlay. Bars = 50μm.


A
75
50
25
0
Percentage of SS cells Co-localized with PCSK
PCSK1
PCSK2
PCSK3
Duodenum
Jejunum
Ileum
B
C
D

4. Discussion

The localization of the PCSKs in the SI via immunohistochemistry is a crucial step in studying the roles of PCSKs in the endocrine and paracrine functions of the gut. Establishing intestinal hormone/protease co-localization is an essential step in the investigation of the potential processing of said hormones by this group of enzymes.

Chromogranin A is a precursor to many bioactive peptides with a variety of roles (45). It is found in an array of endocrine cells throughout the body including sympathetic neurons, adrenal medulla chromaffin cells, as well as EE along the gastrointestinal (GI) tract (92). In this study, Chr A was used as a marker of EE cells to compare regional expression of PCSKs in the EE system of the SI. The results indicated that PCSK1 was the most widely expressed PCSK as its levels were highest in all three regions of the SI (Figure 7). PCSK2, although less abundant than PCSK1, was highest in duodenum but gradually decreased towards the ileum. Despite being present in all three regions of the SI, PCSK3 was much less abundant when compared to the other two PCSKs examined. The elevated amounts of PCSKs in the upper regions of the SI are in concordance with many studies showing intestinal hormones in the duodenum and jejunum. As the duodenum is the first region to come into contact with chyme being released from the stomach, many regulatory hormones controlling motility and glycemia may be required (81). Correspondingly, elevated amounts of PCSKs may be required to mediate the proteolytic activation of the precursors to these hormones.

Our histological co-localization CCK, GIP, SP, and SS with PCSK1, 2 and 3 has provided suggestive hints on possible enzymatic links between these hormones and these convertases.

Pro-CCK is a 115-amino acid (aa) prohormone that undergoes several endoproteolytic cleavages to produce a variety of active CCK peptides (98). It is considered to be one of the most abundant neuropeptides in the brain (7) but it is also found in gut neurons and EE cells (97). Studies have shown that both PCSK1 and PCSK2 are important mediators of mature CCK production in the brain (7). Our study shows co-localization of CCK with these two PCSKs, but not with PCSK3 (Figure 8). This co-localization was only observed in the upper two regions of the SI as no CCK was found in the ileum. The nearly 80% of CCK immunoreactivity found to co-localize with PCSK1, suggests the potential involvement of this enzyme in pro-CCK processing.

GIP is a potent regulator of insulin secretion. When a meal is consumed, the chyme entering the SI triggers the release of GIP from EE cells inward to the circulation of the villus or lamina propria (LP). This GIP then travels to the pancreas where it can augment β cell proliferation, as well as insulin secretion (127). Previous studies have implicated PCSK1 in pro-GIP processing (136). Our co-localization study shows that PCSK1 is detectable in only half of the GIP cells. On the other hand, PCSK3 is found in nearly 75% of GIP cells (Figure 9). The proposed cleavage sites within pro-GIP are $RGPR_{22}\downarrow Y$ at its N-terminus and $QR_{65}\downarrow EAR$ at its C-terminus (136). PCSK3 is known to cleave its substrate after a RXXR↓ motif, which corresponds to the sequence at the N-terminus of GIP. Collectively, these consensus sequence features and our localization data support

the view that PCSK3, alone or in conjunction with another PCSK, may be involved in the processing of pro-GIP and warrants further investigation.

Substance P is an 11-aa peptide produced through proteolytic cleavages of its precursor, protachykinin, by an unknown endoprotease. One of the intestinal functions of SP appears to be the acceleration of nutrient intestinal transit (123, 134). Our study shows that all three PCSKs are expressed in SP cells (Figure 10). Interestingly, essentially all SP cells in the SI express PCSK1 (Figure 10). To a lesser extent PCSK2 and PCSK3 are detectable but only in the duodenum and jejunum (Fig. 10). The strong co-expression of PCSK1 and SP in all three regions suggests that the former might mediate the production of the latter. The sequence preceding the cleavage sites in pro-SP ($RIAR^{57}\downarrow R$ at the N-terminus and $MGKR_{67}\downarrow D$ at the C-terminus) strongly resembles those recognized by PCSK1 in other prohormones/proneuropeptide (64).

Pro-SS proteolytic processing generates active peptides known to inhibit intestinal contractility (80), as well as inhibit secretion of digestive enzymes such as maltase and lactase (69). Previous studies have also shown that the maturation of pro-SS to SS is almost totally abolished in PCSK2 KO mouse brain cortex (149). In our colocalization study, PCSK2 was the convertase most detectable in SS cells of the duodenum only. PCSK3 was detectable in fewer cells, and PCSK1 in none. Based on these observations, PCSK2 is the most likely pro-SS convertase in the SI.

In summary, this is the first detailed examination of PCSK regional expression in SI. The hormone/PCSK co-localization we have conducted can only provide suggestive or supportive evidence on the possible involvement of one PCSK or the other in the proteolytic activation of a particular prohormone. Clearly more experiments are needed to

establish this relationship and its degree of exclusivity. For example, a prohormone and a convertase can be co-transduced in an appropriate cell line and the resulting peptides characterized. In addition, with the availability of viable mouse models of heritable PCSK1 and PCSK2 deficiency, one can determine whether or to what extent lack of a PCSK causes impaired processing of prohormone and how this affects the physiological responses (e.g. gastric emptying, intestinal transit) normally elicited by the mature hormone in the GI tract.

5. Acknowledgments

The authors thank Mr. Andrew Chen for all his aid in the collection of mouse samples for this study. This work was funded by a grant from Canadian Institutes of Health Research

