NESFATIN-1 AND NESFATIN-1-LIKE PEPTIDE: NUCLEOBINDIN-1/2-ENCODED INSULINOTROPIC AND ENTEROTROPIC PEPTIDES

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ABSTRACT

Nucleobindins are a class of secreted, multi-domain Ca^{2+} binding proteins that interact with nucleic acids. Two nucleobindins, nucleobindin-1 (NUCB1) and nucleobindin-2 (NUCB2) have been identified so far. In 2006, nesfatin-1, an 82 amino acid peptide encoded in *NUCB2* was discovered. Nesfatin-1 is an anorexigenic and insulinotropic peptide found abundantly in hypothalamus, pancreas and stomach. Meal responsive insulin secretion is regulated by glucagon like peptide-1 (GLP-1), glucose dependent insulinotropic polypeptide (GIP), peptide YY (PYY) and cholecystokinin (CCK) secreted by intestinal mucosal cells. Since both nesfatin-1 and intestinal hormones modulate insulin secretion, nesfatin-1 could regulate intestinal hormones to elicit its insulinotropic action. Nucleobindin-1 primarily regulates Ca^{2+} homeostasis. Like NUCB2, NUCB1 is also present in the pancreas, stomach, intestine and pituitary. NUCB2 has a high similarity (62% in humans) to NUCB1. Both proteins also retain their prohormone convertase cleavage sites. However, no information exists on whether *NUCB1* encodes bioactive peptides. The fact that NUCB1 is a secreted protein suggests an endocrine function for NUCB1 and/or its encoded peptide. This research hypothesizes that nesfatin-1 is enterotropic, and NUCB1 encodes an insulinotropic nesfatin-1-like peptide (NLP). Nesfatin-1 protein expression was found in STC-1 cells and it co-localized GLP-1, GIP, CCK and PYY in mouse enteroendocrine cells. Treatment of STC-1 cells with nesfatin-1 stimulated GLP-1, GIP, CCK mRNA expression and protein secretion, while opposite effects were found for PYY. *In silico* analysis of the NUCB1 amino acid sequence found a 77 amino acid NLP. Mouse pancreatic islets and MIN6 cells express *NUCB1* mRNA and protein. NUCB1 was co-localized with insulin in mouse pancreatic islets. While treatment of cells with synthetic NLP increased preproinsulin mRNA expression and secretion, a scrambled peptide based on NLP was ineffective, indicating that the specific amino acid sequence is crucial for its insulinotropic action. Overall, the data presented supports the hypotheses. The studies reaffirm NUCB2 expression in intestine and provide the first set of evidence for nesfatin-1 regulation of enteric hormones. It also found a novel NUCB1 encoded insulinotropic NLP that could elicit other functions of nesfatin-1.

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A

ATP Adenosine Triphosphate

ARC Arcuate Nucleus

AgRP Agouti-related Peptide

ATF6 Activating Transcription Factor 6

C

CAPN10 Calpain-10

CCK Cholecystokinin

CNS Central nervous system

CDKAL1 CDK5 regulatory subunit associated protein 1-like 1

CDKN2A Cyclin-Dependent Kinase Inhibitor 2A

CDKN2B Cyclin-Dependent Kinase Inhibitor 2B

CART Cocaine Amphetamine Related Transcript

CRH Corticotropin Releasing Hormone

CRF Corticotropin Releasing Factor

CSF Cerebrospinal Fluid

D

DMN Dorsomedial Nucleus of Hypothalamus

DPP-4 Dipeptidyl Peptidase - 4

DMV Dorsal Motor nucleus of the Vagus

DPBS Dulbecco's Phosphate Buffered Saline

DMEM Dulbecco's Modified Eagles Medium

DAPI 4', 6-Diamidino-2-Phenylindole

E

ENS Enteric Nervous System

ER Endoplasmic Reticulum

EF Elongation Factor

ELISA Enzyme Linked Immunosorbent Assay

F

FFA Free Fatty Acid

FTO Fat mass and Obesity associated protein

FBS Fetal Bovine Serum

G

GLP-1 Glucagon-Like peptide-1

GLP-1R Glucagon-Like Peptide-1 Receptor

GIP Glucose-dependent Insulinotropic Polypeptide

GLUT4 Glucose Transporter 4

GSK3β Glycogen Synthase Kinase 3 β

GWAS Genome Wide Association Studies

GPCR G Protein Coupled Receptors

GIPR Glucose-dependent Insulinotropic Peptide Receptor

GSIS Glucose Stimulated Insulin Secretion

GI Gastrointestinal

GT Glucose Transporter

G3P Glyceraldehyde-3-Phosphate

G6P Glucose-6-phosphatase

H

HHEX Hematopoietically-expressed Homeobox protein

HeLa Henrietta lacks

hrNUC Human Recombinant Nucleobindin

hrCOX-2 Human Recombinant Cyclooxygenase 2

I

IGF2 Insulin-like Growth Factor 2

IRS-1 Insulin Receptor Substrate 1

IRS-2 Insulin Receptor Substrate 2

IGF2BP2 Insulin-like Growth Factor 2 mRNA Binding Protein 2

IR Immunoreactivity

I.C.V Intracerebroventricular

I.P Intraperitoneal

IgG Immunoglobulin G

K

KCNJ11 Potassium inwardly-rectifying channel, subfamily J, member 11

KCNQ Potassium inwardly-rectifying channel, subfamily Q

L

LHA Lateral Hypothalamic Area

LC-MS Liquid Chromatography – Mass Spectroscopy

M

MC4R Melanocortin 4 Receptor

MCH Melanin Concentrating Hormone

MHC Major Histocompatibility Complex

N

NUCB2 Nucleobindin-2 gene

NUCB1 Nucleobindin-1

NLP Nesfatin-1-Like Peptide

NTS Nucleus Tractus Solitarius

NPY Neuropeptide Y

NOD Nodose Ganglion

NEFA Nuclear EF-hand acidic

NRK Normal Rat Kidney Epithelial Cells

P

PEPCK1 Phosphoenolpyruvate carboxykinase 1

PIP_3 Phosphatidylinositol triphosphate

PI3K Phosphatidylinositol 3-kinase

PKA Protein Kinase A

PKB Protein Kinase B

PDK1 Pleckstrin Dependent Kinase 1

PPARG Peroxisome Proliferator-Activated Receptor Gamma

PC Prohormone Convertases

PVN Paraventricular Nucleus

PP Pancreatic Polypeptide

POMC Proopiomelanocortin

R

RBP-4 Retinol Binding Protein 4

RIA Radioimmunoassay

S

SLC30A8 Solute Carrier Family 30, member 8

SON Supraoptic Nucleus

SRP Signal Recognition Particles

S1P Site 1 Protease

SV Simian Virus

T

T1D Type 1 Diabetes

T2D Type 2 Diabetes

TNFα Tumor Necrosis Factor Alpha

TCF7L2 Transcription factor 7-like 2

TRH Thyrotropin Releasing Hormone

3T3 Cells 3-day transfer, inoculum 3 x 10^5 cells

CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION

The prevalence of metabolic diseases is reaching pandemic proportions worldwide, of which obesity and diabetes are the primary diseases of concern. In 2010, more than 285 million people worldwide had diabetes [1]. This estimate is 67% higher than those predicted in 2000 and with this trend, 439 million people worldwide will have diabetes by 2030. Since 2010, efforts are underway to combat the costs resulting from these debilitating metabolic complications. In the majority of cases of diabetes and obesity, the defects lie in glucose metabolism and energy homeostasis. Several lines of research are in progress, including the use of naturally occurring hormones to treat diabetes and obesity [2-4]. In this regard, the role of intestinal hormones in regulating glucose and energy homeostasis is well reported. Some examples of such hormones include the incretins glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), peptide YY (PYY), leptin, orexin, cholecystokinin (CCK), amylin and oxyntomodulin. All these hormones modulate insulin, which is secreted in a meal responsive manner [5-8]. In addition to these peptides, a recent addition to the growing list of pancreatic islet beta cell endocrine modulators is nesfatin-1 [9].

Nesfatin-1 (**N**EFA/nucleobindin-2 **E**ncoded **S**atiety and **FAT** Influencing prote**IN-1**) is a recently identified 82 amino acid anorexigenic (food intake inhibitory) peptide that is derived from the post-translational cleavage of its secreted precursor peptide nucleobindin-2 (NUCB2) at its N-terminal by prohormone convertases [9]. Plasma nesfatin-1 concentrations are inversely correlated with glucose levels in rats and diabetic (T1D and T2D) humans [10, 11]. Our research group for the first time showed that nesfatin-1 and insulin are co-localized in the β cells [12] of pancreatic islets and its stimulatory role in insulin secretion [5, 13], and glucose homeostasis [13]. Since its discovery in 2006, nesfatin-1 has emerged as a multifunctional peptide having a variety of tissue-specific functions. Besides hypothalamic feeding centers and endocrine pancreas, nesfatin-1 was reported in rat gastric oxyntic mucosa [14] and was found to be

co-secreted from distinct cytoplasmic vesicles in ghrelin producing X/A like cells. Our research recently determined that NUCB2 and nesfatin-1 are expressed in the small and large intestines of mice [15]. Previous research has shown that intestinal hormones GLP-1, PYY, GIP and CCK modulate insulin secretion [6-8, 16]. Nesfatin-1, similar to these intestinal hormones, has a crucial role in modulating insulin secretion [5, 12, 13]. In addition to its insulinotropic actions, does nesfatin-1 also modulate intestinal hormones that regulate insulin release? The main focus of Chapter 2 of this thesis is to address this question. Maintenance of insulin secretion is crucial *in vivo* as insulin stimulates glucose uptake by cells, thereby regulating glucose homeostasis. Insulinotropic peptides i.e. peptides that increase insulin secretion are therefore much in focus for their therapeutic use in diabetes treatment. Nesfatin-1, as an insulinotropic peptide, is also pursued as a therapeutic target for diabetes.

The third chapter of this thesis aims to investigate whether NUCB1 encodes a nesfatin-1-like peptide (NLP) and whether NLP has insulinotropic action similar to nesfatin-1. This is crucial, as no information exists regarding NUCB1 encoded insulinotropic peptide, although *NUCB1* was identified more than a decade earlier to nesfatin-1. Is NUCB1 also a precursor of an insulinotropic peptide? NUCB1 is a 55 KDa multi-domain protein first discovered in cultured supernatant of a B lymphocyte cell line identified from mice prone to systemic lupus erythematosus, an autoimmune disorder [17]. It was identified as a golgi resident protein [18], secreted protein [19] and a nuclear protein [20]. Previous studies have shown that the secreted extracellular NUCB1 contributes to matrix maturation in bones [21]. NUCB1 plays a crucial role in Ca^{2+} homeostasis [22] and was reported to interact with G proteins [22] and cyclooxygenases [23]. These studies indicate the important role of *NUCB1* in multiple cellular processes. The nucleobindin genes (both *NUCB1* and *NUCB2*) will be addressed in uppercase italicized letters, to distinguish from NUCB1 and NUCB2 proteins (uppercase non-italicized), in rest of the thesis. Given the cytoplasmic location of NUCB1 and that it is secreted is indicative that it or its encoded peptides could have endocrine functions.

1.2. LITERATURE REVIEW

1.2.1. Energy Metabolism and Type 2 Diabetes Mellitus

Humans obtain energy from three classes of fuels: carbohydrates, lipids and proteins. Energy metabolism is the process of generating adenosine triphosphate (ATP) from these nutrients. It is comprised of linked pathways that function either in the presence or absence of oxygen. Typical aerobic metabolism will convert one molecule of glucose to 30-32 ATP molecules [24]. The three classes of energy rich fuels are acquired by food ingestion. Based on the nature of the ingested food, the percentage of energy fuels in them varies. The digestion of food in the alimentary tract and its subsequent absorption in blood stream makes it possible for cells and tissues to transform the chemical energy generated into useful work. Energy intake in the form of food, and energy expenditure in the form of cellular energy metabolism and exercise are precisely coupled over longer intervals. In healthy adults, this tight balance between intake and expenditure results in energy homeostasis and stable body fat reserves [25].

Amongst carbohydrate fuels, glucose is a major energy source for cells in the body. Glucose in cells is crucial for its proliferation, growth and survival. Therefore, maintenance of glucose levels in blood is an essential physiological process [26]. Insulin is a major peptide hormone secreted by β cells in the pancreas to regulate blood glucose levels. An elevation in blood glucose level during feeding stimulates insulin release, resulting in the uptake of glucose into skeletal muscles and adipose tissues. Diabetes or diabetes mellitus is a complex metabolic disease characterized by several metabolic abnormalities, especially elevated blood glucose levels over an extended period. The two major types of diabetes are type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D primarily arises from the lack of insulin secretion from pancreatic islets. T2D is due to the loss of insulin sensitivity in glucose responsive cells, resulting in inhibition of glucose uptake in response to insulin [27]. The disruption of energy metabolism in T2D is well reported in literature with significant research focusing on the mechanism of insulin action and its receptor kinetics.

The onset of elevated blood glucose levels or hyperglycemia arises from three key defects: unregulated hepatic glucose production, diminished insulin secretion and impaired insulin action [28]. Insulin resistance is defined as the delayed response of cells to insulin. Human insulin receptor contains an two extracellular α subunit and two transmembrane β subunits [29]. The α subunits or the ligand-binding domain controls the activity of β subunits [30]. The gene coding for insulin receptor in mammals has 22 exons that generates two isoforms resulting from the alternative splicing of exon 11. The isoform-a called insulin receptor a (IRa) retains exon 11, whereas b-isoform (IRb) omits exon 11. In typical insulin responsive tissues including liver, adipose tissues and skeletal muscles the IRb expression is higher as it binds strongly to insulin. In contrast, the IRa binds with equal affinity to both insulin and insulin-like growth factor 2 (IGF2) and is expressed in the majority of fetal tissues and central nervous system (CNS) [31]. The insulin receptor resides in the plasma membrane and is activated by binding of insulin (ligand). Ligand binding increases the flexibility of the activation loop, which allows ATP to bind and autophosphorylate, thereby stabilizing the activation loop [32].

In humans and other mammals, activated insulin receptor phosphorylates the tyrosine residues in intracellular insulin receptor substrates (IRS-1, IRS-2). Previous studies on transgenic mice suggests that the majority of insulin action is mediated through either IRS-1 or -2. Activation of the insulin receptor leads to the production of phosphatidylinositol triphosphate (PIP_3) by phosphatidylinositol 3-kinase (PI3K). PIP_3 then recruits the phosphatidylinositol-dependent protein kinase 1 (PDK1) and protein kinase B (AKT) to the plasma membrane where AKT is activated by phosphorylation. AKT then phosphorylates a variety of proteins including glycogen synthase kinase 3β (GSK3β) in liver and AS160 protein which is involved in the translocation of the glucose transporter (GLUT4) [33]. The necessity of the IRS-PI3K-AKT-AS160 axis in insulin stimulated glucose uptake is well documented [34]. Depending on the tissue site, dysregulation of any of these signaling molecules, disrupts insulin signaling cascade resulting in sub-optimal or lack of insulin responsiveness in cells. This progresses to hyperinsulinemia, glucose intolerance and insulin resistance seen in T2D.