2.2 PCSK2 Deficiency Causes Altered Gastrointestinal Physiology and Associated Hormones Profiles

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Abstract

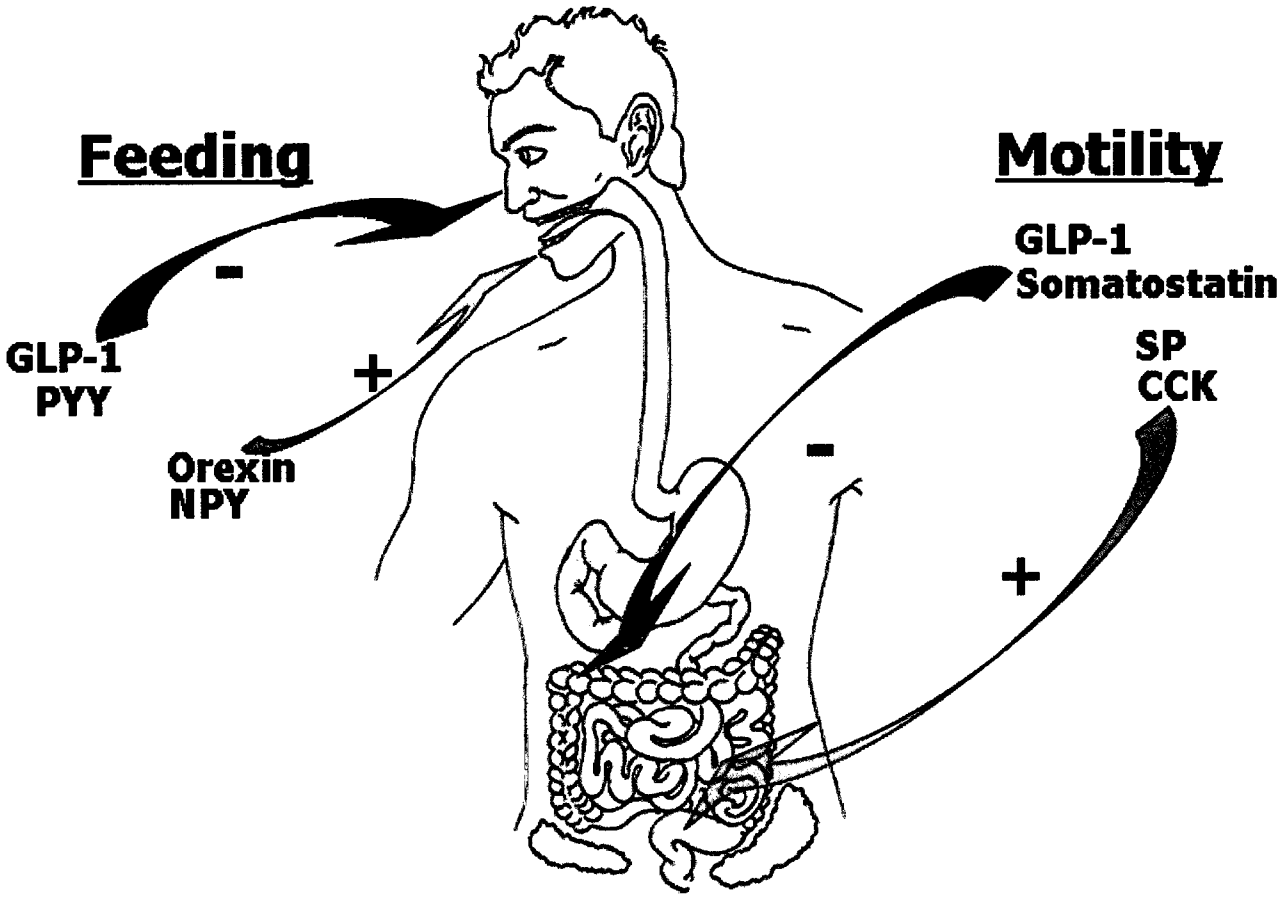
Proprotein Convertase Substilisin Kexin type 2 (PCSK2) plays a vital role in the maturation of prohormones into their biologically active forms. A deficiency in PCSK2 has been shown to alter the intestinal levels of the orexigenic neuropeptide Y (NPY) as well as increase the brain levels of the intestinal motility attenuating somatostatin (SS). As these peptides and several others are important in modulating feeding and gastrointestinal (GI) physiology we hypothesize that an absence of PCSK2 should manifest abnormalities in GI physiology. We examined feeding response after fasting and the rate of intestinal transit in 10 week old PCSK2 KO and WT mice. Female PCSK2 KO animals exhibited an attenuated feeding response after being fasted, which was significant following or during the first hour of feeding. Male PCSK2 KOs demonstrated a similar feeding trend but this lacked significance. Both male and female PCSK2 KO mice had significantly slower (or delayed) intestinal transit than WT littermates. To establish if these observations were associated with altered circulating hormone levels we assayed hormones including NPY, peptide YY (PYY), glucagon like peptide 1 (GLP-1), and Orexin A for feeding and SS, Substance P (SP) and cholecystokinin (CCK) for motility in the fed and fasted state. The levels of each of the hormones coupled to their known functions complemented the physiologies observed. In the fasted state only GLP1 and PYY remained elevated in the KO animals illustrating PCSK2's involvement. Our findings suggest that the altered physiology of feeding and motility occur from a PCSK2 deficiency that leads to the altered processing and levels of several important regulatory peptides.

Introduction

The regulation of gastrointestinal (GI) physiology (or function(s)) is complex involving feedback from both paracrine and endocrine systems. Hormones like substance P (SP)(87), cholecystokinin (CCK)(130) and somatostatin (SS)(34, 143) play important roles in how the motility of the GI tract is regulated. At the level of feeding there are many hormones including stimulators like neuropeptide Y (NPY)(67) and orexin-A (18), and attenuators like peptide YY (PYY)(135) and glucagon like peptide-1 (GLP1)(75) that accomplish balance of energy intake (Figure 12). These hormones, and many others like them, are derived from larger inactive precursors. They must undergo limited proteolysis to release their active forms. The proprotein convertase substilisin kexin-type (PCSKs) are a group of serine proteases that are produced in the cellular secretory pathway (113). The PCSK family contains 9 members that are collectively responsible for limited proteolysis of precursor proteins leading to the production of such bioactive molecules as growth factors, cytokines, hormones, cell surface proteins, receptors and transcription factors (113). PCSK 1-7 cleave their substrates following dibasic residues at the motif RX(R/K)(R/K)↓ (114) while PCSK8 (35) and PCSK9 (9) prefer hydrophobic and acidic residues, respectively.

PCSK2, one of the first discovered PCSKs (1990), was found to largely localize in the secretory granules of endocrine and neuroendocrine tissues (88, 118). In these tissues PCSK2 is synthesized as an inactive 74kDa precursor and during its maturation, autocatalytically cleaves off its prodomain to become an active 64kDa enzyme(84). The majority of the work in the field of PCSK2 has been focused on its processing of

Figure 12. Cartoon depiction of gastrointestinal hormone effects on feeding and transit of man. Red arrows indicate lowering effect on feeding and motility. Green arrows indicate stimulatory arrows indicate effect on transit being either accelerating (+) or slowing (-).


Feeding
Motility
-
GLP-1
PYY
+
Orexin
NPY
GLP-1
Somatostatin
SP
CCK
-
+

proopiomelanocortin (POMC) (11), proinsulin(125) and proglucagon (42). A recent study has shown PCSK2 to be genetically linked to a reduction in obesity, energy storage and food intake (20). In addition, PCSK2 has been associated with the processing of several propeptides involved in GI physiology (82). In our recent study, several PCSKs were localized to the enteroendocrine (EE) cells of the small intestine (SI), and PCSK2 was shown to co-localize with SS,CCK and SP (all known regulators of intestinal motility) (43). Over a decade ago a PCSK2 KO mouse was developed (41) and the authors focused their research efforts on the pancreatic effects of the PCSK2 deficiency. The resulting viable mouse exhibited many abnormalities within the scope of glucose homeostasis including elevated levels of proglucagon lower levels of circulating mature glucagon and altered islet morphology (41) .

Based on prior knowledge of PCSK2 processing of hormone precursors that are required for feeding and GI physiology, we decided to use the PCSK2 KO mouse model to examine the effects of PCSK2 deficiency on the GI physiologies of hunger response and intestinal transit. In order to correlate our physiological observations we then assayed the circulating levels of regulatory hormones known to impact these physiologies using the enzyme linked immuno assay (ELISA) method on plasma from both fed and fasted animals.

Materials And Methods

1. Animal Physiology

The animal experiments were carried out on female and male 9 week old C57/Bl6 mice that were WT or homozygous for the gene disruption insert (production of animals is explained previously (42)). All experiments carried out were approved by the CCAC institutional animal care committee.

1.1 Refeeding response.

In this experiment the animals were placed on an overnight (ON) fast to stimulate a hunger response. Animals were given a pre-weighed meal of regular chow at time 0. Food consumption was measured at 1, 2, and 4 hour time points These time points were selected to enable greater detail on the immediate refeeding and have be used in other studies(124). Special care was taken by removing bedding from cages during feeding to ensure bedding material was not consumed and all the uneaten food was available for weighing. In addition, at the 24 hour time point due to mixing of food particles with animal waste, special care was taken to dry then separate animal waste from leftover food for accurate measurement. Results from WT and KO animals were analyzed using the repeated measures ANOVA.

1.2 Intestinal Transit.

Animals were fasted ON and given a charcoal meal by oral gavage with a 22 gauge feeding needle in the morning. This meal was comprised of water with 5% w/v fine

powder wood charcoal and 10% w/v gum Arabica. The mixture was sonicated to produced a smooth homogenous solution. The meal was allowed twenty minutes to travel. The animals were then sacrificed by decapitation. Chest cavities were opened and the edges of the charcoal meal were tied off. The intestine was then cut out after the stomach at the pyloris and before the ceacum. Intestines were removed and laid out on a flat surface where the length measurements could be taken. Intestinal transit was measured as a percentage of the intestine traveled; 100x (pyloris to meal front/ total length of SI). Results from WT and KO animals were analyzed using the Student's t-test.