The pathophysiology of T2D is highly tissue specific. The skeletal muscles that contribute to 75% of glucose uptake is highly sensitive to blood glucose and plays a major

role in glucose homeostasis in patients with T2D [34]. As explained above IRS-1 and IRS-2 are crucial in insulin responsive tissues like skeletal muscles. Studies have shown that IRS2 knockout mice progressively develop T2D with insulin resistance in skeletal muscles [35]. T2D subjects have impaired insulin stimulated tyrosine phosphorylation of IRS-1 in skeletal muscles. However, this was not due to a decreased protein expression of IRS-1. A similar impairment was observed at the level of PI3K in skeletal muscles of T2D subjects [34]. The dysregulation of the insulin receptor constitutes a common feature of insulin resistance in skeletal muscles of T2D patients. High levels of free fatty acids were known to contribute to insulin resistance by reducing the insulin stimulated glucose uptake via accumulation of lipids inside muscle cells [36]. T2D was also associated with impaired switching from fatty acid oxidation to glucose oxidation in response to insulin [37]. However, the cause of these derangements in skeletal muscles of T2D patients remains to be elucidated.

In adipose tissues, the *GLUT4* expression is downregulated in patients with T2D [38]. Owing to this, hyperglycemia associated with decreased glucose uptake cannot be explained in adipose tissues. The adipocyte-selective knockout of *GLUT4* in mice resulted in insulin resistance similar to muscle selective knockout of *GLUT4* [39]. Adipocyte GLUT4 deficiency results in the generation of circulating factors that are responsible for organ communication [40]. Of particular interest recently is the retinol-binding protein-4 (RBP-4). Insulin resistant mice, obese and diabetic humans had increased RBP-4 protein levels. Also overexpression of *RBP-4* gene or injection of RBP-4 recombinant protein caused insulin resistance in mice. Conversely *RBP-4* knockout mice had enhanced insulin sensitivity [41]. Adipocytes secrete a wide array of factors that may alter insulin action in adipose tissue e.g. adiponectin, resistin, leptin and TNFα [42].

T2D has been reported to have direct effects on the pancreatic β cells, contributing to apoptosis and reduction in mass and ability to compensate for insulin resistance [43]. A wide variety of mechanisms for these effects of T2D on pancreatic islets have been proposed in literature which includes endoplasmic reticulum stress [44], chronic hyperglycemia [45], chronic hyperlipidemia [46], oxidative stress [47] and inflammatory cytokines [48]. Reduced insulin sensitivity of β cells has been attributed to the early failure of insulin secretion in T2D pathogenesis [49]. However, many questions remain open on

the effect of T2D on β cells. What is the mechanism of β cell compensation in hyperinsulinemia? At which point in T2D pathogenesis this compensation fails? Nonetheless, the consensus in the literature is that the main adverse effects of T2D on β cells are its loss of insulin sensitivity and insulin secretion later in the disease as β cell mass decreases.

An organ with the ability to absorb, store and produce glucose is liver. It maintains glucose and lipid homeostasis in the body. Owing to its role in maintaining glucose levels, it is a key target for insulin and its catabolic counterpart glucagon. Increased blood glucose causes release of insulin from β cells and this effect is amplified by the presence of FFA. Lack of insulin sensitivity and disrupted insulin action in liver has a significant contribution towards hyperglycemia and dyslipidemia [50]. Similar to skeletal muscles IRS-1 and -2 are complementary key players in liver, regulating insulin signaling, expression of genes in gluconeogenesis, glycogen synthesis and lipid metabolism [50]. Dysfunctional *IRS* in liver leads to postprandial hyperglycemia, increased hepatic glucose production both of which have a major contribution in the development of insulin resistance in T2D [51-53]. Liver specific *IRS-2* knockout mice have T2D, increased adiposity, female infertility, insulin resistance in liver and skeletal muscles and lack of β cell compensation for peripheral insulin resistance [35, 54].

Above all these tissue specific contributions to T2D pathophysiology, it has a very strong genetic component to it. The lifetime risk of T2D is about 7% in people with a healthy medical history. However, the chances of T2D are as high as 40 and 70% for offspring of a single or double parent T2D respectively [55]. Twenty common genetic variants of T2D have been identified recently using linkage analysis, candidate gene approaches and by genome wide association studies (GWAS) [56]. The genetic mapping results showed that insulin sensitivity is related to eight candidate genes TCF7L2, KCNJ11, HHEX, SLC30A8, CDKAL1, CDKN2A/2B, IGF2BP2, and KCNQ; PPARG, glucose transport to CAPN10 gene and melanocortin receptor gene MC4R and FTO to obesity [56]. This explains the complex nature of T2D, due to multi-faceted genetic background and varied gene-environment interactions. These lead to defective insulin-mediated glucose uptake, β cell dysfunction, dysregulation of adipocyte and liver. An overall state of dysregulated metabolism over time also causes extensive T2D related

complications in nearly all tissues and organs. Recent advances have led to many interesting animal models for studying T2D. This includes the *ob/ob* (spontaneous mutation in ob (obese) Lep (leptin) gene) [57] and *db/db* mouse (point mutation in db (diabetes) LEP-R (leptin receptor) gene) [58], Zucker fatty rats, Zucker diabetic fatty rat and obese rhesus monkey. Current research has focused on exploring the mechanism of pathogenesis of T2D using these animal models [59, 60].

1.2.2. Several Hormones Regulate Appetite and Energy Metabolism

As discussed in the previous section, energy homeostasis refers to two distinct processes *in vivo*: 1) the maintenance and regulation of readily available supply of energy metabolites in circulation; and 2) the regulation of adipose tissue mass that serves as a major energy store. Both these aspects are linked to eating, which eventually results in the entry of sufficient energy metabolites from the intestine into circulation. Meal size, to a large extent maintains the above two processes of energy homeostasis. Previous studies have shown that the total amount eaten correlates with the average meal size but not meal frequency [61]. Diluting the energy content of diet results in an immediate increase in meal size [62]. Diabetic rats that cannot metabolize glucose normally increase meal size rather than frequency [63]. The effect of meal size on two distinct parameters of energy homeostasis arises from two different classes of feedback signals. The first, described by Smith *et al*., [64] consists of negative feedback signals that are generated from the GI tract in response to nutrient stimuli. The primary effect of these signals is to produce satiation. Circulating energy metabolites and hormones that arise from organs such as liver, pancreas and adipose tissue and from nutrient absorption are the second class of feedback signals that act on “metabolic sensing neurons” in the CNS [65]. The current understanding is that the gastrointestinal nutrient signals and metabolic sensing neurons converge in the caudal brainstem that also regulates motor control of satiation. A variety of hormones have direct and/or indirect effects on feed intake and cellular energy metabolism. Leptin, the satiety hormone secreted from adipose cells, adiponectin secreted from adipose tissues that regulates glucose levels and fatty acid breakdown, amylin co-secreted with insulin from β cells that slows gastric emptying and promotes

satiety and oxyntomodulin secreted from the oxyntic cells in colon that suppresses appetite are few examples of such hormones.

The GI tract over the years has evolved as a major endocrine organ in the body. Specialized enteroendocrine cells present here secrete a variety of peptides that act as short and long-term peripheral signals for regulation of feed intake and energy balance [66]. Upon release from the GI tract in response to an ingested meal, the enteric hormones modulate the brain orexigenic and/or anorexigenic neuropeptides either directly or through nervous signaling from periphery to brain, thereby regulating feed intake [67]. Since this thesis research focuses on four major peptides i.e. GLP-1, GIP, CCK and PYY primarily produced from the GI tract, literature review will be limited to these four intestinal hormones [68-70].

During early 1900s, the concept of certain factors secreted from the intestinal mucosa in response to ingested nutrients that are capable of releasing substances from endocrine pancreas leading to a reduction in blood glucose levels was introduced [71]. The term “incretins” was then used to denote these glucose lowering factors derived from intestine [72]. With the advent of radioimmunoassay (RIA), it was shown that oral glucose administration is associated with a much greater increase in plasma insulin when compared to the same amount given intravenously [73]. This result further solidified the concept that intestinal insulinotropic factors exist. The phenomenon of intestinal factors enhancing insulin secretion is called the *“incretin effect”*, and it contributes to approximately 70% of insulin secretion in response to oral glucose.

The first described incretin hormone was isolated from the crude extracts of porcine small intestine and had the ability to inhibit gastric acid secretion in dogs. It was hence named gastric inhibitory polypeptide (GIP) [74]. However, studies using more purified extracts showed that GIP stimulates insulin secretion in animals and humans [75] even at a physiological dose, it was renamed as glucose dependent insulinotropic polypeptide (GIP). GIP is a 42 amino acid peptide secreted by the K-cells in the upper small intestine in response to carbohydrates and lipids. However, it was found that immunoneutralization of endogenous GIP activity attenuated but did not abolish the incretin effect in rodents and surgical resection of ileum in humans was associated with

diminished incretin activity, despite normal plasma GIP levels [76]. This observation led to the discovery of second incretin hormone, glucagon-like peptide-1 (GLP-1) after cloning and sequencing of the mammalian proglucagon cDNAs. Since, the proglucagon gene in addition to glucagon also encoded two other peptides that were 50% homologous to glucagon they were named glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2). Of these, GLP-1 alone was insulinotropic. GLP-1 is a highly tissue-specific post-translational product of the proglucagon gene, secreted and released from the intestinal L cells in response to nutrients and glucose-stimulated insulin secretion (GSIS) [77]. GLP-1 and GIP are the only known incretin hormones and contribute in an additive manner towards the incretin effect.

The long arm of chromosome 2 encodes the proglucagon gene which has 6 exons and 5 introns, with the entire coding sequence of GLP-1 present within exon-4 [78]. The GLP-1 gene is predominantly expressed in pancreatic α cells, the L-cells of the intestine and neurons in the caudal brainstem and hypothalamus. The mRNA generated by the transcription of this gene is structurally identical in all of the above-mentioned three cells [77, 79]. There are similarities in proglucagon gene expression in α cells and the intestine. Similar to α cells, intracellular cAMP and activation of cAMP/PKA pathway is critical for intestinal proglucagon gene expression [80]. Increase in cAMP levels also upregulates proglucagon gene transcription by activation of PKA. Nutrient ingestion is a primary regulator of proglucagon expression in intestine [81]. Fasting decreases, whereas refeeding increases proglucagon expression in rat intestine [82]. Meals containing high levels of fibers and short chain fatty acids also increase its expression [83, 84]. Gastrin-releasing peptide and GIP are also known to increases proglucagon mRNA expression in intestine. Overexpression of transcription factor Pax-6 via adenovirus in primary cells cultures of intestine, enhances the expression of proglucagon gene expression [85]. An opposite effect is seen in mice that express the dominant negative form of Pax-6, showing a reduction in proglucagon expression [86]. These studies show the importance of Pax-6 in proglucagon expression in both pancreas and intestine. Transfection of rodent islets with human proglucagon promoter-reporter plasmids shows that the sequence within the first 6 kb of human proglucagon gene 5'-flanking region is required for its pancreas-specific expression [87]. Subsequent cell transfection studies revealed a conserved

region within intron 1 named “ECR3” that is critical for its expression in pancreatic islet α cells and intestine. These studies reveal that the human proglucagon gene uses a distinct set of transcription factors to specify tissue-specific proglucagon gene transcription. The proglucagon mRNA is translated into a single protein (180 amino acids) which then undergoes tissue-specific posttranslational processing yielding specific peptides in α cells, intestine and brain (**Figure 1.1**). Of several prohormone convertases (PC), PC 1/3 and 2 alone were shown to be important in processing proglucagon [88].

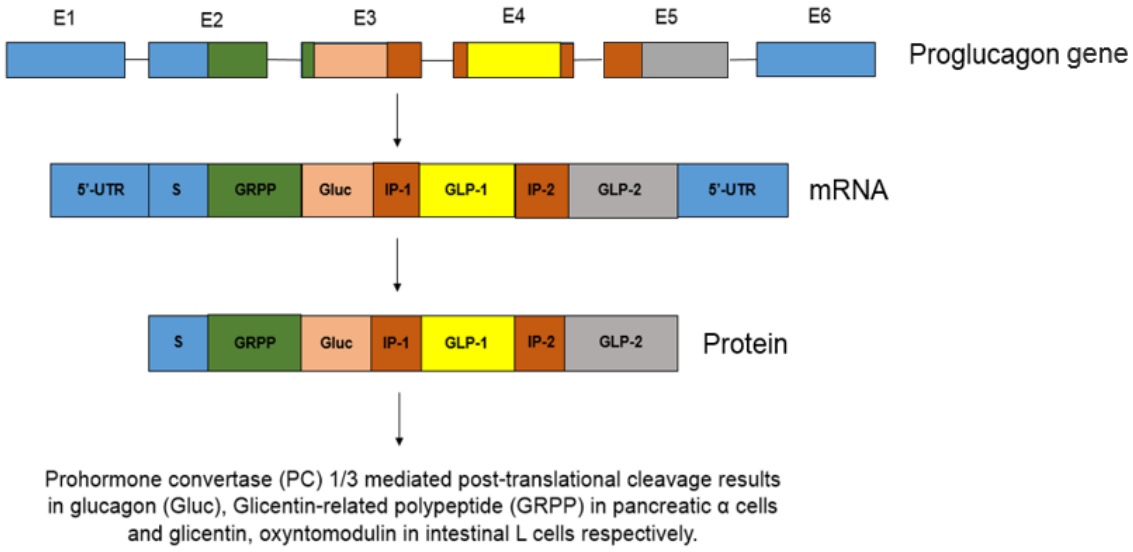

Figure 1.1. A depiction of the proglucagon gene, transcribed proglucagon mRNA and the translated protein. The protein is then post-translationally processed to glucagon (Gluc), glicentin-related polypeptide (GRPP), intervening peptide-1 (IP-1) and major proglucagon fragment (MPGF) in pancreas. In intestinal L cells and central nervous system the protein is processed into glicentin, oxyntomodulin (OXM), intervening peptide-2 (IP-2), GLP-1 and GLP-2.