2. Hormone Assays

Hormones known to be important in the regulation of feeding and intestinal motility were assayed using the competitive enzyme immuno assay method. Kits were purchased as described in Table 3. Assays were conducted on 20ul plasma aliquoted that had been partially purified using solid phase extraction on SEP PAK columns according to manufactures' instructions. The eluted peptides were dried by speedvac, reconstituted in assay buffer provided in the kits and immediately assayed. Plasma samples were collected from fed animals for the complete panel of assays and additional fasted plasma was collected for assay of hormones related to feeding and hunger. All samples were run in duplicate with $n > 6$ on male and female mice. Absorbance readings were completed at wavelengths indicated in assay kits on Thermo Electron © *Multiskan Spectrum* plate reader and data was analyzed using 4 parameter logistics in Prism Graph Pad software. Results from WT and KO animals were analyzed using the Student's t-test.

Table 4. List of assay kits used for ELISA with manufacturer and product number indicated.

Assay	Manufacturer	Product #
GLP1	Bachem	S-1141
NPY	Bachem	S-1145
PYY	Bachem	S-1150
SS	Bachem	S-1179
CCK	Bachem	S-1205
Orexin A	Phoenix Peptides	EK003-30
SP	Assay Designs	900-018

Results

1. Animal Experiments

1.1 Refeeding Response

Figure 13 depicts the amount of food consumed between each time point for PCSK2 KO and WT littermates. The female KO mice consumed significantly less food than the WT littermates during the first hour post fasting. At each of the time points thereafter there was a trend toward less eating in the KO but the significance was lost. Although the male KO mice showed a trend toward less food consumption during the first hour of re-feeding, their response was not significantly different from WT littermates (Figure 13).

1.2 Intestinal Transit

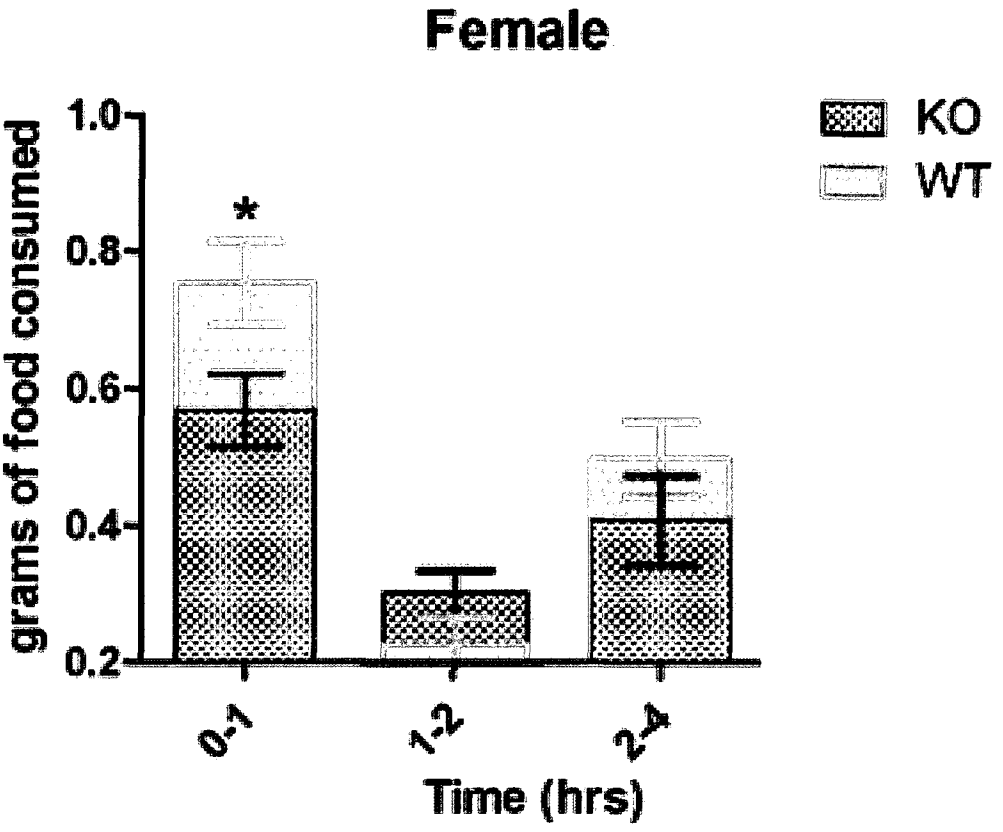
The distance a measured meal travels through the SI is depicted in Figure 14. This figure illustrates the striking difference between the PCSK2 KO and WT animals for both males and females. Female KO animals have 30% slower intestinal transit then their WT counterparts ($P<0.001$). Male PCSK2 KO have 10% slower intestinal transit than WT littermates ($P<0.05$) .

2. Hormone Assays

2.1 Fed Assays

Figure 15 shows the varying levels of hormones assayed in the fed state for both male and female, WT and PCSK2 KO mice for feeding represented in set A and motility in set B. The assays examined for female feeding show that in all cases the there is a

Figure 13. Food consumption over 4 hour period in male and female WT and PCSK2 KO mice. Y-axis indicates the amount of food consumed in grams. X-axis indicates the time interval where measurement is taken. Blue bars represent WT animals and red bars represent KO. Bars are overlaid to indicate elevated eating. * indicates $p<0.05$. Values indicated are means ± SEM n=11 per each sex and genotype.


Female
KO
WT
grams of food consumed
1.0
0.8
0.6
0.4
0.2
*
0-1
1-2
2-4
Time (hrs)

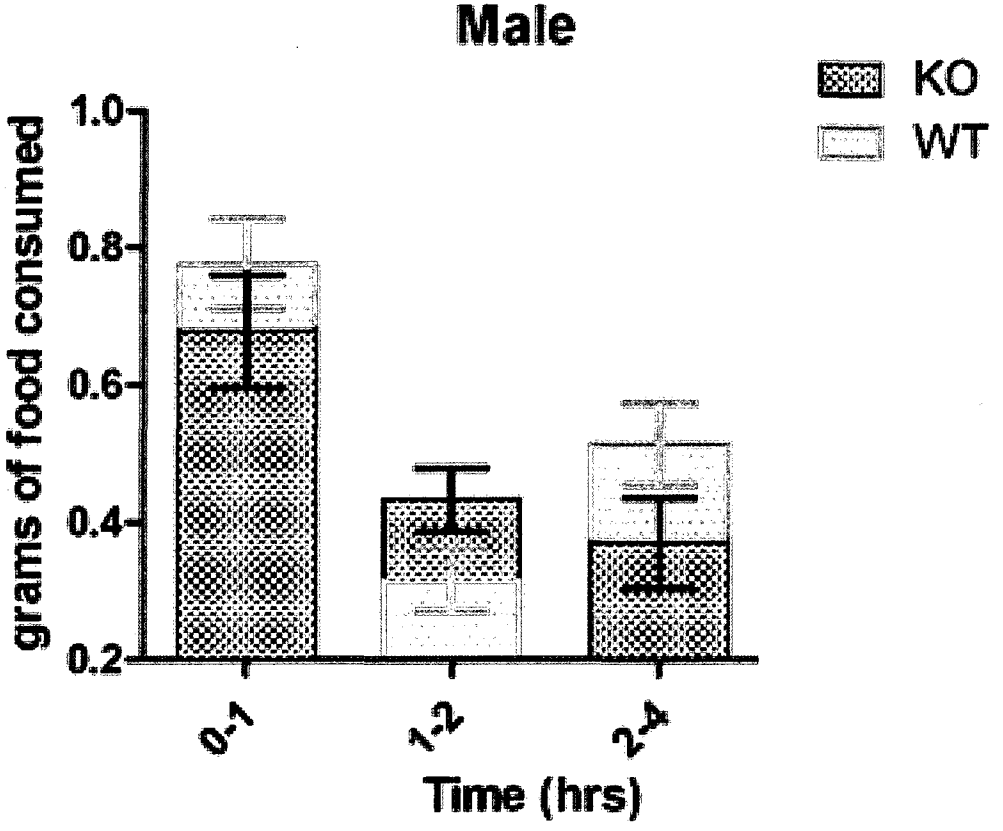

Male
KO
WT
grams of food consumed
1.0
0.8
0.6
0.4
0.2
0-1
1-2
2-4
Time (hrs)

Figure 14. Intestinal transit in male and female PCSK2 KO and WT mice. Bar graphs represent percentage of dye travel relative to total length of intestine (X-axis) for each sex (Y-axis) with WT animals in blue and KO in red. *** indicate $p<0.001$ and * indicate $p<0.05$. Bars indicate mean ± SEM $n>9$.