GLP-1 is synthesized within and secreted by the L cells in the distal ileum and colon. The L cells are open type intestinal epithelial enteroendocrine cells. That is, they are in direct contact with luminal nutrients on the apical side and neural and vascular tissues through their basolateral side. Meal rich in fats and carbohydrates are primary physiologic stimuli for GLP-1 secretion in rodents [89]. GLP-1 secretion occurs in a

biphasic pattern with an early 10-15 minute phase and a longer 30-60 minute second phase in rats [90]. Multiple forms of GLP-1 in secretion includes GLP-1(1-37) and GLP-1(1-36)NH_2 which are inactive, GLP-1(7-37) and GLP-1(7-36)NH_2 that are bioactive. In humans majority of GLP-1 in circulation is GLP-1(7-36) NH_2 [91]. The half-life of bioactive GLP-1 in circulation is less than 2 minutes as it is rapidly inactivated by proteolytic enzyme dipeptidyl peptidase-4 (DPP-4) that cleaves GLP-1 at the penultimate alanine residue [92]. The central and peripheral administration of GLP-1 strongly stimulates insulin release. Intracerebroventricular administration of GLP-1 reduces food intake in rodents and its peripheral administration inhibits appetite in humans [93].

The GLP-1 receptor (GLP-1R) is a 7-transmembrane-spanning heterotrimeric G-protein coupled receptor (GPCR) which acts as receptor for glucagon, GLP-2 and GIP [94]. In rodents and humans, a single GLP-1R that is structurally identical has been characterized in a wide variety of tissues ranging from pancreatic islets (α, β and δ cells), liver, heart, kidney, stomach, intestine, pituitary, skin, brainstem, nodose ganglion neurons and vagus nerves. In humans secretion of GLP-1 throughout the day strongly correlates with the release of insulin. The effect of GLP-1 on insulin secretion is strictly glucose-dependent and no effects on insulin secretion for glucose concentration below a certain threshold level (4.5 mmol/L) was observed. GLP-1 has also been shown to stimulate insulin gene transcription and all steps of insulin biosynthesis in isolated β-cells [8]. It stimulates β-cell proliferation and neogenesis from rat and human pancreatic duct [95]. GLP-1 inhibits glucagon secretion (glucagonostatic) in pancreatic islets. Administration of GLP-1 in T1D patients inhibited glucagon secretion and decreased blood glucose level suggesting that it suppresses the hepatic production of glucose induced by glucagon [96]. The inhibitory effects of GLP-1 are also extended to the GI tract wherein it slows down gastric emptying and glucose absorption [97]. This inhibitory role is crucial in pathological conditions like diabetes because they potentially reduce postprandial glucose excursions. The secretion of GLP-1 is impaired in patients with T2D. However, the insulinotropic response of GLP-1 and its glucagonostatic activity is preserved as in normal subjects [98]. A study involving a lizard, the gila monster (*Heloderma suspectum),* resulted in the isolation of exendin-4, a peptide that is a potent DPP-IV degradation-resistant agonist of the mammalian GLP-1 receptor [99]. This

analogue of GLP-1, under the trade name Byetta™, is currently commercially available in the United States as a therapeutic agent for T2D [100].

In rat hepatocytes and skeletal muscles, GLP-1 increases glucose incorporation and enhances insulin-stimulated glucose metabolism in 3T3 L1 adipocytes and primary rat adipocytes. GLP-1 stimulates cAMP production in isolated rat primary hepatocytes and exendin-4 improves insulin sensitivity and aids in reversal of hepatic steatosis in *ob/ob* mice [101]. GLP-1 and exendin-4, increases glycogen synthase activity in rat soleus muscles and human skeletal muscles. In addition, GLP-1 also has lipolytic effects in rat adipocytes and has both lipolytic and lipogenic actions in human adipocytes [102, 103]. Intravenous administration of GLP-1R agonist increases systolic, diastolic, mean arterial pressure and heart rate in rodents. GLP-1 also exhibits cardioprotective effects in models of cardiac injury and failure [104]. In humans, a 72 hour GLP-1 perfusion in angioplasty and myocardial infarction patients improved their left ventricular function [105]. Despite all these tissue-specific biological actions of GLP-1, an ongoing question is whether these are solely due to its endocrine action as a circulating hormone or whether recruitment of CNS GLP-1R mediates some of peripheral actions of endogenous GLP-1? Also whether native GLP-1 exerts its metabolic actions (weight loss and decreased feed intake) by communicating with brain or by ascending neural pathways or in part by direct access to brain GLP-1R remains unclear. Recent trials have shown remarkable results using high molecular weight GLP-1R agonists. Studies using 4 structurally unique GLP-1R analogues CJC113, CJC114, albiglutide and CNTO736 showed successful entry into brain showing their full range of actions, despite their large size. This includes, activation of neuronal c-Fos expression, inhibition of gastric emptying, reduction of food intake and weight loss with chronic administration [106-109].

The GIP gene in humans consists of 6 exons with the majority of GIP encoding sequences found in exon 3, being localized to the long arm of chromosome 17. GIP is expressed predominantly in stomach and intestinal K cells in rodents and humans. There is little information about the regulation of GIP gene expression. Recent studies stress the importance of the transcription factor *Pdx-1* and that it could mediate the cell-specific GIP gene expression. *Pdx-1* was also detected in the nucleus of the mouse K cells secreting GIP, and the number of intestinal GIP secreting cells is drastically reduced in

Pdx-1 -/- mice [110]. Chromatin immunoprecipitation in the same study also revealed Pdx-1 binding to GIP promoter region and its overexpression in transfection assays increased the activity of GIP promoter/reporter gene constructs [110]. GIP is derived from a larger ProGIP prohormone precursor that encodes N-terminal peptide, GIP and C-terminal peptide (**Figure 1.2**). PC knockout mice study and overexpression of PC enzymes *in vitro* showed that mature 42 amino acid GIP is released from 153 amino acid ProGIP precursor via PC 1/3 posttranslational cleavage [111].

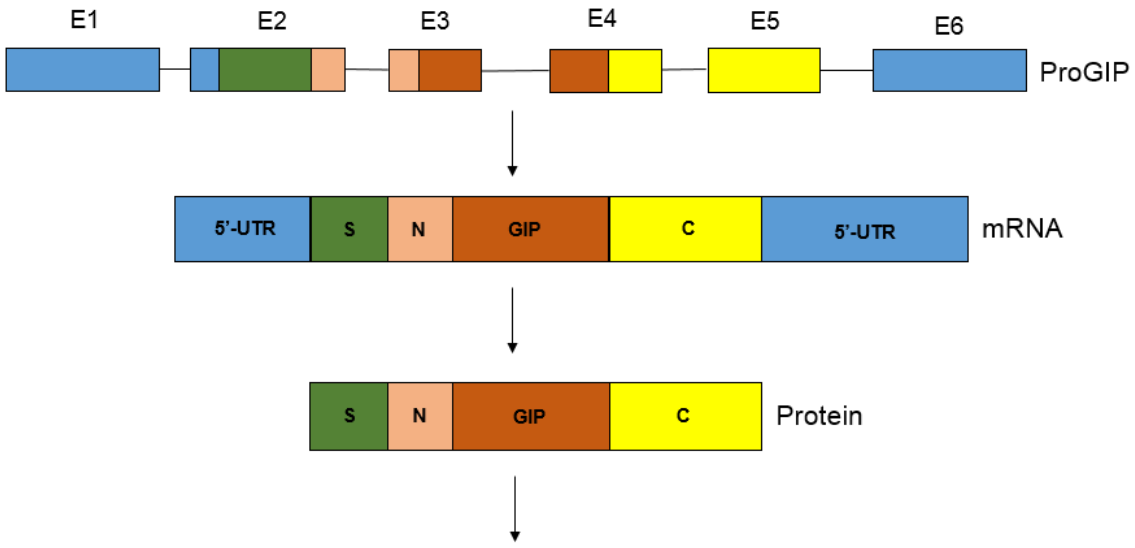

Figure 1.2. Processing of mature GIP from the ProGIP gene. The precursor protein is post-translationally cleaved by PC 1/3 at a single arginine residue flanking the GIP mature peptide region.

GIP is synthesized and released from K cells in the duodenum and proximal jejunum [112]. Rather than the presence of nutrients, it is the rate of nutrient absorption that primarily stimulates GIP secretion. Thus, GIP secretion is reduced in patients with intestinal malabsorption or after administration of exogenous agents that reduce nutrient absorption [113]. There is a species-specific difference to the nutritional regulation of GIP secretion, as fat stimulates it in humans and carbohydrates are most effective in stimulating them in rodents and pigs. The half-life of intact bioactive GIP is 2 minutes in rodents [92] and 7 to 5 minutes in healthy human subjects and T2D patients [114]. Similar

to GLP-1, since GIP has an alanine residue in position 2, it is a target for DPP-4 mediated inactivation, which cleaves it into an active GIP (1-42) and GIP (3-42) that are inactive. However, intravenous infusion of GIP in humans found 40% bioactive GIP remains intact when compared to 20% active GLP-1. This shows that it is less susceptible to DPP-4 cleavage *in vivo* than GLP-1 [92].

GIP receptor (GIPR) is a 7-transmembrane spanning heterotrimeric GPCR similar to GLP-1R [115]. The human GIP receptor (GIPR) contains 14 exons and is localized in chromosome 19. GIPR is expressed in pancreas, small intestine, stomach, adipose tissue, adrenal cortex, heart, pituitary, testis, bone, spleen, thymus, lung, kidney and in several regions of the CNS. Relatively little is known on the factors regulating GIPR expression. However, GIPR mRNA and protein levels are reduced in the islets of diabetic rats, consistent with defective GIP action in diabetic animals and humans [116]. GIP exerts glucose-dependent stimulatory effect on insulin secretion in isolated perfused rat pancreas and humans [6]. GIP also upregulates insulin gene transcription and biosynthesis in β cells [117]. Elimination of GIPR signaling via *GIPR* knockout mice results in impaired oral glucose tolerance and a defective glucose stimulated insulin secretion [118]. Infusion of exogenous GIP into diabetic rats for 2 weeks reduces β cell apoptosis, by decreasing the expression of the pro-apoptotic *bax* gene [119]. GIP also reduces the expression of biochemical markers responsible for endoplasmic reticulum (ER) stress in cells of the islets, after inducing ER stress *in vitro* [120].

GIP in the CNS has been shown to play a crucial role in neural progenitor cell proliferation and behavior modification, as transgenic mice that overexpress *GIPR* have enhanced sensorimotor co-ordination and increased memory compared to wild types [121]. Besides cerebral cortex, hippocampus and olfactory bulb in the CNS, GIPR is also expressed in isolated rat adipocytes and 3T3 L1 cells [122]. GIP has been reported to have lipogenic and lipolytic actions *in vivo*. It stimulates fatty acid synthesis and re-esterification, increases insulin-stimulated incorporation of fatty acids into triglycerides and upregulates lipoprotein lipase synthesis [123]. On the other hand, *GIPR -/-* mice are resistant to diet induced obesity and have less adipocyte mass even after high fat diet feeding [124]. Leptin deficient *ob/ob* mice with *GIPR* knockout gain less weight and have improved glucose tolerance, insulin sensitivity and expend more energy than wild type

mice thereby preventing fat accumulation in adipocytes [124]. GIP increases bone mineral density in a rodent model of post-menopausal osteoporosis [125]. Subsequently *GIPR* knockout mice have reduced bone mass and size, altered bone turnover and abnormal bone microarchitecture. On the contrary, mice over-expressing GIP have greater bone mass than wild-type controls [126]. In stomach, GIP increases gastric acid secretion and upregulates specifically intestinal hexose transport [127]. It also attenuates glucagon stimulated hepatic glucose production, although the presence of GIPR in liver was not convincingly shown. The abnormal expression of *GIPR* in adrenocortical adenomas could contribute to the development of food-dependent Cushing's syndrome [128]. The GIPR is also present in endothelial cells and increases intracellular Ca^{2+} in these cells [129]. Depending on the type of vascular bed involved GIP infusion can stimulate either vasoconstriction or vasodilation in dogs. This opposing effect is believed to be due to differential activation of GIP's signal transduction pathways in distinct endothelial cell types. Besides all tissues discussed here, GIPR mRNA is also expressed in heart, testis and lung. However, the function of GIP on these tissues remains to be elucidated.

Cholecystokinin (CCK) is a peptide hormone secreted from the I-cells in the intestine. The action of this hormone was first characterized in 1973 [130]. CCK was the first gastrointestinal hormone found to act as a hunger suppressant. Upon nutrient stimulus, CCK from intestine stimulates pancreatic hormone secretion, bile secretion [131] and inhibits gastric emptying [132]. CCK levels in blood increases approximately 15 minutes after meal initiation, with a half-life of 1-2 minutes in circulation [131]. There are several bioactive forms of CCK such as CCK-8, -22, -33 and -58 that differ in the number of amino acids. All forms are derived from a 95 amino acid precursor peptide pro-CCK [133] The predominant form is CCK-33 which is found in plasma and intestine [134]. CCK is also expressed in central and enteric nervous system specifically in the dorsomedial nucleus (DMN) and median eminence of the hypothalamus [135, 136].

Two types of CCK receptors have been characterized, CCKA and CCKB. The CCKA receptor subtype predominates in the GI system, but occurs also in highly localized areas of the rat CNS, where it modulates feeding and dopamine-induced behavior [137, 138]. CCKB receptor predominates the CNS and are involved in the modulation of anxiety, neuroleptic activity and arousal [138]. CCK is reported to reduce appetite. In rats,

administration of CCK-8 reduces meal size and duration while leaving water intake unchanged. Similar findings were also reported in humans, with the infusion of CCK-8 reducing meal intake and initiating meal termination [139]. Recent evidences also suggests that when the stomach is distended, the satiety-inducing effect of CCK-8 is significantly increased, possibly involving a synergistic action [132]. Rats lacking *CCKA* expression present with high food intake, obesity and hyperglycemia [132]. However, this is not reiterated by the studies on knockout mice, as the data is not suggestive of any long-term effects on body weight [140]. CCKA receptors are expressed in the pancreas, nucleus of the solitary tract (NTS), afferent and efferent neurons of the vagus nerve and DMN in hypothalamus which are crucial centers for regulating food intake [141].

In vitro studies have demonstrated that CCK causes insulin exocytosis [16] and promotes growth of pancreatic β cells [139]. CCK exerts a protective effect on β cells and islets of *ob/ob* mice. The islets from these mice expressed higher amounts of CCK compared to lean mice and helps modulate insulin expression by preventing cell death from stress-mediated pathways [142]. The action of CCK on pancreatic exocrine secretion is either direct via the receptors expressed on the pancreatic acinar cells, or indirect via receptors expressed on the vagal afferents, which when depolarized relays the signal through the efferent nerves in the pancreas. Two paradigms have emerged regarding the action of CCK on pancreatic secretion. 1) CCK could act directly on the neurons located in the dorsal motor nucleus of the vagus (DMV) [143] or 2) CCK could act simultaneously on the pancreatic stellate cells stimulating pancreatic exocrine secretion [144].

The influence of exogenous CCK on reduction of food intake is dose-dependent in both rats and humans [130, 145]. Gastric and abdominal vagatomy abolished the satiety induced by CCK-8 administration peripherally, indicating that the CCKA receptors in the vagus nerve plays a crucial role in its regulation of feed intake [146]. CCK administered centrally also decreases food intake and the effects are potentiated by concomitant administration of leptin, indicating that CCK along with leptin could play an important role in long-term weight regulation [147]. Studies have evaluated the use of CCK as a treatment for obesity. Infusion of CCK for 6 days reduces ingested meal size by 44%. However, meal frequency is increased by 162% with no effect on body weight

[148]. Furthermore, 2 week intraperitoneal infusion of CCK resulted in development of tolerance for the peptide, thus having a lack of effect on feed intake or body weight [149]. The increase in postprandial CCK levels was high and quicker in lean individuals, possibly leading to an early occurrence of satiety, while in obese individuals, postprandial CCK levels remained higher for longer periods [150]. Postprandial CCK levels was also demonstrated to be sex-specific with females showing higher CCK levels than males [151].

PYY, like neuropeptide Y (NPY) and pancreatic polypeptide (PP), belongs to the "PP-fold" family of proteins. Similar to all PP fold family proteins, intracellular post-translational C-terminal amidation of PYY in L cells of large intestine increases its half-life in circulation and is crucial for its biological activity. Endogenously PYY exists in two forms: PYY_{1-36} and PYY_{3-36} [152]. Similar to GLP-1, PYY_{1-36} is cleaved at the amino terminal by cell surface enzyme DPP-4 giving rise to PYY_{3-36}, the predominant circulating form [153]. PYY is detected in low levels in stomach and the levels increases distally along small and large intestine, reaching highest levels in the cells of colon and rectum [154]. PYY is secreted from the L cells in proportion to feed intake and the circulating level is lowest in the fasting state. Plasma levels raise within 30 minutes post-prandially and plateaus within 1-2 hours, remaining elevated for 6 hours [155]. Among different types of nutrients, protein rich meals cause the greatest increase in PYY levels [156]. Administration of PYY_{3-36} reduces feed intake and weight gain in rodents [157-159]. Demonstration of PYY's anorectic actions is highly dependent on full acclimatization of animals to handling and injection procedures as even mild stressors affects baseline feeding, making it difficult for anorectic agents to further suppress appetite. Intravenous administration of PYY reduces food intake in humans and was shown to be equally effective in normal and obese subjects [155]. The anorectic actions of PYY_{3-36} are mediated via arcuate nucleus (ARC) in the hypothalamus as the early gene marker, c-fos expression increases in this nucleus in response to peripheral PYY administration. There are conflicting results on the effect of PYY_{3-36} on propiomelanocortin (POMC) and AgRP/NPY neurons in ARC. Peripheral administration of PYY has been shown to decreases expression and release of NPY whilst activating POMC neurons [160]. However, reports have shown that PYY_{3-36} inhibits POMC neurons via postsynaptic Y_2R

(Y_2 receptor; PYY receptor) [161]. This was clarified by studies in POMC knockout mice, which maintained their anorectic response to peripheral PYY_{3-36} administration showing that POMC neurons are not critical for the anorectic response of PYY [162].

Both PYY_{1-36} and PYY_{3-36} exert their effect via neuropeptide family of receptors [162]. PYY_{1-36} binds with equal affinity to all Y receptors. However, PYY_{3-36} selectively binds with high affinity to Y_2R subtype and its effect on food intake is mediated via this receptor [160]. This was further confirmed by studies using antagonists of Y_2R that attenuated the anorectic effects of PYY_{3-36} [163]. Besides, PYY is present in myenteric nervous plexus neurons that innervate the GI tract and the Y_2R has been identified on the vagus nerve [164]. Furthermore, total vagotomy or transectoning of brainstem-hypothalamic pathways abolished PYY_{3-36} effect on food intake and ARC activation in rodents, suggesting a crucial role for vagus in appetite regulation [165]. PYY has been shown to have an effect on intestinal motility. Intra-arterial PYY administration in cats causes jejunal and colonic motility [166]. PYY also delays gastric emptying, decreases pancreatic secretions, and increases absorption of fluids and electrolytes from human ileum [167, 168]. More recently, it was shown that the acute effects of gastrointestinal bypass surgery on weight loss is lost in PYY knockout mice and that wild-type mice losing weight after surgery exhibit increased colonic PYY expression and circulating fasting PYY levels [169], highlighting the crucial role of PYY in mediating early weight loss in GI bypass surgery.

Apart from the intestinal cells, PYY has been demonstrated to be localized in the pancreatic α cells [7]. Electron microscopy and immunohistochemistry studies have revealed that PYY and glucagon are co-stored in the secretory granules of α cells, suggesting a possible role as an intraislet regulatory peptide. Major studies so far have focused on the role of PYY in T1D, which results from sub-optimal levels of insulin secretion from the pancreatic islets. PYY inhibits stimulated insulin secretion under *In vivo* conditions in rat, dogs and mouse [7, 170, 171]. The number of rectal PYY cells in patients with T1D was found to be significantly higher than in healthy volunteers [172]. Also, the number of colonic PYY cells was reduced in non-obese diabetic (NOD) mice. However, no significant decrease in the cell number was observed in pre-diabetic NOD mice. Surprisingly, radioimmunoassay of tissue extracts showed lower concentrations of

colonic PYY cells in both pre-diabetic and diabetic mice [173]. Thus, it can be inferred that the synthesis of PYY decreases prior to the onset of diabetes, though the number of PYY cells remain unaffected. After the onset of diabetes, even the number of PYY cells declines, paving the way for PYY secretion as a possible biomarker for diabetes diagnosis. PYY has also been studied in two animal models of T2D, namely *ob/ob* and *db/db* obese diabetic mice. In *ob/ob* mice the number of colonic PYY cells and tissue concentrations of PYY were found to be lower than those in controls [174]. By contrast, the number of colonic PYY cells in *db/db* mice was reported to be higher than in controls [175]. This contradiction between the studies could be attributed to the difference in the duration of the diabetes in the *ob/ob* mice.

Bertrand et al., [176] have reported a direct inhibitory effect of PYY on insulin secretion in rats. PYY caused a dose-dependent and sustained reduction of insulin secretion in the presence of 8.3 mM glucose. At a concentration of 10^{-9} M, PYY inhibits both phases of insulin secretion. In contrast, higher concentration of 10^{-8} M elicited a decrease in insulin output rate only during the first phase and no inhibitory effect was noted thereafter. Further studies on isolated islets showed that PYY at a concentration of 10^{-7} M inhibited stimulated insulin secretion by 27% at a glucose concentration of 15 mM. However, this same concentration of PYY was ineffective in the presence of 3 mM glucose. The inhibitory effect of PYY on insulin secretion is comparable to the results previously observed with the neuropeptide Y in perfused rat pancreas [176, 177] although in contrast to NPY, PYY at 10^{-8} M inhibited insulin secretion only during the first 2 minutes, after which the inhibitory action disappeared. This might be due to an indirect action of PYY via stimulation of glucagon secretion. However, this can also be ruled out, since glucagon secretion was not increased by PYY at 10^{-8} M in the same study. It has been proposed that the inhibition of insulin secretion by PYY could be mediated via decrease in the pancreatic flow rate [178]. As reviewed in this section, the list of major hormones regulating appetite and energy metabolism includes but are not limited to GLP-1, GIP, CCK and PYY. A recent addition to this list is nesfatin-1.

1.2.3. Nesfatin-1 – A Novel Metabolic Peptide

It has been shown that a secretory protein NEFA (Nuclear EF-hand acidic, Ca^{2+} binding) or NUCB2, which has a calcium-binding domain (EF domain) and DNA-binding domain, is present in the hypothalamic appetite regulating nuclei [179]. In order to find new satiety substances, Oh-I and colleagues [9] surveyed genes that are activated by troglitazone, a peroxisome proliferator activated receptor γ (PPARγ) agonist. They found the *NEFA/NUCB2* gene upregulated both in the brain and adipocytes. *In silico* analysis predicted the proteolytic processing of NUCB2 by prohormone convertases (PC), resulting in an N-terminal fragment of 82 amino acids. The expression of the N-terminal fragment of NUCB2 in the hypothalamic PVN and cerebrospinal fluid was decreased by fasting in rats. As the intracerebroventricular (I.C.V) injection of this fragment in rats suppressed food intake, they named this processed peptide nesfatin-1 (**N**EFA/nucleobindin-2 **E**ncoded **S**atiety and **FAT** influencing prote**IN-1**) [9]. The 396 amino acid precursor NUCB2 is highly conserved among rodents, humans and non-mammals, indicative of its physiological importance [180]. In addition, the processing also could result in two C-terminal peptides nesfatin-2 (85-163) and nesfatin-3 (166-396), which have no known biological function [9]. The active site of nesfatin-1 is located in the mid-segment of 30 amino acids (23-53 amino acid, **Figure 1.3**) as the injections of C-terminal (1-23) and N-terminal regions (54-82) had no anorectic effect in mammals [9, 181]. The mid segment is similar to the amino acid sequence of α-MSH and agouti-related peptide (AgRP) and this sequence is critical for its anorexigenic activity [182].

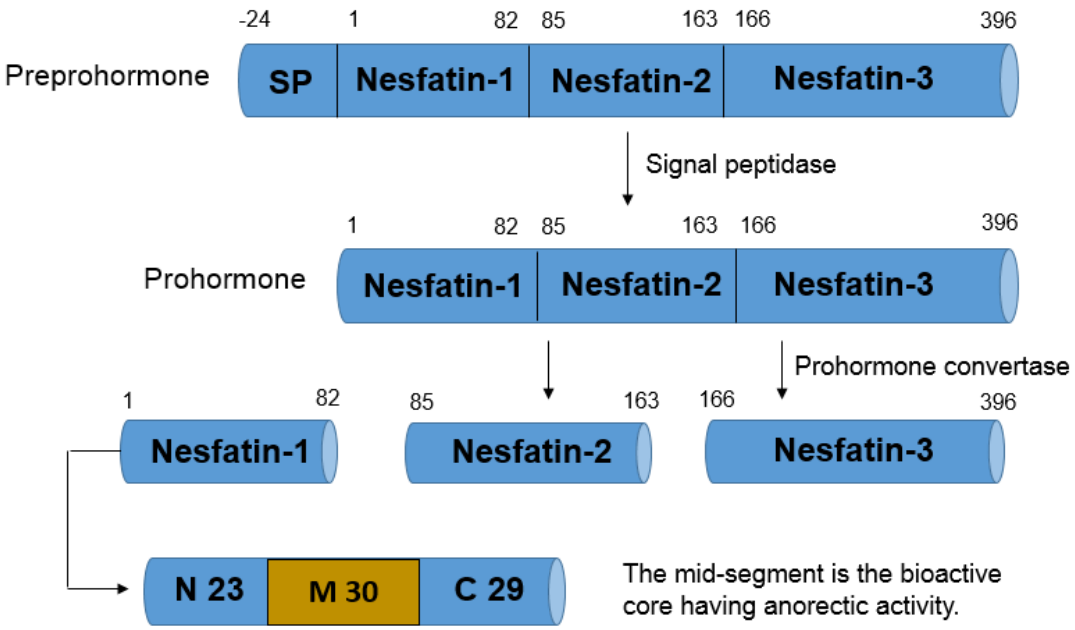

Figure 1.3. A schematic of nesfatin-1 processing from its precursor NUCB2. The preprohormone is cleaved by signal peptidase, yielding the prohormone. This sequence is then post-translationally cleaved by PC 1/3 and PC 2 resulting in 3 distinct fragments including nesfatin-1 (82 amino acids). Nesfatin-2 and -3 have no known biological functions. The mid segment of nesfatin-1 (30 amino acids) is critical for its anorexigenic action.

Oh I *et al*., found the presence of NUCB2 in hypothalamus by immunohistochemical analysis in rats using NUCB2 Ab-1 antibody. They also found the secreted nesfatin-1 protein in rat hypothalamic extracts and cerebrospinal fluid [9]. NUCB2 is expressed in various hypothalamic regions like supraoptic nucleus (SON), paraventricular nucleus (PVN), arcuate nucleus (ARC) lateral hypothalamic area (LHA) and in the nucleus tractus solitarius (NTS). In spinal cord, nesfatin-1 is expressed in both sympathetic and parasympathetic preganglionic nerve cells and dorsal vagus nerve. Its presence was also confirmed by RT-qPCR in nucleus accumbens, cerebellum and lumber spinal cord [183]. Nesfatin-1 was co-localized with PC 1/3 and PC 2 in these neurons, suggesting the processing of NUCB2 by these enzymes [184]. Besides PCs, nesfatin-1 expressing neurons often were found to express other bioactive substances. In the PVN and SON of hypothalamus, nesfatin-1 is co-expressed with oxytocin and

vasopressin. Nesfatin-1 positive neurons of PVN contained 24% oxytocin, 18% vasopressin, 13 and 18% of corticotrophin and thyroptrophin releasing hormones respectively (CRH and TRH). In SON, 35% of nesfatin-1 positive neurons contained oxytocin and 28% contained vasopressin [185]. In ARC, nesfatin-1 coexists with cocaine-amphetamine regulated transcript (CART) and tyrosine hydroxylase, and in LH with CART and melanin-concentrating hormone (MCH) [186, 187]. Nesfatin-1 also co-localizes with choline acetyl transferase in dorsal vagus nerve, and with tyrosine hydroxylase in NTS and with 5-hydroxytryptamine in caudal raphe [186]. It is worth noting that all brain-mapping studies have been performed with antibodies specific to nesfatin-1 that also recognizes full length NUCB2. Therefore, the expression pattern cannot distinguish whether the distribution reflects the precursor and/or processed nesfatin-1. A consistent feature of NUCB2/nesfatin-1 immunostaining is its confinement to the cell-body cytoplasm and primary dendrites, whereas immunolabeling is completely absent in axons and nerve terminals. Such specific cellular localization of nesfatin-1 is at variance with other neuropeptides that it co-expresses, and points towards an intracellular rather than an extracellular mode of action for central NUCB2/nesfatin-1. Also, large set of evidence suggests that the neuronal effects of nesfatin-1 are mediated predominantly through the G-protein coupled receptors, especially GPCR12, GPCR3 and/or GPCR6 [188].