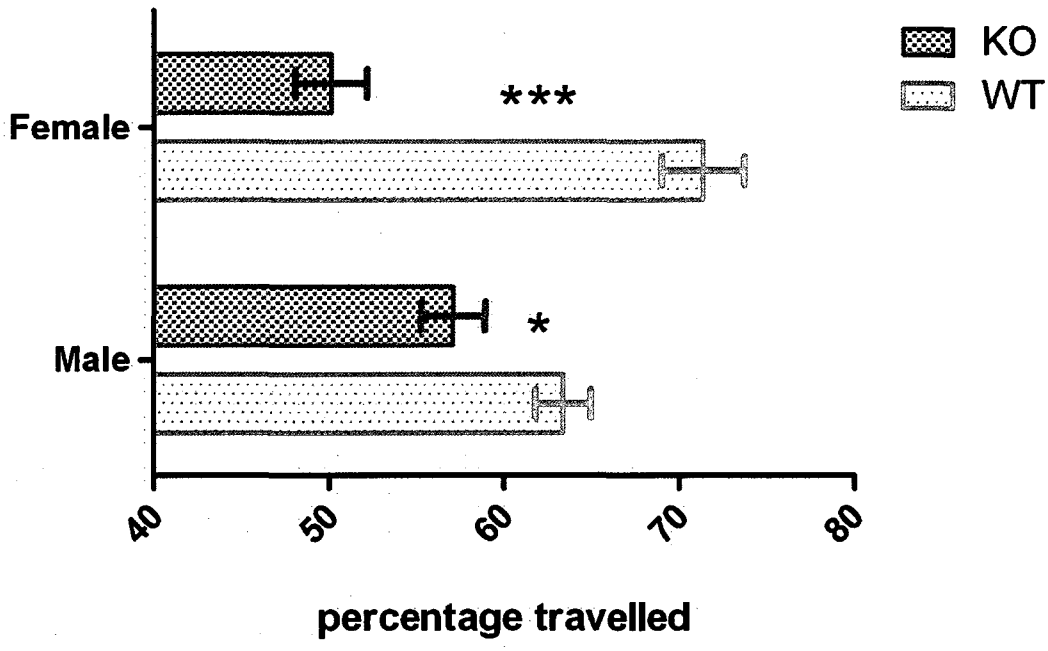
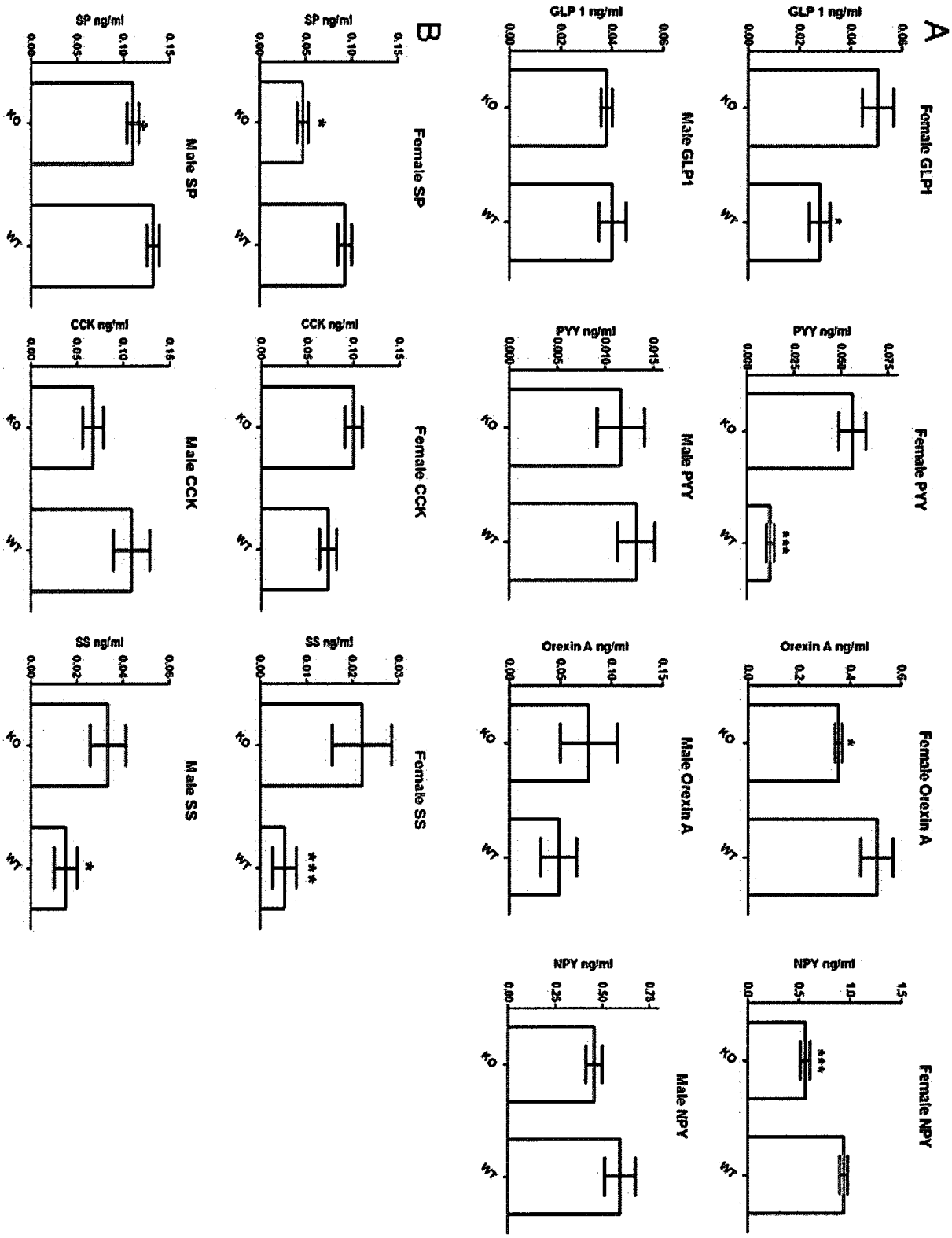

KO
WT
Female
Male
***
*
40
50
60
70
80
percentage travelled

Figure 15. Fed state assay of feeding and motility related hormones in circulation. Panel A represents feeding based assays and panel B motility based assays. Sexes and hormone assayed are indicated above each column. Significance is indicated as follows; *= $P<0.05$, **= $P<0.01$ and ***= $P<0.001$. Bars indicate mean ± SEM n >6 animals for each column.