The presence of NUCB2/nesfatin-1 was recently characterized in non-mammalian vertebrates. The *NUCB2* gene structure was found remarkably conserved among teleosts (ray-finned fishes). In zebrafish two paralogues of *NUCB2* gene namely *NUCB2A* and *NUCB2B* exists due to teleost-specific whole genome duplication. The *NUCB2* in goldfish and zebrafish were closely aligned and were highly similar. The nesfatin-1 region of goldfish NUCB2A had high percent similarity to nesfatin-1 region in zebrafish (94%), medaka (91%), stickleback (86%) and green pufferfish (84%). Phylogenetic analysis showed that the goldfish NUCB2A amino acid sequences is clustered with NUCB2A sequences of other teleost fishes while showing strong clustering with zebrafish NUCB2A. These data provided the first set of evidence for the expression and modulation of *NUCB2*/nesfatin-1 in non-mammalian models [180].

In peripheral tissues, nesfatin-1 is expressed in ghrelin producing X/A like cells in the stomach gastric oxyntic mucosa and to a smaller extent in enterochromaffin like cells

of gastric corpus oxyntic mucosa [14]. Subsequent studies have confirmed the presence of a circulating hormone [189]. Expression of *NUCB2* mRNA is found to be 10 fold higher in gastric mucosa than in brain, suggesting stomach as the main source of circulating nesfatin-1 [14]. Confocal microscopy of single X/A like cell revealed that NUCB2/nesfatin-1 immunoreactivity (IR) is localized in different pool of cytoplasmic vesicles from ghrelin. Nesfatin-1 has been reported to cross the blood-brain barrier via a non-saturable mechanism, providing the possibility that nesfatin-1 produced in stomach may act centrally [190]. The half-life of nesfatin-1 in circulation is 6-7 minutes [191]. Our lab, for the first time showed that nesfatin-1 and insulin are co-localized in the pancreatic islet β-cells, suggesting its viable role in insulin secretion and glucose homeostasis *in vitro* [12]. Similar to X/A like cells, the sub-cellular distribution of NUCB2/nesfatin-1 and insulin are not identical [10]. These data along with the distinct responses of circulating NUCB2/nesfatin-1 to a meal compared with that of insulin or acyl ghrelin in healthy subjects [192] support a differential regulation and release of these hormones. Recent studies also have confirmed the presence of NUCB2/nesfatin-1 in intestine and colon via immunohistochemical staining and Western blot analysis [15, 193]. The prominent and consistent immunolabeling of nesfatin-1 in endocrine cells [193] and adipose tissue [9] strongly suggests a role as a circulating hormone involved in various homeostatic processes. This consensus is supported by studies in mice showing that circulating nesfatin-1 similar to other gut hormones influences food intake [189], can cross blood-brain barrier bi-directionally and via a non-saturable mechanism [190]. A similar evidence was shown indirectly in a recent clinical study, where a significant linear relation between plasma NUCB2/nesfatin-1 and cerebrospinal fluid NUCB2/nesfatin-1 levels in both lean and to a lesser degree in fasted obese subjects [194]. Besides stomach, pancreas and intestine nesfatin-1 was also reported in other peripheral tissues like testis, and cardiac muscles [11, 195] of rodents and in goldfish [180]. All these studies collectively reiterate nesfatin-1 as a novel peptide expressed equally in CNS and periphery, which is involved in regulation of appetite and feed intake.

1.2.4. Nesfatin-1 Regulation of Appetite and Metabolism

The first biological action described for NUCB2/nesfatin-1 was reduction of dark phase (phase where nocturnal rats/mice ingest most of their diet) feed intake after injection into the third brain ventricle in rats and reduction in body weight gain and fat pads upon I.C.V administration [9]. Compelling evidences via functional tests showed that injection of nesfatin-1 into brain elicits an anorexigenic response in rodents [196-198] as well as in goldfish [180]. Microstructure analysis of meal patterns in mice documented that the anorexigenic action of nesfatin-1 is due to induction of satiation, involving both reduction in meal size and meal frequency when injected into the lateral brain ventricle, 4 hours post-administration in dark phase [198]. Nesfatin-1 injected into the cisternae magna or the fourth ventricle also reduces dark-phase food intake within the first hour after injection, indicative of specific hindbrain sites of its action [199]. Since most hypothalamic peptides that influences food intake also affect digestive processes peripherally [200] a direct modulation of gastrointestinal propulsive function was proposed for nesfatin-1. Accordingly, it was demonstrated that injection of nesfatin-1 into lateral brain ventricle delayed gastric emptying in rats [200] as well as in mice [197] and affected gastroduodenal motility in mice [201]. These gut motility effects could contribute to the satiety actions.

The mechanism of nesfatin-1 action in the brain has been established to be independent of leptin action in a variety of studies [9, 197, 202]. However, it involves several hypothalamic pathways regulating feed intake. In rats, lateral brain ventricle injection of nesfatin-1 caused a decrease in food intake in dark period by 87% and 45% at 2-3 hours and 3-5 hours after injection, respectively. This action of nesfatin-1 involved the activation of anorexigenic CRF receptor 2 (CRF_2) signaling system, as the injection of corticotrophin releasing factor (CRF_2) antagonist astressin$_2$-B into lateral brain ventricle blocked the decrease in food intake by nesfatin-1 [199]. However, astessin$_2$-B did not modify the effect of nesfatin-1 on food intake when nesfatin-1 was administered into the brain medulla at the level of the fourth ventricle, further supporting the notion that differential forebrain and hindbrain downstream signaling cascades exist for nesfatin-1. Also, intraperitoneal (I.P) injection of CCK induced c-fos expression in 43% of nesfatin-1

neurons in the PVN and 24% of nesfatin-1 neurons in NTS. This indicates that nesfatin-1 acts centrally to reduce dark phase food intake through CRF_2 receptor-dependent pathway after forebrain injection and its acts via CRF_2-independent pathway after hindbrain injection. The activation of nesfatin-1 neurons in PVN by CCK suggested a possible role of gut-derived peptides in its satiation effect. In the PVN oxytocinergic neurons, nesfatin-1 stimulated oxytocin release. Oxytocin release from these neurons was suppressed when endogenous nesfatin-1 was neutralized immunochemically. Both melanocortin 3/4 antagonist SHU9119 as well as oxytocin antagonist blocked the forebrain nesfatin-1 induced reduction in food intake in rats [203, 204]. Whether nesfatin-1 acts in series or parallel activates CRF_2, melanocortin 3/4 and oxytocin pathways remains to be elucidated. In addition to the activation of anorexigenic pathways, nesfatin-1 was also shown to suppress orexigenic pathways such as NPY signaling based on an *in vitro* study and whole cell clamp recordings from rat ARC. The study showed hyperpolarization of NPY neurons when nesfatin-1 was applied to the cell [205]. Besides, in non-mammalian vertebrates, nesfatin-1 had an appetite regulatory effect on goldfish [180]. *NUCB2* mRNA was detected centrally in hypothalamus, olfactory bulbs, telencephalon, midbrain and hindbrain. A 2 to 3 fold increase in hypothalamic NUCB2 mRNA expression was observed in fed goldfish when compared to fish deprived of food for 3 to 7 days. Consequently, the levels of circulating nesfatin-1 were also found to be lower in food-deprived goldfish compared to fed controls [180]. I.C.V injection of 25 ng/g synthetic goldfish nesfatin-1 reduces food intake by approximately 50%.

Convergent sets of evidence support the physiological role of nesfatin-1 as a negative modulator of feed intake. First, the most abundant mRNA/protein expression of NUCB2/nesfatin-1 is detected in hypothalamic nuclei and brainstem areas that contributes to the regulation of feed intake [9]. Functional studies established that injection of nesfatin-1 into cerebrospinal fluid at picomolar doses induces an anorexigenic effect in rodents and goldfish [180, 199]. Second, other food intake suppressing signals such as α-MSH and CCK increases *NUCB2* mRNA expression to activate NUCB2/nesfatin-1 immunopositive neurons in hypothalamus and brainstem. Third, injection of anti-NUCB2 antisense oligonucleotide or anti-nesfatin-1 antibody into third ventricle increases food intake in male rats [9]. However, a recent report showed that

knocking-down hypothalamic NUCB2 had no effect on feed intake and weight gain in female rats [206]. Whether the effects of nesfatin-1 are sex specific needs to be clarified. Also, the intracellular mechanism of nesfatin-1's anorexigenic action remains to be elucidated owing to the lack of identification of its receptor. One recent reports suggests that the neuronal effects of nesfatin-1 are mediated predominantly through the G-protein coupled receptors, especially GPCR12, GPCR3 and/or GPCR6 [188].

A majority of studies have focused on its central effects. However, reports investigating its peripheral effects are limited and are less consistent. One group of investigators has shown that I.P injection of a large dose of nesfatin-1 in *ad libitum* fed mice reduces dark phase food intake through leptin-independent mechanisms [182]. Based on *in vitro* data that shows nesfatin-1 activating Ca^{2+} influx in primary cultured nodose (tightly grouped) ganglion neurons from mice, the above effects are likely mediated via the vagus nerve [207]. However, in separate studies, I.P injection of nesfatin-1 did not decrease the dark phase feed intake in mice as well as in rats [198, 199]. In goldfish either no effect or an 18% decrease in food intake was observed at doses 10 fold higher than those injected intracerebroventricularly, leading to a striking food intake reduction [180]. Therefore, data so far suggests that the satiety action of nesfatin-1 is more readily inducible centrally than peripherally. This warrants further investigation in the context of high levels of gastric *NUCB2* mRNA expression and the circulating levels being regulated according to nutritional status.

A recent clinical study with healthy non-obese subjects showed negative correlation between BMI and fasting plasma levels of nesfatin-1 measured using nesfatin-1 specific ELISA. However, healthy humans do not show a nutrient-related fluctuation in plasma nesfatin-1 levels under conditions of decreased acyl-ghrelin and increased insulin [11, 192]. Other studies in humans have reported a positive correlation between BMI and fasting plasma nesfatin-1 levels under conditions of anorexia nervosa [208] and higher levels in obese subjects, indicating that circulating nesfatin-1 levels are possibly regulated by sustained changes in adipose tissue mass. A recent report showing a lower ratio of CSF/plasma NUCB2/nesfatin-1 levels in obese compared to lean subjects, suggests a reduced uptake from circulation to brain and therefore a reduced central action from peripheral sources of nesfatin-1 [194]. Moreover, the reported difference in eight distinct

single nucleotide polymorphism in *NUCB2* gene being associated with obesity in large cohort of male subjects provides strong evidence for the crucial role of *NUCB2*/nesfatin-1 in susceptibility or protection against development of obesity [209]. Interestingly, NUCB2 is highly homologous to nucleobindin-1 (NUCB1), a homologous multi-domain calcium and DNA binding protein similar to NUCB2.

1.2.5. Nesfatin-1 Regulation of Insulin Secretion in Mammals

Normal insulin secretion contributes to glucose uptake and use in insulin-responsive cells. The secreted monomer of insulin is 51 amino acids long with molecular weight of 5.8kDa. The insulin gene also encodes a 110 amino acid precursor for insulin called preproinsulin. Preproinsulin has a hydrophobic N-terminal signal peptide. This signal peptide interacts with the cytosolic signal recognition particles (SRP), which aids in the translocation of preproinsulin mRNA across the rER (rough endoplasmic reticulum) membrane into the lumen [210]. This process occurs via the peptide-conducting channel where the signal peptide at the N-terminal end of preproinsulin is cleaved by signal peptidase to yield proinsulin [211]. Proinsulin then undergoes folding and formation of three disulfide bonds. Subsequent to the maturation of the 3D confirmation, proinsulin is transported from ER to Golgi apparatus where it enters the secretory vesicles and is cleaved into insulin and C-peptide. Both the peptides are stored in these granules along with other endogenous peptides (e.g. amylin) until translocation and release in response to glucose stimulus. Insulin secretion from the β-cells of pancreatic islets is regulated by a variety of intracellular signals, enteroendocrine hormones as well as by hypothalamic centers in the brain.

Of many modulators of insulin secretion, nesfatin-1 was recently reported to have anti-hyperglycemic and insulinotropic effects besides its anorexigenic action [5, 212]. Our lab, for the first time showed that nesfatin-1 and insulin are co-localized in the pancreatic islet β-cells of rodents, suggesting its crucial role in insulin secretion and glucose homeostasis [12]. A four-fold increase in nesfatin-1 levels was observed, when MIN6 (mouse insulinoma cells) were incubated at high glucose concentrations (16.7 mM). Nesfatin-1 stimulates glucose stimulated insulin secretion (GSIS) from rat pancreatic islets in a dose-dependent manner [13]. In *db/db* mice, nesfatin-1 had antihyperglycemic

effects by lowering blood glucose levels [212]. Nesfatin-1 increases glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells by direct action involving Ca^{2+} influx through L-type calcium channels [5]. It was also shown to increase the preproinsulin mRNA expression in MIN6 cells. The circulating levels of nesfatin-1 are regulated by nutritional status as fasting decreases, and refeeding normalizes its level. Also, nesfatin-1 in circulation increases in response to oral glucose ingestion when compared to saline treated groups [198]. In Goto-Kakizaki (GK) rats with T2D, the levels of NUCB2 immunoreactivity in islet homogenate is reduced when compared to Wistar rats and plasma NUCB2 concentrations showed an inverse relationship with circulating glucose levels during glucose tolerance tests [10]. This negative association between blood glucose level and nesfatin-1 was also observed in human subjects with T2D [11]. Similarly, nesfatin-1 levels were lower in the breast milk of subjects with gestational diabetes [213]. These studies highlight the role of nesfatin-1 in regulating whole body glucose homeostasis.

Simultaneously, in studies involving rats with diet-induced obesity, ICV injection of nesfatin-1 regulated hepatic gluconeogenesis to inhibit hepatic glucose production. It was postulated that nesfatin-1 does this by either decreasing the synthesis of phosphoenolpyruvate carboxykinase enzyme, which is a rate limiting enzyme in gluconeogenesis in liver or by increasing the activity of insulin hormone that causes a decrease in hepatic glucose production [214]. In addition, peripheral insulin injection also increases the activation of ARC, PVN, LHA and NTS nesfatin-1 protein expression [215]. Nesfatin-1 administration to hyperglycemic rats reduces blood glucose levels. Therefore, the anti-hyperglycemic effects of nesfatin-1 results not only from its endocrine function increasing insulin secretion, but also from its inhibition of hepatic glucose production by way of regulating glycogen synthesis and gluconeogenesis.

An overall assessment of the available literature on nesfatin-1 shows that it plays a crucial role in energy metabolism via regulation of feed intake, metabolism and glucose homeostasis via anti-hyperglycemic and insulinotropic actions. The regulation of energy metabolism involves careful coordination between neurons in the hypothalamus and their regulation by peripheral signals including nesfatin-1. The regulation of two classes of neurons i.e. the orexigenic AgRP/NPY neurons and the anorexigenic POMC/CART/α-

MSH neurons is the essence of energy metabolism. *In vitro* and *in vivo* studies have shown the effects of centrally arising nesfatin-1 and nesfatin-1 from adipocytes and GI tract on this distinct population of neurons. Nesfatin-1 activates the POMC neurons, while simultaneously inhibiting the AgRP/NPY to bring about a reduction in food intake by directly modulating energy balance.

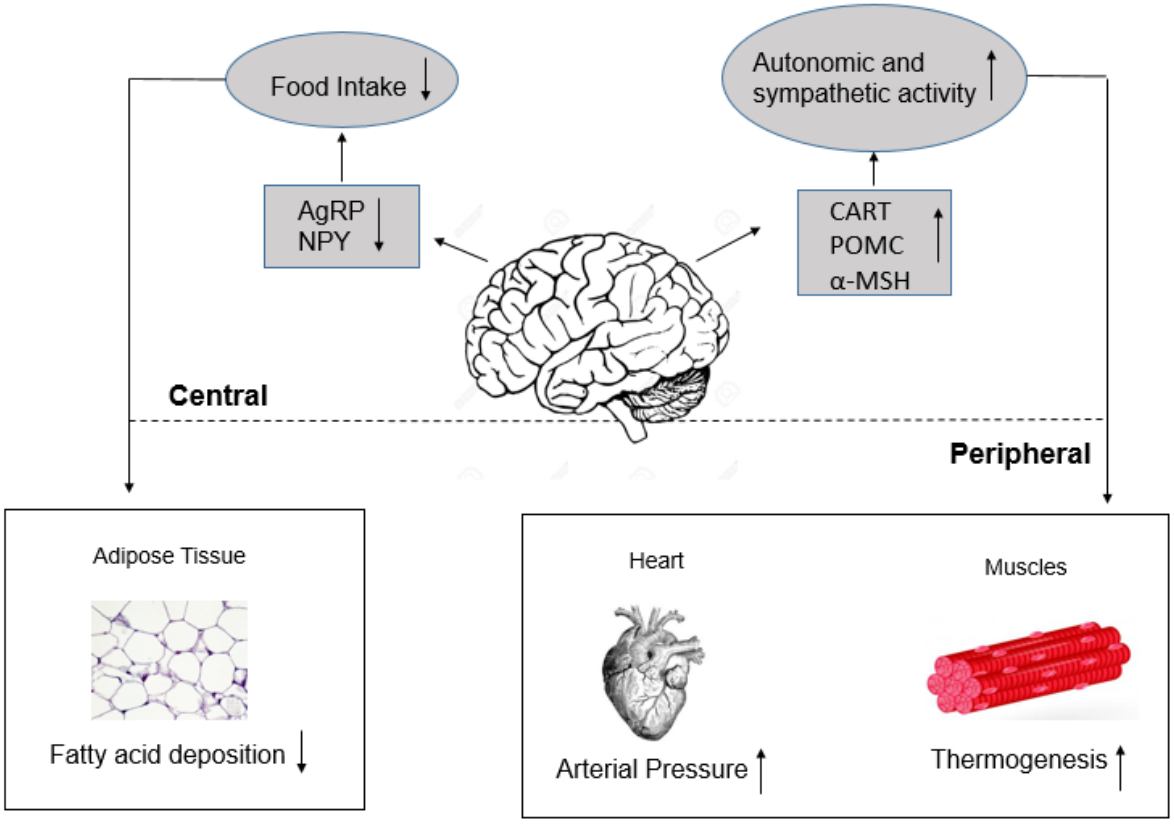

Figure 1.4. Some of the major effects of nesfatin-1 in regulating food intake and energy balance, by integrating its peripheral and central actions.

1.2.6. Nucleobindin-1 – Expression and Function

Nucleobindin-1 (NUCB1) is a 55 kDa multi-domain protein identified first in a culture supernatant of B lymphocyte cell line identified from mice prone to systemic lupus erythematosus, an autoimmune disorder. The first paper that reported this peptide indicated that it has a potential role in autoimmunity and apoptosis [17]. It is also called CALNUC owing to its Ca^{2+} and DNA-binding ability. The DNA binding domain of basic residues (172-218) lies in the N-terminus following the signal sequence. The Ca^{2+} domain

is at the core of the protein sequence consisting of two EF hand motifs with an intervening acidic region (residues 253-316). The Ca^{2+} domain is followed by a leucine zipper domain (residues 347-389) that has been proposed to induce its dimerization [19]. The C-terminal region that follows the leucine zipper region is intrinsically disordered and unstructured. NUCB1 sequence is strongly conserved across species from flies to humans [216] and is widely distributed among golgi, nucleus, endoplasmic reticulum and cytoplasm [17, 22, 23, 217]. NUCB1 immunoreactivity has been reported in several primary cells and cell-lines of rats, mice, monkeys and humans (HeLa cells). NUCB1 displayed partial co-localization when co-labeled with α-Man II (a Golgi marker) and ERGIC-53 (*cis*-Golgi network marker) [18]. Immunogold labelling on ultrathin cryosections of ArT-20, normal rat kidney epithelial (NRK) cells and rat anterior pituitary, liver and kidney tissues demonstrated that NUCB1 was concentrated on the cisternae and vesicles present on the *cis* side of the golgi stack [20, 218].

The endogenous expression profile of NUCB1 was evaluated extensively, showing that NUCB1 is present in pancreatic islets as well as in other endocrine tissues including stomach, intestine, adrenal gland, pituitary, ovary and testis [219]. Immunofluorescence staining performed on formalin fixed sections showed NUCB1 to be localized in the endocrine pancreas with no signals in the surrounding exocrine acinar cells or pancreatic ducts. NUCB1-IR was broadly distributed in pancreatic islets being detected along with glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin immunopositive cells. This cellular localization of NUCB1 is also suggestive of the proposed insulinotropic action. Subcellular localization of NUCB1 using organelle specific protein stains showed NUCB1 to be co-distributed with golgi apparatus protein giatin. NUCB1-IR was found all through the GI tract but not in all endocrine cell types. In stomach, the NUCB1 signal was concentrated in the fundus region of gastric glands, whereas neck and gastric pits displayed no immunoreactivity for NUCB1. NUCB1-IR was found in duodenum, jejunum and colon being primarily present in the duodenal enterocytes. Strong IR was observed in deep intestinal glands wherein cells immunopositive for somatostatin and ghrelin showed NUCB1-IR. NUCB1-IR was also observed in principal cells of parathyroid, thyroid follicles, adrenal medulla and zona glomerulosa of adrenal cortex, seminiferous tubules in testes and follicular cells of ovary. NUCB1-IR was detected in corticotropes,

somatotropes, thyrotropes, lactotropes and gonadotropes in the anterior pituitary. In addition, it has been shown that extracellular NUCB1 to be secreted into bone osteoid (unmineralized organic portion) possibly modulating matrix maturation via paracrine action [21]. These studies indicate the role of NUCB1 in multiple cellular processes. Given the cytoplasmic location of this protein and that it could be secreted, points towards a potential endocrine function for NUCB1 and/or encoded proteins.

NUCB1 has been reported to play an important role in the maintenance of Ca^{2+} homeostasis [22] and to interact with G proteins and cyclooxygenases [22, 23]. The C terminal of *NUCB1* is highly acidic, giving it a low affinity and high capacity binding to Ca^{2+}. Previous studies have reported that *NUCB1* changes its conformation when Ca^{2+} binds to the EF hand domains with the intervening acidic domain, that represents a coiled-coil region [220]. *NUCB1* has been demonstrated with G_{α} subunit excluding $G_{\alpha i}$, $G_{\alpha 12}$ and $G_{\alpha 13}$ [217]. *NUCB1* specifically binds to α5 helix region of $G_{\alpha i3}$ subunit and this interaction is dependent on Ca^{2+} and Mg^{2+} divalent cations. Studies have reported that high expression of NUCB1 results in redistribution of $G_{\alpha i1}$ subunit to the plasma membrane and secreted granules [217]. Results from *in vitro* pull down assays shows that NUCB1 interacts strongly with $G_{\alpha i3}$ subunit only in the presence of both ions and that the interaction is weak with presence of either Ca^{2+} or Mg^{2+} [221]. A soluble form of NUCB1 called sNUCB1 has also been identified that binds to Ca^{2+} and exists as a dimer in solution [221]. sNUCB1 in the unbound state (without Ca^{2+}) interacts with inactive GDP bound state of $G_{\alpha i1}$ subunit, thus inhibiting GDP release. This is reversed when sNUCB1 is bound to Ca^{2+} [221]. Also, overexpression of sNUCB1 in HEK293 cell lines results in decreased receptor mediated $G_{\alpha i1}$ inhibition of adenylyl cyclase, explaining the physiological relevance of sNUCB1 and GDI activity.

NUCB1 was reported to be COX-2-associated (cyclooxygenase-2) protein that has an impact on prostanoid (cyclooxygenase metabolites of arachidonic acid including prostaglandins) biosynthesis. In golgi and ER of human neutrophils, NUCB1 is localized with COX-2. It interacts with COX-2 with high affinity resulting in an increase of prostaglandin E_2 (PGE_2) generation [23]. This was further reiterated by the study wherein the addition of human recombinant NUCB1 (hrNUC) to hrCOX-2 increased PGE_2 production up to 5-fold from the basal levels independent hrNUC of concentration [23].

This modulation of COX-2 by NUCB1 is important because COX-2 activity is involved in the development of cancer by promoting cell division [222]. NUCB1 acts as a negative regulator of unfolded protein response (UPR) that is involved in inhibiting the site 1 protease (S1P) mediated cleavage of activating transcription factor 6 (ATF6) in golgi apparatus during ER stress [223]. Results obtained so far reiterate the importance of NUCB1 as an intracellular regulatory protein.

On comparing NUCB1 and NUCB2, both proteins are found encoded by two unlinked genes. However human NUCB1 and NUCB2 exhibit 62% amino acid sequence identity. Compared to NUCB1, NUCB2 is 40 residues shorter and is approximately a 50kDa protein in humans and rodents [224]. While NUCB2 encodes nesfastin-1, no information exists regarding NUCB1 encoded peptides so far. Similar to the primary structure of NUCB2, NUCB1 also has 24 amino acid signal peptide sequence (**Figure 1.5,** in italics) followed by a signal peptidase cleavage site between position 26 and 27. This is followed by a region that has high similarity to nesfatin-1 region of NUCB2 (shown in bold) containing a mid-segment of 30 amino acids (underlined) and a prohormone convertase cleavage site. In comparison to NUCB2 the proposed mid segment of 30 amino acids in NUCB1 has less similarity to active sites of AgRP and α-MSH.

E.g. Human NUCB1 precursor (**NP_006175.2**)

1 *mppsgprgtl lllpllllll lravla***vple rgapnkeetp atespdtgly yhrylqevid**
61 **vletdghfre klqaanaedi ksgklsreld fvshhvrtkl del***kr*qevsr lrmllkakmd
121 aeqdpnvqvd hlnllkqfeh ldpqnqhtfe ardlelliqt atrdlaqyda ahheefkrye
181 mlkehe*rr*ry leslgeeqrk eaerkleeqq rrhrehpkvn vpgsqaqlke vweeldgldp
241 nrfnpktffi lhdinsdgvl deqelealft kelekvydpk needdmreme eerlrmrehv
301 mknvdtnqdr lvtleeflas tqrkefgdtg egwetvemhp ayteeelrrf eeelaareae
361 lnakaqrlsq etealgrsqg rleaqkrelq qavlhmeqrk qqqqqqqghk apaahpegql
421 kfhpdtddvp vpapagdqke vdtsekklle rlpevevpqh l

Figure 1.5. The amino acid sequence of human NUCB1 precursor.

1.3. RATIONALE

Recent studies using immunohistochemical staining in *ad libitum* fed rats showed no nesfatin-1 immunopositive cells in the intestine [14]. However, this view is challenged by our recent findings wherein we detected both *NUCB2* mRNA and protein expression

in the intestine of *ad libitum* fed C57BL/6J mice [15]. Additional studies that showed *NUCB2* mRNA expression and nesfatin-1 immunolocalization in the small intestine of rats are also supportive of this [193, 219]. Does nesfatin-1 has a direct role in modulating the secretion of enteric hormones that regulate insulin secretion and energy homeostasis? The cytoplasmic presence of NUCB1, and that it is a secreted protein (found in bones, cerebrospinal fluid and rat hypothalamic extracts) suggests a possible endocrine function for NUCB1 and a potential NUCB1-encoded peptide called nesfatin-1-like peptide (NLP). Interestingly, the nesfatin-1 region of NUCB1 and nesfatin-1 region in NUCB2 are highly conserved in humans, with 68% amino acid sequence identity. In addition, both NUCB1 and NUCB2 sequences retain the prohormone convertase cleavage sites [225]. Therefore, the possibility of NUCB1 and/or NLP eliciting biological actions similar to that of NUCB2/nesfatin-1 could not be ruled out. Does NUCB1 encode a bioactive nesfatin-1-like peptide (NLP)? Is NLP insulinotropic?

1.4. HYPOTHESES

Based on the information discussed above, the central hypothesis of this thesis research is that (1) nesfatin-1 has a direct role in modulating enteric hormone secretion, and (2) a nesfatin-1 like peptide is encoded in NUCB1 and this peptide has insulinotropic action.

1.5. SPECIFIC OBJECTIVES

The *specific aims* of this thesis research are to:

(1) determine the presence and co-localization of endogenous NUCB2/nesfatin-1 with major enteric hormones in mouse intestine and *in vitro* in a mouse intestinal cell line (STC-1).

(2) test whether nesfatin-1 regulates enteric hormones *in vitro* in STC-1 cells, and *in vivo* in male mice.

(3) identify the nature of NUCB1 encoded NLP *in silico*, and *NUCB1* expression in various nutrient states.

(4) elucidate the *in vitro* effects of NLP on insulin expression/secretion, and *in vivo* effects on dark and light phase food intake in male mice.

TRANSITION

The following chapter focuses on objectives 1-2, to determine the nesfatin-1 regulation of enteric hormone secretion. As discussed earlier, we observed endogenous *NUCB2/Nesfatin-1* mRNA in the intestine of C57BL/6J mice by RT-qPCR and gel electrophoresis. Subsequently, we also observed NUCB2 protein expression in intestine by Western blotting of total protein samples from intestinal sections of *ad libitum* fed male C57BL/6J mice. This chapter will elaborate the studies carried out, major techniques used for answering my specific research question mentioned above and the results obtained. This is followed by a discussion and conclusion of my findings pertaining to the research question.

Contribution of co-authors: Naresh Ramesh conducted the studies, analyzed the results and wrote the first draft of the manuscript. Ms. Sima Mortazavi helped with the *in vivo* studies and Western blotting, immunoassay, and immunohistochemistry. Dr. Suraj Unniappan provided the infrastructure, funding, research ideas, assisted with experimental design, in vivo study, data analysis and manuscript writing.