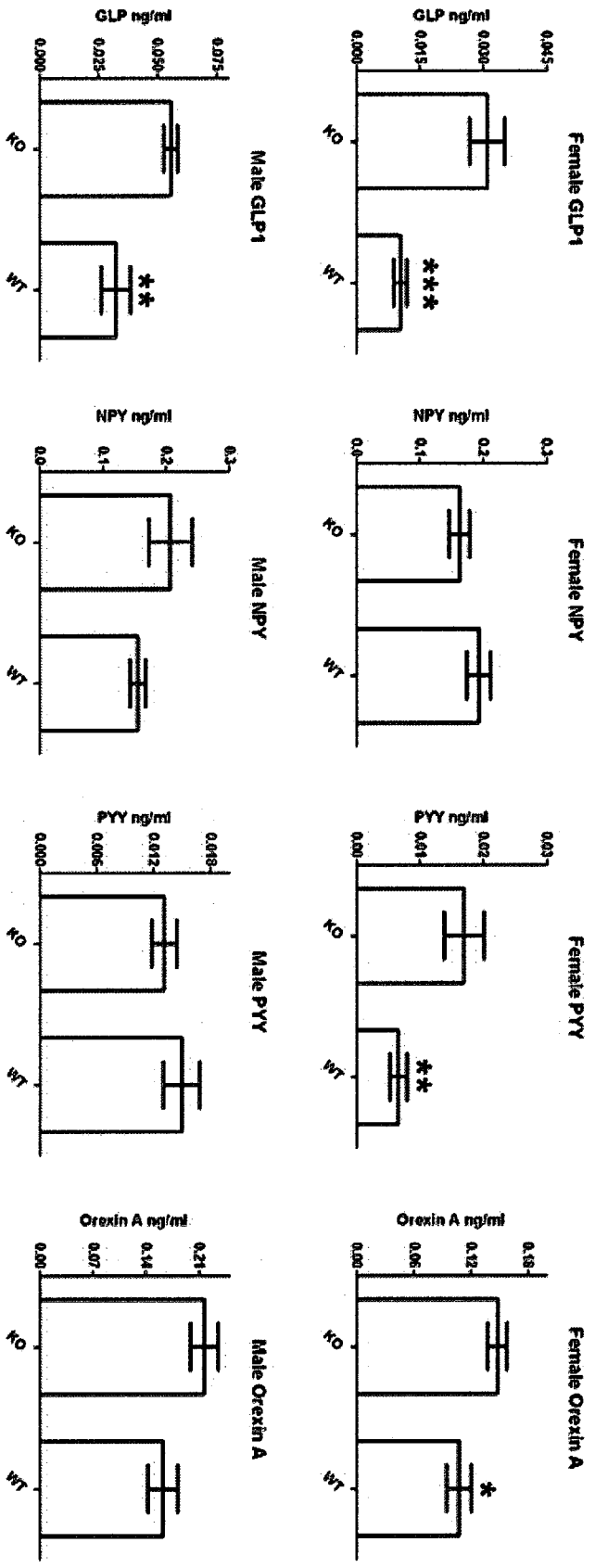

A
Female GLP1
GLP 1 ng/ml
0.00
0.02
0.04
0.06
KO
WT
*
Male GLP1
GLP 1 ng/ml
0.00
0.02
0.04
0.06
KO
WT
Female PYY
PYY ng/ml
0.000
0.025
0.050
0.075
KO
WT
***
Male PYY
PYY ng/ml
0.000
0.005
0.010
0.015
KO
WT
Female Orexin A
Orexin A ng/ml
0.0
0.2
0.4
0.6
KO
WT
*
Male Orexin A
Orexin A ng/ml
0.00
0.05
0.10
0.15
KO
WT
Female NPY
NPY ng/ml
0.0
0.5
1.0
1.5
KO
WT
***
Male NPY
NPY ng/ml
0.00
0.25
0.50
0.75
KO
WT
B
Female SP
SP ng/ml
0.00
0.05
0.10
0.15
KO
WT
*
Male SP
SP ng/ml
0.00
0.05
0.10
0.15
KO
WT
Female CCK
CCK ng/ml
0.00
0.05
0.10
0.15
KO
WT
Male CCK
CCK ng/ml
0.00
0.05
0.10
0.15
KO
WT
Female SS
SS ng/ml
0.00
0.01
0.02
0.03
KO
WT
***
Male SS
SS ng/ml
0.00
0.02
0.04
0.06
KO
WT
*

difference between PCSK2 KO and WT. Specifically, the hormones known to stimulate feeding, NPY and orexin A, are lowered and hormones known to inhibit feeding, GLP1 and PYY, are elevated in the PCSK2 KO animals compared to WT littermates. This significant difference in feeding hormones was not observed in the males (Figure 15 A). For both female and male mice, the intestinal motility related hormones known to increase intestinal transit including SP were lowered in the PCSK2 KOs while those that are known to slow intestinal transit like SS were increased in the PCSK2 Kos (Figure 15 B). No significant difference between genotypes was observed for the motility hormone CCK (Figure 15 B)

2.2 Fasted assays

To gain a better understanding of the endocrine state at the beginning of the experiment, hormones for feeding regulation were assayed in fasted male and female PCSK2 KO and WT mice (Figure 16). In the fasted state female PCSK2 KO animals had the same increase in appetite lowering GLP1 and PYY levels; However, for appetite stimulating hormones a significant difference between WT and KO was lost with NPY , and switched orientation for orexin A levels with an elevated hormone level in the PCSK2 KO compared to the WT (Figure 16). Assays for PYY, NPY and orexin A in males still had no significant difference between WT and PCSK2 KO, however GLP1 now showed increased levels in the KO compared to WT.

Figure 16. Fasted state assay of feeding related hormones in circulation. Male and female PCSK2 KO and WT are indicated above each set comparison. Significance is indicated as follows; *= $P<0.05$, **= $P<0.01$ and ***= $P<0.001$. Bars indicate mean ± SEM n >7 animals per assay.


Female GLP1
GLP ng/ml
0.000
0.015
0.030
0.045
KO
WT
***
Male GLP1
GLP ng/ml
0.000
0.025
0.050
0.075
KO
WT
**
Female NPY
NPY ng/ml
0.0
0.1
0.2
0.3
KO
WT
Male NPY
NPY ng/ml
0.0
0.1
0.2
0.3
KO
WT
Female PYY
PYY ng/ml
0.00
0.01
0.02
0.03
KO
WT
**
Male PYY
PYY ng/ml
0.000
0.006
0.012
0.018
KO
WT
Female Orexin A
Orexin A ng/ml
0.00
0.06
0.12
0.18
KO
WT
*
Male Orexin A
Orexin A ng/ml
0.00
0.07
0.14
0.21
KO
WT

Discussion

The regulation of both feeding and gastric motility is known to be controlled by several hormones produced in endocrine tissues in the body. The production and controlled secretion of these peptide hormones depends heavily on proper processing of their precursors. It is known that the PCSKs are involved in the bioactivation of said molecules and that, in particular, PCSKs 1 and 2 are largely involved in endocrine prohormone processing. Given the involvement of PCSKs in the maturation of prohormones known to regulate feeding and gastric function, PCSK2 deficiency may affect these processes. To examine the importance of this enzyme in the mentioned physiologies, PCSK2 KO and WT mice were compared in hunger response and intestinal transit experiments.

Under normal situations of negative energy balance orexin A and NPY are up-regulated and stimulate appetite (104, 146). Conversely, PYY and GLP-1 are involved in satiety signals and will lower the feeding response. In our hunger response experiments, we show that the PCSK2 deficient animals consume less following an overnight fast. Most notably, PCSK2 deficient females in comparison to WT littermates had the greatest difference in food consumed during the first hour following the fast. When examining the circulating levels of the hormones known to regulate appetite, it was clear that the decreased appetite corresponded to the altered levels of these hormones in female mice: with the PCSK2 KO having lower levels of the appetite stimulating hormones and higher levels of the appetide lowering hormones compared to WT animals. When we examined the levels of these same hormones in male circulation we found no significant difference between WT and KO littermates which correlated with our observation as the male

PCSK2 KO and WT animals did not have a significant difference in the hunger/ feeding response but only a trend.