Publication: A part of this chapter has been published *(Article in Press)*.

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CHAPTER 2

NESFATIN-1 REGULATION OF ENTERIC HORMONE SECRETION

2.1. INTRODUCTION

The GI tract is the primary center for nutrient absorption and assimilation. Besides, GI tract is also a major endocrine organ secreting a variety of enteroendocrine hormones. The vagus nerve is a main communication route between the central nervous system (CNS) and the enteric nervous system (ENS) that is responsible for proper GI functions including gastric motility, enzyme secretion and nutrient sensing [226]. The enteric hormones are secreted from specialized enteroendocrine cells in response to chemical and mechanical stimuli in the gut, resulting from food intake. Upon secretion they bind to their receptors in vagal afferent neurons communicating the peripheral nutritional status to CNS [227]. As discussed elaborately in chapter 1, GLP-1, GIP, CCK and PYY are major enteric hormones secreted from the GI tract.

Nesfatin-1 is a recent addition to the list of anorexigenic peptides capable of inducing satiety [9]. **N**EFA/nucleobindin-2 **E**ncoded Satiety and **FAT** influencing prote**IN-1**, abbreviated as nesfatin-1 is an anorectic peptide cleaved from the N-terminal of nucelobindin-2 (NUCB2). The prohormone of NUCB2 is then post-translationally cleaved into three distinct fragments named nesfatin-1 (1-82 amino acids), nesfatin-2 (85-163 amino acids) and nesfatin-3 (166-396 amino acids). The 82 amino acid nesfatin-1 has a 23 amino acid N-terminal segment, 29 amino acid C-terminal segment and a 30 amino acid mid segment (**Figure 1.3**). Of the three segments, the mid segment is the bioactive core having anorectic activity [9]. Nesfatin-1 has been reported to be expressed in peripheral tissues ranging from stomach [14], pancreas [12], gut [193] and cardiac muscles [195]. I.C.V injection of nesfatin-1 reduces dark phase food intake and body weight gain in rats [9]. Opposing evidences exist regarding the expression of NUCB2/nesfatin-1 in the intestine. Although nesfatin-1 is expressed predominantly in gastric oxyntic mucosa in X/A like cells, the original study that reported this in *ad libitum* fed rats showed no nesfatin-1 immunopositive cells in intestine [14]. This view is challenged by our recent findings where both *NUCB2* mRNA and protein expression was

confirmed in the intestine of *ad libitum* fed C57BL/6J mice [15]. This is supportive of previous observations by Zhang *et al*., [193] that showed NUCB2/nesfatin-1 immunoreactivity and protein expression in various sections of intestine. Nesfatin-1, besides being a modulator of food intake, also stimulates GSIS from rat and mouse pancreatic islets in a dose-dependent manner [5]. Also, the role of intestinal hormones on insulin secretion and food intake has been studied extensively [89, 117, 141, 142, 160, 176, 228].

Taken together, the main hypothesis behind the research in this chapter is that NUCB2/nesfatin-1 is expressed in the intestinal enteroendocrine cells and that it regulates intestinal hormone secretion. This study determined that the enteroendocrine cell line STC-1 expresses *NUCB2* mRNA and also show NUCB2/nesfatin-1 immunoreactivity when incubated with a highly specific NUCB2 antibody. Further, immunohistochemical analysis of intestinal sections from *ad libitum* fed mice showed NUCB2 immunoreactivity to be co-localized with GLP-1, GIP, CCK and PYY immunoreactive cells. Subsequently, treatment of STC-1 cells dose-dependently with nesfatin-1, upregulated GLP-1, GIP and CCK, while downregulating PYY expression and secretion. Osmotic mini-pump infusion of 100 µg/kg body weight nesfatin-1 into C57BL/6J mice showed no changes in glucose handling between controls and treatments. Whether or not nesfatin-1 infusion causes an increase in circulating levels of the respective hormones remains to be elucidated via immunoassays.

2.2. MATERIALS AND METHODS

2.2.1. Cell Culture

In this study, a mouse enteroendocrine cell line, STC-1 (**C**ells derived from tumors in transgenic mice carrying transgenes consisting of rat insulin promoter 2 (RIP2) linked to polyoma small T antigen [Py**ST**], hence **STC**) was used. Studying the gene expression of gut peptides has a major limitation in that their distribution is diffuse *in vivo*. Hence primary cell isolation and culture are tedious. Ideally, therefore the studies on intestinal peptides are being undertaken in immortalized cells that express these peptides. Cell lines are regularly used by several laboratories to investigate basic questions related to

organization and function of intestinal cells. However, intestinal enteroendocrine tumors are very rare and consequently are not a ready source of permanent cell lines that secrete all the major gut peptides [229]. The STC-1 cell line was derived from intestinal endocrine tumors that developed in mice carrying transgenes containing the rat insulin promoter linked to the potent viral oncogene SV40 T antigen and to the polyoma virus small T antigen [230].

STC-1 cells were previously used to study the release of CCK, secretin and GLP-1 and it was then concluded that this cell line can serve as a model system to investigate the secretory mechanisms for these peptides [231-233]. In addition, in the presence of glucose, a concentration-dependent increase in GIP gene expression was observed in STC-1 cells, suggesting that these glucose responsive cells express GIP in a regulated fashion similar to those observed in rats [228, 234]. CCK secreted from STC-1 cells was shown to have the same HPLC retention time as naturally occurring peptide [232]. The HPLC retention time for the immunoreactive GIP secreted by these cell was also shown to be the same as GIP derived from intestine [234]. In addition, STC-1 cells were shown to secrete PYY [235]. Therefore, STC-1 cells are ideal models for studying the synthesis and/or secretion of GLP-1, GIP, CCK and PYY.

STC-1 cells used here were a generous gift from Dr. Timothy Kieffer (University of British Columbia, Vancouver, Canada). The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen, Catalog #11995-040) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Catalog #12484), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Invitrogen, Catalog #15140-122). Cells were incubated at 37°C and 5% CO_2 culture conditions in a humidified incubator to promote growth. Cell culture media was changes every 48 hours after washing twice with Dulbecco's Phosphate-Buffered Saline (DPBS, Life Technologies, Catalog #14190-250). The cells were sub-cultured once they reached 85-90% confluency using 0.25% trypsin-EDTA (Life Technologies, Catalog #25200-056).

2.2.2. Animals

Age matched (5 weeks old, average body weight: 20 grams) male C57BL/6J mice (Charles River Laboratories, Quebec, Canada) were housed individually in a 12 hours light: 12 hours dark cycle (lights off at 7 PM and on at 7 AM), temperature and humidity controlled vivarium. Mice had *ad libitum* access to standard mouse chow and water. Male mice were preferred owing to the sexual dimorphisms observed in endogenous *NUCB2* expression and biological functions (previous lab observations) and also to maintain consistency. All protocols strictly adhered to the guidelines of the Canadian Council for Animal Care, and were approved by the University of Saskatchewan Animal Research Ethics Board (Protocol # 2012-0033).

2.2.3. Detection of Endogenous NUCB2/Nesfatin-1 in Mice Intestinal Cell Line and in Mice Intestine

2.2.3.1. Immunocytochemistry and Immunohistochemistry

STC-1 cells were cultured on LabTek™ chamber slides to look at the indirect immunofluorescence. The cells were fixed in 4% paraformaldehyde for 15 minutes at 4°C and then washed with Dulbecco's Phosphate Buffered Saline (DPBS, Life Technologies, Catalog #14190-250). After fixation, the primary antibody was added with dilution ratio 1:200 using DAKO antibody diluent (DAKO, Catalog# S0809) in room temperature. The primary antibody used was rabbit anti-NUCB2 (Custom antibody, Pacific Immunology, Catalog# 1312-PAC-01). The primary antibody pre-absorbed in 10 µg of nesfatin-1 (200 µg stock, >95% purity) overnight was used for pre-absorption controls to confirm the specificity of antibody. After six hours, the slides were rewashed with DPBS. Following this secondary antibody was added with dilution ratio of 1:200. Goat anti-rabbit Texas-Red IgG (Vector Laboratories, Catalog# TI-1000) diluted with DAKO antibody diluent was used as secondary antibody. The slides were maintained at 37°C in a humidified incubator for 1 hour. The chambers were then peeled off and were allowed to dry in room temperature. After drying, slides were mounted with Vectashield mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Catalog# H-1200).

For confirming the presence of NUCB2/nesfatin-1 immunoreactivity in intestine and to assess its co-localization with intestinal hormones, *ad libitum* fed 3 months old male C57BL/6J mice were used. Animals were euthanized using cervical dislocation. Briefly both large and small intestines were collected for immunohistochemistry. The tissues collected were fixed in 4% paraformaldehyde for 24 hours at 4°C and were processed and sectioned at the center for modeling human disease (CMHD, Toronto, Canada). Paraffin sections of 4 µm thickness were prepared for immunostaining. These sections were deparaffinized with xylene (incubated twice in 100% xylene; 5 minutes at 25°C) and rehydrated in graded ethanol series (incubated twice in 100% ethanol, once in each 95% ethanol, 70% and 50% ethanol, 2 minutes each at 25°C). The sections were then incubated with 3% hydrogen peroxide in distilled water to block endogenous peroxidase activity (30 minutes at room temperature). The sections were then blocked with serum-free protein block reagent (DAKO, Catalog# S0809) for 10 minutes before being incubated with primary antibodies. The primary antibodies were diluted using DAKO antibody diluent at different dilution ratios. The primary antibodies used are rabbit anti-NUCB2 (Custom antibody, Pacific Immunology, Catalog# 1312-PAC-01, 1:500 dilution) and mouse monoclonal anti-GLP-1 (Abcam, Catalog# ab26278, 1:500 dilution) for GLP-1 sections, Mouse polyclonal NUCB2/nesfatin-1 (ENZO Life Sciences, Catalog# ALX-804-854-C100, 1:100 dilution) and rabbit polyclonal anti-GIP (Abcam, Catalog# ab22624, 1:500 dilution) for GIP sections, Goat anti-nesfatin-1 (SantaCruz, Catalog# SC65160, 1:500 dilution) and Rabbit anti-$PYY_{(3-36)}$ (Phoenix pharmaceuticals, Catalog# H-59-04, 1:200 dilution) for PYY sections, Goat anti-nesfatin-1 and rabbit anti-CCK (Abcam, Catalog# ab43842, 1:500 dilution) for CCK sections. After 24 hours all slides were washed three times with 1X PBS and incubated with secondary antibodies. The secondary antibodies were diluted using DAKO antibody diluent at different dilution ratios. The secondary antibodies used are goat polyclonal anti-mouse FITC (Abcam, Catalog# A-11034, 1:500 dilution) for GLP-1, goat polyclonal anti-rabbit Alexa Flour-488 (Invitrogen, Catalog# A11037, 1:500 dilution) for GIP, goat polyclonal anti-mouse Alexa Flour-594 (Abcam, Catalog# ab150108, 1:500 dilution) for NUCB2/nesfatin-1 and goat anti-rabbit Texas-Red IgG (Vector Laboratories, Catalog# TI-1000, 1:100 dilution) for nesfatin-1, PYY and CCK. The slides were then washed three times with 1X PBS and

seven times with distilled water. Finally, the slides were mounted with Vectashield medium that contains the nuclear dye DAPI.

2.2.3.2. Fluorescence Microscopy

Once the cells and tissues were fixed and stained for NUCB2/nesfatin-1 and GLP-1, PYY, CCK (tissues alone), the slides were viewed under a Nikon Eclipse-Ti inverted fluorescence microscope (Nikon, Canada) to detect indirect immunofluorescence. The slides were viewed in 40X magnification with blue, green and red filters specific for the stains used. DAPI stains the nuclei of the cells, and this was imaged under the presence of blue filter. Green and red filters were used for imaging cells and tissues stained with Texas Red and FITC green respectively. The images were captured using a Nikon DS-Qi1 MC camera. Images were analyzed using NiS-Elements basic research software on a Lenovo ThinkPad workstation.

2.2.4. Qualitative Analysis – Expression of *NUCB2* mRNA in STC-1 Cells

NUCB2 mRNA was detected in STC-1 cells using RT-qPCR followed by gel electrophoresis of the PCR product. The following sections will describe the procedure and materials used for carrying out these techniques.

2.2.4.1. Total RNA Extraction

STC-1 cells were grown on 6 cm plates as described in section 2.2.1. Upon confluence, TRIzol® Reagent (Life Technologies, Catalog# 15596-026) was added to the plate kept over an ice bath. The reagent and lysed cells were collected in 1.5 mL tubes for total RNA extraction. Approximately 200 µL of chloroform was added to the tube followed by centrifugation at 13,000 rpm for 15 minutes. The supernatant containing the RNA was separated from DNA and protein layers. Isopropanol was added in order to precipitate RNA in solution. 70% ethanol was added to remove any phenol impurities from the pellet. Purified RNA was isolated using RNAse free water (35 µL). The quality and quantity of RNA extracted was determined using Nanodrop™ 2000C (Thermo Scientific, Canada). Total RNA was quantified by measuring the optical density (OD) and absorption ratio (A260 nm/A280 nm) of diluted RNA. Pure RNA will have a ratio of approximately

2.0. The ratio of the samples used for my study ranged from 1.9 - 2.0. All RNA samples used for cDNA synthesis and RT-qPCR had high quality. Isolated RNA was stored at -80° C until further analysis.

2.2.4.2. cDNA Synthesis

Synthesis of cDNA was conducted using iScript™ Reverse Transcription Supermix (Bio-Rad, Catalog# 170-8840) for RT-qPCR as directed by the manufacturer. The reaction set up for cDNA synthesis is described in **Table 2.1**, and the conditions used in Thermo cycler (Bio-Rad, Canada) is described in **Table 2.2**. cDNA samples were stored at -20°C until required for PCR analysis.

Table 2.1. Reaction setup for cDNA synthesis using iScript DNA synthesis kit (as provided by manufacturer; Bio-Rad).

Components	Volume Used
Nuclease Free Water	14 µL – 1/Conc of RNA
5X iScript reaction mixture	4 µL
RNA Template	1/Conc
iScript Reverse Transcriptase	1 µL
Total Volume	20 µL

Table 2.2. Reaction protocol optimized for cDNA synthesis using thermo cycler.

	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	42°C	85°C	4°C
Time	5 minutes	30 minutes	5 minutes	Infinite Hold

2.2.4.3. Reverse-Transcription Polymerase Chain Reaction

The cDNA synthesized was used as template to determine the qualitative expression of *NUCB2* in STC-1 cells. The primer sequences were obtained from National Center for Biotechnology Information Gene Bank (NCBI) and the primers were synthesized using Primer-BLAST™, primer designing tool from NCBI (www.ncbi.nlm.nih.gov/tools/primer-blast/). The GenBank accession number is given under each gene. The forward and reverse primers are provided in **Table 2.3**.