Conducting the assays under fed conditions gives us information on these hormones in the steady state. The feeding experiment, although involving food consumption during measurement, was conducted after an ON fasting to illicit the hunger response. It was therefore important to investigate hormones levels in the fasting state to get clear picture of their profile immediately before the animals enter the experiments. As such the assays related to feeding were repeated in fasted animals. When comparing female WT and KO animals the levels of GLP-1 and PYY remained similar in the fed and fasting state, however the levels of orexin A and NPY did not. Orexin A switched orientation with higher circulating amounts being present in the PCSK2 KO. In the NPY assays the significant difference was lost. The changes (or lack of changes) observed in these assays under a different feeding state gives us information regarding the importance of PCSK2 in their respective levels. The observation that the satiety hormones GLP1 and PYY remained elevated in PCSK2 KO animals regardless of energy state strengthens the possibility of PCSK2 being directly involved in their processing. As orexin A and NPY levels varied depending on energy state and not necessarily genotype of the animal it is likely that other proteases are involved in their maturation. Already studies have been completed in the pancreas indicating that in the absence of PCSK2 proglucagon is processed (by PCSK1) to GLP1 instead of glucagon (147, 148). Little is known about PYY's processing and this represents an excellent opportunity for future research.

PCSK2 has been implicated via colocalization (43) and other in vitro studies in the maturation of hormones involved in intestinal motility including SP (63) SS (34), GLP1 and CCK (70). In addition to its appetite lowering effects GLP1 is known to delay intestinal transit in dogs (4). In our transit experiments, we observed a markedly decreased rate of intestinal transit in both male and female PCSK2 KO mice. Again in these experiments this phenotype was stronger in the females versus males. When examining the circulating levels of hormones known to regulate intestinal motility we saw a significant difference between WT and PCSK2 KO animals. Hormones known to stimulate motility like SP were lowered in the KO and hormones known to slow motility like SS and GLP-1 increased in the KO animal. Again we have a correlation between the phenotype observed and the circulating hormones examined. How the absence of PCSK2 is causing the increased levels of SS has not been elucidated. It is possible that due to impaired processing of one form of mature SS a feedback mechanism is increasing the overall production of pro-SS. The elevated SS levels in the PCSK2 KO animal present an opportunity for future investigation.

The variability of PCSK effects between sexes has been observed in several studies. In a PCSK1 KO recently produced by Mbikay et al there was a significant difference between female and male KO animal with respect to catch up growth rate (72). A similar gender dichotomy was observed in humans with PCSK9 and LDL cholesterol (71). The gender dichotomy observed in the GI physiology may be related to PCSK2-mediated processing on sex hormones and their downstream effects

These studies indicate that aside from its impaired glycaemia aspects, the PCSK2 deficient animals have altered feeding and gastrointestinal transit physiologies. To establish what may be contributing to these phenotypes we went on to examine globally the levels of hormones known to be effectors in these functions. Based on these results we saw that the levels of several important hormones are clearly altered in the PCSK2 KO animals and this may contribute to the altered physiology. Previous work with co-localization studies has made suggestive links to PCSK2-substrate processing in a number of organs including the SI. However, the exact cause of the altered circulating hormone levels can not be directly attributed to the PCSK2 deficiency in these animals. These altered hormones levels may be arising from secondary effects of the convertase deficiency. In particular the orexigenic and anorexigenic neurons may be affected by the alterations of neuropeptides in the hypothalamus of the PCSK2 KO animal. To clarify this, an intestinal specific KO of PCSK2 should be developed. Additionally in vitro experiments could indicate if PCSK2 can process these substrates. And finally, with the availability of a mouse model for PCSK1 deficiency (64) these experiments can be repeated to determine its role in GI physiology.

3 Extended Discussion

3.1 PCSK intestinal localization

Establishing the localization of the PCSKs by immunohistochemistry was an essential first step in this study. The first studies examining PCSK localization were done with in situ hybrization experiments describing each of these PCSKs levels in a variety of mouse tissues including heart, brain, lung and pancreas (13, 117, 119). Several years later and broad examination of RNA expression indicated PCSK1 2 and 3 expression in the GI tract (115). Other studies examining individual PCSK roles in the processing of specific proproteins have been conducted as well, as is the case with PCSK1 and pro-GIP (138). What was lacking was a comprehensive localization study at the protein level for several PCSKs known to be in the SI. In the first set of experiments we were able to show that PCSK1 2 and 3 are present inside the EE cell population in different levels depending on the region being examined. PCSK1 was abundant along the rostrocaudal extent of the SI, PCSK2 in the duodenum and tapering off towards the ileum, and PCSK3 similarly present across all regions but at lower levels. Observing this differential expression across the length of the SI suggests the enzymes may have specific roles in each region. The duodenum has the highest amount of convertase containing EE cells. Accordingly, it is also the first region of the SI to come into contact with partially digested food leaving the stomach. It is already established that many signaling cascades begin once food enters the SI including secretion of the incretins, GIP and GLP-1, which stimulate insulin release; and the bile releasing and intestinal motility effector CCK (77). The increased levels of these and other pro-hormones may explain why there are elevated levels of

cellular PCSK expression as these converting enzymes could be responsible for their maturation. This is perhaps best illustrated with the levels of PCSK2 being highest immediately after the stomach in the duodenum and eventually less along the length of the SI towards the large intestine. Seeing PCSK3 immunoreactivity strictly in the EE cell population and not ubiquitously expressed in all cell types was unexpected as it is typically known to be ubiquitously expressed. However its expression in EE cells is essential as it processes the chaperone 7B2 which is required for the maturation of PCSK2 (49). To verify this, experiments using both PCSK2 and PCSK3 double labeling should be done. As antibodies providing adequate detection in immunofluorescence were raised in the same animal in these studies, this was not possible. However, as new antibodies for either PCSK become available this can be revisited.

With the established regional expression of these 3 PCSKs and knowledge of the abundance of prohormones in the SI, our next goal was to establish potential enzyme-substrate links between the convertases and several SI hormones. Based on their relative abundance and evidence of potential processing from previous literature we selected CCK, SP, SS and GIP. In cases where a high degree of colocalization with a given PCSK was observed, there was a primary PCSK cleavage consensus site in the substrate. This observation is described in detail in the above manuscript discussion (chapter 2.1) and adds strength to the potential intestinal substrate – convertase link. These studies suggested several avenues of further research. One such observation was GIP colocalization with PCSK3. Previous research has indicated that PCSK1 is the convertase responsible for its maturation (138). However in our experiments over 50% of the GIP immunoreactivity was not found in PCSK1 positive cells. We observed another nearly

70% of GIP immunoreactivity co-localizing with PCSK3. These data, taken together, indicate some convertase overlap, but more important, underline the potential involvement of another PCSK in pro-GIP maturation. As immunoflourescense represents a "snapshot" of the cells at a given time it cannot be said by these experiments alone that PCSK1 is not essential for the maturation of proGIP. However, it does tell us there may be other enzymes involved and that further in vitro investigation with PCSK3 and pro-GIP to explore whether the former can process the latter needs to be examined.

In some situations, we observed a very strong degree of colocalization for a given hormone and a given PCSK. Having strong convertase- substrate colocalization provides us with a very strong probability that the PCSK is playing a role in the prohormone's maturation (36) . For example, PCSK1 was found in over 80% of CCK positive cells. Even more striking, this same convertase was found in nearly all SP positive cells. In both cases, lesser amounts of PCSK2 and 3 were found to colocalize (respectively). As these links are the strongest they should be among the first candidates for the detailed *in vivo* and *in vitro* examination on PCSK processing of GI hormones.

The completion of this study has shed some light on the importance of the PCSKs in the SI. Knowing that they are expressed at the protein level in a region specific manner and that they are colocalized (in several cases to a very high degree) with GI prohormones has generated several questions to be addressed in further studies. If the PCSKs are present in the SI and if they are found in the same cells as prohormones then how might their levels impact GI physiology? I addressed this question in part 2 of my thesis for PCSK2.

3.2 PCSK2 KO and intestinal physiology

In the second part of this thesis the PCSK2 complete gene knock out mouse was used to examine its role in GI physiology. The initial focus was to observe several aspects of GI physiology including feeding response and intestinal transit time in these mice. Upon observing differences in WT and KO mice the focus was then shifted to assaying the circulating levels of hormones known to be effectors of these processes. After being fasted overnight both female (significant) and male KO mice consumed less food than WT. Interestingly when the levels of orexic and anorexic hormones were assayed there was a positive correlation with their levels and the observed physiology. Orexin A, also known as hypocretin 1, is produced from pre-pro-orexin, exclusively in orexin neurons of the brain (79). It then acts on its receptor to stimulate both wakefulness and feeding activity (79). As the circulating levels of orexin-A were lower in KO females, the diminished feeding response was expected however there may be additional phenotypes including sleep cycle alterations in these PCSK2 KO mice. Another hormone known to stimulate feeding that was found to be lower in KO females was NPY. Again, NPY has multiple effects on physiology in addition to feeding including angiogenesis in ischemic heart muscle (60), As such it may be possible that these PCSK2 KO have impaired cardiovascular repair abilities.

When examining anorexic peptide hormones including GLP-1 (47) and PYY (6), an increase of the circulating levels was observed in the KO females. Seeing increased levels of the mature hormone in the PCSK2 deficient animal may initially appear

perplexing; however, one must consider the complete prohormone to understand how this arises. GLP-1 is produced from the larger proglucagon precursor which is differentially processed in a tissue specific manner by PCSK1 and PCSK2 to produce glucagon, GLP-1 and GLP-2 (148) Figure 17. In the absence of PCSK2 the proglucagon precursor will produce more of the PCSK1 cleaved product, GLP-1, and less of glucagon and GLP-2 (148). This agrees with the original observation whereby PCSK2 KO animals being glucagon deficient (41).

Assays for hormones related to feeding on animals in the fasted state provided some insight on the roles PCSK2 may play in their processing. Previous research examining the roles of the satiety hormones were often conducted in the fasting state so as to stimulate the required response to an energy deficit. Additionally, conducting assays in the fasted state ensure that both groups (WT and PCSK2 KO) are in the same energy deficient state an enables level ground for comparison. Some the hormones examined in the fed state produced different results in the fasted state. With regards to Orexin A we now saw a shift from the expected lowered level in the fed PCSK2 KO to an elevated level in the fasted PCSK2 KO. Upon re-examining the literature pertaining to the PCSK2 KO we see that this animal has impaired proglucagon processing which leads to cronic fasting hypoglycemia (42). This hypoglycemia will lead to the stimulation of orexin neurons and the production of orexin A and B (111). And as orexin A is capable of crossing the blood brain barrier (56), and may explain the increased levels in the fasted PCSK2 KO assay.

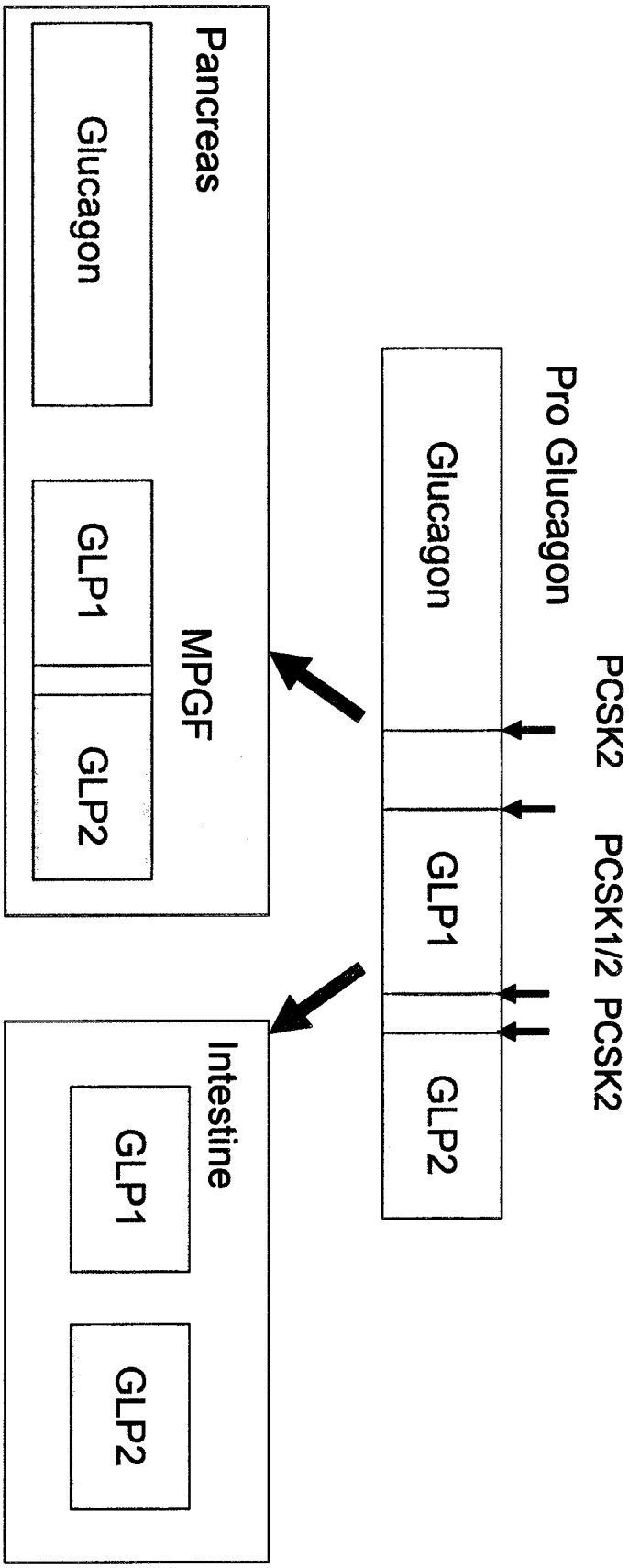
With regards to NPY being similar between the KO and WT in the fasted state may not have as much meaning as NPY activity in relation to feeding is secreted and signals in the

arcuate nucleus of the brain and is not derived from circulating NPY (57). In the future it would be beneficial to assay NPY from brain tissue.

Only GLP 1 and PYY maintained their elevated levels in the KO regardless of feeding state. Like PCSK2 deficiency has been shown to increase GLP1 levels in the pancreas, our observations in circulation increased GLP1 as a result of PCSK2 deficiency. The PYY assay showed a similar result of increased levels in the KO regardless of feeding state. The PYY was also only significantly higher in the fasted PC2KO females. As only the females observed a significant decrease in refeeding, perhaps PYY is even more important in determining feeding than GLP1

The observation of a sexual dimorphism with respect to physiologies examined in the PCSK2 KO is not the first time PCSKs have been implicated in a gender dimorphism. Previous studies examining PCSK1 KO mice (72) , PCSK3 in the submadibular gland of developing mice (36) and PCSK9 in cholesterol metabolism have all indicated differences between sexes. The differences observed in the PCSK2 KO model may suggest different requirements of this convertase in GI physiology. It is also equally plausible that the female differences in PCSK2 WT and KO feeding are arising from a sex specific hormonal effect. One example is the energy homeostasis hormone leptin, which can be 40% higher in human females (102) and may be eliciting its satiety response. This fact in combination with the observed hormone levels may explain why only the female KO animals have decreased refeeding. Additional assays on these animals would clarify this.

Figure 17. The differential processing of pro glucagon by PCSK1 and PCSK2 to yield multiple active hormones in the pancreas and intestine. PCSK2 cleavage site indicated for the production of glucagon and major pro glucagon fragment (MPGF) in pancreas. PCSK1 causes production of mature GLP1 and GLP2 in intestine. Adapted from **(30)**


Pancreas
Glucagon
GLP1
GLP2
MPGF
Pro Glucagon
Glucagon
GLP1
GLP2
PCSK2
PCSK1/2
PCSK2
Intestine
GLP1
GLP2

This increased level, like GLP1 and proglucagon, may result from processing events. However unlike GLP1, proPYY is not known to be processed by PCSK2 and instead dipetidyl peptidase IV is thought to be responsible (76). One possibility of why PYY levels are increased in the PCSK2 KO may be dependant on their decreased feeding as PYY is required in the cessation of digestive gastric acid secretion (145).

Another key component of intestinal physiology is small intestinal motility. Proper absorption of nutrients in the SI is maintained in part by the time spent in contact with the mucosal membrane of the SI (152). More time spent in SI may indicate a need for greater levels of absorption(152). In our transit experiments there was significantly slower transit in both male and female mice lacking PCSK2. This difference was greater in the females showing similar gender-based sensitivity to PCSK2 deficiency as observed in the feeding experiments. Using the same approach as in the feeding experiments we went on to examine the levels of circulating hormones known to impact the physiology of intestinal transit including CCK, SS and SP. Although there was no significant difference in CCK levels between WT and KO, both SP and SS levels were affected. Substance P is both a neurotransmitter(87) and endocrine hormone (144). In the intestine it functions to stimulate intestinal motility and in both male and female KO animals the levels of SP were lower than WT. In the previous manuscript we showed that SP, although largely colocalized with PCSK1, was colocalized with PCSK2. These two pieces of evidence linking SP and PCSK2 together need to now be examined in greater detail. In vitro studies expressing both the convertase and prohormone in cell culture and analyzing the cleavage products could be one such avenue. Conversely SS, the intestinal motility attenuator, was increased in the KO animals correlating again with the observed

physiology. It should be mentioned that there are 2 forms of SS derived from the pro-somatostatin precursor, SS-28 and a smaller SS-14. The larger form is thought to be processed by PCSK3 and the smaller form by both PCSK1 and 2 (15). The SS-14 ELISA used in this study also indicated 100% cross reactivity with SS -28. The increased levels detected in the KO could indicate a compensatory mechanism. Potentially, in the absence of PCSK2 the SS precursor is primarily processed to its SS-28 thus creating a demand for SS-14. To answer this question immuno precipitation/ Mass Spectrometry should be completed with the antibody used in this ELISA.

4 Conclusions

The many roles of the endocrine PCSKs have been studied extensively throughout the body in several endocrine organ systems including the brain, pancreas, adrenal gland and liver. One area that has not received as much attention is the SI, and represents a frontier in PCSK research. Perhaps this is due to its diffuse (never the less large) endocrine cell population. In this body of work we were able to show the region specific expression along with colocalization with potential hormone substrates of several of the PCSKs including PCSK1, 2, 3. We made important connections with these potential substrates that will foster future work. The close examination of the PCSK2 KO animal gave even more information linking the convertase deficiency to GI abnormalities. There is no question the GI tract is an important endocrine organ based on both the total amount of endocrine cells and the number of hormones produced. A greater understanding of how hormones like GLP 1 are matured and secreted will enable new therapies as there is accumulating evidence linking the GI tract's signaling roles to diseases like type 2 diabetes mellitus and obesity. By understanding how the family of PCSKs functions in this organ, we can begin to make important associations that may form the basis for the development of biologically rational therapies.

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6 Contributions of Collaborators

In the first study I completed the collection, histochemistry and quantification of the intestinal sections. Dr. Mayne and Dr. Mbikay helped plan the method of quantification. Dr. Mayne also established control experiments for the immunohistochemistry. Dr Woulfe provided guidance on how immunohistochemical manuscripts are completed, and Dr Chrétien provided the general goal design of this project.

In the second manuscript I completed the animal physiology experiments and all of the assays and subsequent analysis. Dr. Mayne aided in the collection of feeding data as well as well as the method to present the data. Mrs. Raymond aided in the general collection of blood samples and food measurements. Mr. Chen aided in the collection of plasma for the hormone assays. Dr. Mbikay helped with the assay conditions and analysis. Dr. Woulfe and Dr. Chrétien helped design the manuscript.

7 Curriculum Vitae

Publications:

- Prichett W, Milman P, **Gagnon J**, Munoz DG, Woulfe J. Intranuclear rodlets in human pancreatic islet cells. Pancreas. 2007 Oct; 35(3):207-11.
- **Gagnon J**, Mayne J, Mbikay M, Woulfe J, Chrétien M. Expression of PCSK1 (PC1/3), PCSK2 (PC2) and PCSK3 (furin) in the Mouse Small Intestine. Regulatroy Peptides. Online July 22, 2008.
- **Gagnon J**, Mayne J, Raymond A, Chen A, Mbikay M, Woulfe J, Chrétien M. PCSK2 Deficiency causes impaired GI Function (final stages for submission)

Intellectual Property:

I have submitted a patent application (2546686) on a device used for membrane based biochemistry techniques.

Social/Ethical:

- September 2006-Sept 2007 OHRI Animal Care Committee (protocol review panel)
- September 2007 Graduate Student recruitment info Session

Administrative

- May 2007-September 2008 Procurement Requestor (lab purchaser)

Seminar Symposiums

- February 2008 Dalhousie Universities Department of physiology seminar series. Halifax, Nova Scotia. PCSKs and intestinal physiology.
- January 2008 University of Ottawa BMI research day. Ottawa, Ontario. GI physiology in PCSK2 KO mice
- November 2007 Ottawa Health Research Institure annual seminar symposia. Ottawa, Ontario. The expression and function of PCSKs in GI tract
- April 2007 University of Ottawa BMI poster day. Ottawa, Ontario. The Spatial temporal expression of the proprotein converatases in the Murine small intestine
- November 2006 Ottawa Health Research Institute annual seminar symposia. Ottawa, Ontario. Moderator.

Conferences

- October 2003 Annual Protease Conference. Mont Tremlant, Quebec.
- July 2008 GRC Proprotein Processing, Trafficking & Secretion. New London, New Hampshire

Research Experience

Outlined below is a list of my research experience.

1) **2001-2002** high school co-op student at the Loeb Research Institute. Peptide chemistry laboratory of Dr Ajoy Basak. This was my first experience in laboratory setting. Here I acquired the basics and operation of HPLC, solid phase peptide synthesis and MALDI MS.

2) **2002-2005** Summer research student at the Ottawa Health Research Institute. Molecular biology laboratory of Dr Majambu Mbikay. During this series of summer student positions I developed and honed many biochemistry and molecular biology techniques including mammalian cell culture, and immunology techniques

3) **2005-2006** Honours Research student University of Ottawa under the supervision of Dr Majambu Mbikay. Here I developed my skills in immunohistochemistry and cytochemistry and prepared poster and seminar presentations of my work.

4) **2005-current** Junior Research Technician for Dr John Woulfe at the Ottawa Hospital. Here I was charged with the responsibility of maintaining the histology laboratory as well as carrying out routing histological staining of neuronal and endocrine tissues.

5) **2006-current** Masters in Science Candidate at the University of Ottawa under the joint supervision of Drs. John woulfe and Michel Chrétien. In this thesis based project I combined my previous skills with extensive training in animal based experiments.