Table 2.3. Primer Pairs for mouse *NUCB2* and β-Actin with Respective Annealing Temperatures

Gene	Forward (5' – 3')	Reverse (3' – 5')	Amplicon Size (bp)	Annealing Temperature
Mouse NUCB2 (NM_001130479.2)	ccagtggaaaatgcaaggat	gctcatccagtctcgtcctc	202	61°C
Mouse β-Actin (NM_007393.3)	ccactgccgcatcctcttcc	ctcgttgccaatagtgatgac	77	60°C

Primers were validated and optimized for high primer efficiency and annealing temperatures. The primers were then used for conducting qualitative PCR. The PCR samples were run on a thermo cycler (Bio-Rad, Canada) for 35 cycles. The samples were stored at -20°C until gel electrophoresis. The components of the mastermix are provided in **Table 2.4**. The components for the qualitative PCR are provided in **Table 2.5**. The reaction protocol used is described in **Table 2.6**.

Table 2.4. Components of Reaction Mastermix for qualitative PCR

Components	Volume Required
10X PCR buffer reaction mixture	100µL
25nM $MgCl_2$	60µL
dATP	5µL
dTTP	5µL
dCTP	5µL
dGTP	5µL
Sterile water	820µL

Table 2.5. Reaction Components for qualitative PCR

Components	Volume Required
Mastermix	20µL
Forward Primer	1µL
Reverse Primer	1µL
cDNA template	1µL
Taq Polymerase	0.5µL
Total Volume	23.5µL

Table 2.6. Optimized PCR conditions with respective annealing temperatures for *NUCB2* and β-Actin. (Step 1: Initial Activation; Step 2: Denaturation; Step 3: Annealing; Step 4: Elongation; Step 5: Infinite Hold)

	Step 1	Step 2	Step 3	Step 4	Step 5
Temperature	95°C	95°C	Annealing Temp	73°C	4°C
Time	5 minutes	30 seconds	30 seconds	30 seconds	Infinite Hold

2.2.4.4. Gel Electrophoresis

Following PCR, the samples were run on 1.5% agarose gel. 1.5 gram of agarose (Invitrogen, Catalog# 16500-500) was mixed with 1X TAE buffer (Fischer Scientific, Catalog# BP1332-1, 50 X). Approximately 5 µL of ethidium bromide (Invitrogen, Catalog# 15585-011) was added and the mixture was allowed to set in the electrophoresis tray. Prior to setting, a comb was placed. Once set, the comb was taken out and the electrophoresis chamber was filled with 1 X TAE buffer. This was followed by the addition of 3 µL of 10 X gel loading buffer (Invitrogen, Catalog# 10816-015) to the PCR samples and approximately 20 µL of sample were loaded into each well. One Kb plus DNA ladder (5 µL) (Invitrogen, Catalog# 10787-018) was loaded alongside the samples to detect the amplicon gene. The gel was run from negative to positive, as DNA is negatively charged, for one and half hour at 120 V. The image was obtained using GelDoc™ EZ system (Bio-Rad, Canada) and the expression of genes of interest was qualitatively analyzed.

2.2.5. Dose Dependent Effects of Nesfatin-1 on the Expression of Proglucagon, GIP, CCK and PYY mRNAs in STC-1 Cells

This study was conducted to determine proglucagon (GLP-1), GIP, CCK and PYY mRNA expression in STC-1 cells after treatment with nesfatin-1. Cells were cultured as described in section 2.2.1. Twenty four hours prior to study, cells were seeded into 24-

well plates at 2 X 10^5 cells/well in 1 mL DMEM (80% confluence). On the day of study, wells were washed twice with 1 X DPBS (1 mL/well). The cells were then incubated for 1 hour with DMEM containing 10, 1, 0.1, 0.01 and 0.001 nM (n = 8 wells/treatment) rat full length nesfatin-1 (Abgent Technologies, USA, >95% purity). Controls were incubated with culture media without nesfatin-1 for the same period of time. The studies were repeated twice and data from these independent studies were pooled. Total RNA extraction and relative gene expression was determined as described below.

2.2.5.1. Total RNA Extraction

Cells were collected from each study to assess the relative mRNA expression of enteric hormones. Extraction of RNA was carried out using the TRIzol reagent procedure as described in section 2.2.4.1. The purity of RNA was determined using Nanodrop 2000C. The RNA is stored in -80°C until further required.

2.2.5.2 cDNA Synthesis

cDNA was synthesized using iScript cDNA synthesis kit as described in section 2.2.4.2. The samples were stored at -20°C until used as a template for quantitative PCR.

2.2.5.3. Real Time Quantitative PCR

The relative mRNA expression of enteric hormones (GLP-1, GIP, CCK and PYY) was conducted using iQ™ SYBR® green supermix (Bio-Rad, Catalog# 170-8880) for 35 cycles. All reactions were performed with final volume of 20µL as per **Table 2.7**. The reaction protocol was set up with CFX connect real time PCR detection system (Bio-Rad, Canada) as per **Table 2.8**. Forward and reverse primers for proglucagon, GIP, CCK and PYY were synthesized as described in section 2.2.4.3 and are provided in **Table 2.9** (for β-Actin primers refer **Table 2.3**).

Table 2.7. Components of PCR Reaction Set Up.

Components	Volume per reaction
iQ™ SYBR® Green Supermix	10µL
Forward Primer	0.5µL
Reverse Primer	0.5µL
cDNA template	1µL
Sterile Water	8µL
Total Volume	20µL

Table 2.8. PCR Reaction Set Up in CFX Connect.

	Step 1	Step 2	Step 3	Step 4	Step 5
Temperature	95°C	95°C	Annealing Temp	72°C	4°C
Time	2 minutes	10 seconds	30 seconds	30 seconds	Infinite Hold

Table 2.9. Primer Pairs for preproglucagon, GIP, CCK and PYY with Respective Annealing Temperatures

Gene	Forward (5' – 3')	Reverse (3' – 5')	Amplicon Size (bp)	Annealing Temperature
Mouse Preproglucagon (AF276754.1)	aatcttgccaccagggactt	agtgactggcacga gatgtt	112	56.3°C
Mouse GIP (NM_008119.2)	acaaagaggcacaggaga gc	agccaagcaagcta aggtca	180	60°C
Mouse CCK (NM_001284508)	tttcctgcccgcatttgaac	aatccatccagccc atgtagtc	153	60°C
Mouse PYY (NM_145435.1)	ttcaggccagaaggtttgga	acaccgagatatga agtgccc	122	59°C

A melting curve analysis was carried out at 65°C to 95°C, which is helpful in order to check for any dimer formation or artifacts. C_T was obtained for both reference gene and gene of interest. Reference genes/housekeeping genes are important in order to help normalize the RT-qPCR data. It is important that housekeeping genes be validated separately for each gene of interest and for each test group and control group. A number of housekeeping genes are available, including β-Actin, GAPDH and Elongation Factor (EF). RT-qPCR data is generally analyzed using relative quantification method rather than the absolute quantification. In absolute quantification, the target gene is compared to a standard curve, whereas in relative quantification analysis, the target gene is compared to a reference/housekeeping (HK) genes. The C_T values for both gene of interest and HK genes is determined for control group and the experimental group. The data is normalized

to HK genes ($\Delta C_T = C_{T\,(exp)} - C_{T(control)}$). The reference gene used for this research was β-Actin. Relative mRNA expression of enteric hormones were normalized with β-Actin from the same samples according to Livak method [236].

2.2.6. Dose Dependent Effect of Nesfatin-1 on GLP-1, GIP, CCK and PYY Secretion

This study was conducted to determine the changes in GLP-1, GIP, CCK and PYY secretion from STC-1 cells into the media, after treatment with nesfatin-1. Cells were cultured as described in section 2.2.1. Twenty four hours prior to study, cells were seeded into 24-well plates at 2 X 10^5 cells/well in 1mL DMEM (80% confluence). On the day of study, wells were washed twice with 1X DPBS (1mL/well). The cells were then incubated with DMEM containing 10, 1, 0.1, 0.01 and 0.001 nM (n = 8 wells/treatment) rat full length nesfatin-1 (Abgent Technologies, USA, >95% purity). Controls were incubated with culture media only (without nesfatin-1) for the same period of time. After 1 hour, media were collected. In order to prevent cell debris, samples were centrifuged at 13,000 rpm for 10 minutes at 4°C and top 500 µL was stored at -20°C until measured. The studies were repeated twice and data from these independent studies were pooled. The relative secretion levels of enteric hormones into media were measured using immunoassays as described below.

2.2.6.1. Immunoassays (ELISA and RIA)

GLP-1, GIP and CCK secretion levels into the media were measured using the multispecies GLP-1 total ELISA kit (Millipore Inc., Catalog# EZGLP1T-36K), Rat/mouse GIP (total) ELISA kit (Millipore Inc., Catalog# EZRMGIP-55K) and rat/mouse CCK ELISA kit (Abnova, Catalog# KA1862) respectively. PYY in media was measured using rat/mouse PYY radioimmunoassay kit (Millipore Inc., Catalog# RMPYY-68HK). All procedures were carried out according to the manufacturer's instructions. The limit of assay sensitivity was 1.5 pM, 8.2 pg/mL, 17.74 pg/mL and 15.6 pg/mL for GLP-1, GIP, CCK and PYY respectively. The detectable range was from 4.1 – 1000 pM, 8.2 – 2000 pg/mL, 0.1 – 1000 pg/mL and 5 – 500 pg/mL for GLP-1, GIP, CCK and PYY respectively. The plates were read using SoftMAX® 190 microplate reader equipped to read absorbance at 450nm (Molecular Devices, USA). The amount of immunoreactive material

was determined using non-linear regression curve-fit, which was used to quantify and compare the concentration of enteric hormone secretion in media samples.

2.2.7. *In Vivo* Effects of Nesfatin-1 on Enteric Hormone Secretion in Male C57BL/6J Mice

This study was conducted to determine the changes in GLP-1, GIP, CCK and PYY circulation levels in the blood plasma in response to subcutaneous infusion of nesfatin-1 using an osmotic pump. It was designed in such a way that the effect of nesfatin-1 on intestinal hormone release post-glucose oral ingestion can be compared with saline controls. Age matched (5 weeks old, average body weight: 20 grams) C57BL6J male mice (The Jackson Laboratory, USA) were housed as described in section 2.2.2. Mice were divided into two groups either infused with nesfatin-1 (n = 6) or infused with saline (n = 6). Mice had *ad libitum* access to standard mouse chow and water throughout the experiment. On the day of experiment, osmotic pumps (ALZET®, Catalog# 2001D) were filled either with saline or 100 µg/Kg body weight of nesfatin-1 dissolved in saline, as per the manufacturer's instructions. The pumps were then primed over 10 cm plates containing 0.9% saline in an incubator at 37°C for 2 hours. This was followed by subcutaneous implantation of pumps into animals. The pumps were capable of delivering consistently 8 µL/hour of either saline or nesfatin-1 for 24 hours. During implantation, animals were anesthetized using isoflurane (4-5% + 0.8-1 L/min). Upon induction of anesthesia, the effect was maintained by isoflurane (1-3% + 0.8-1 L/min). Briefly incisions were made on the dorsal side of the animal's skin between the scapulae. Using a hemostat, a small pouch was formed by spreading the subcutaneous connective tissues apart. The pumps were then inserted into the pocket with the flow moderator of the pump pointing away from incision. The incisions were then closed with wound clips. Animals were checked for normal activity and behavior upon recovery from anesthesia.

Blood glucose levels were measured from the tail vein using glucometers (OneTouch™ UltraMini®) six and fifteen hours post-implantation. The animals were then given an oral load of 1 g/kg body weight D-glucose (Sigma-Aldrich, Catalog# G8270) using Popper™ animal feeding needles (Popper and Sons Inc., Catalog# 01-290-10A). The blood glucose levels were measured 15 minutes post oral load of D-glucose. The

animals were then euthanized by decapitation followed by collection of blood, pancreas, whole stomach, liver, large intestine and small intestine from each mice. To maintain consistency, the timing and duration of experiment, and sample collection were kept constant for all animals.

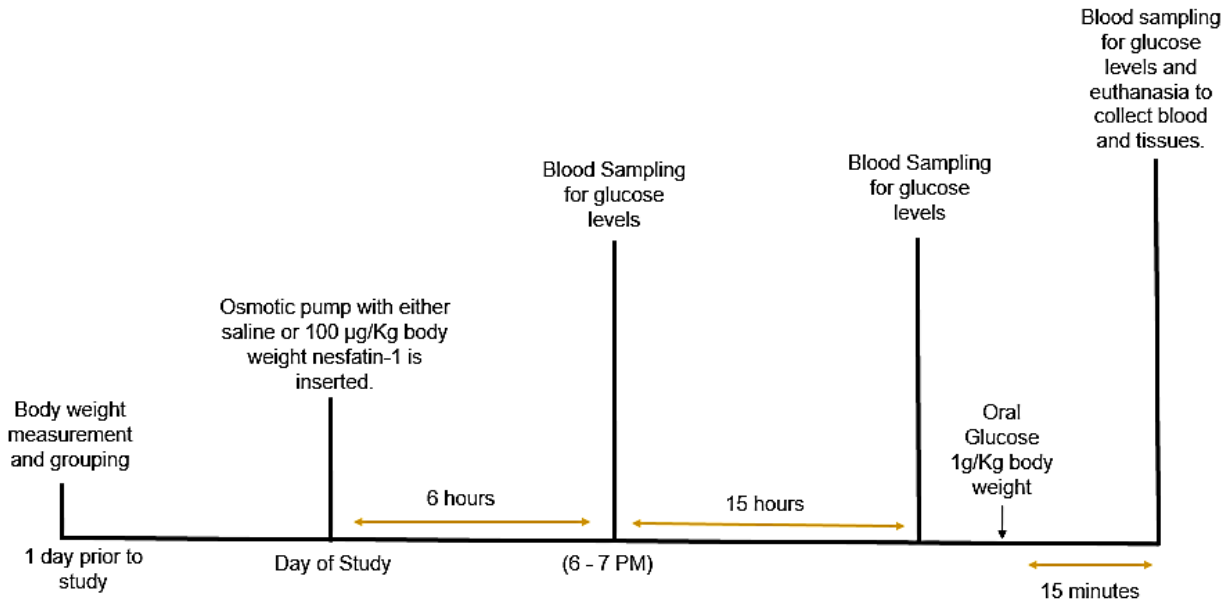

Figure 2.1. A schematic of the animal studies to test the *in vivo* effects of nesfatin-1 on enteric hormone secretions.

2.2.7.1. Immunoassays (ELISA and RIA)

Blood samples collected were allowed to clot on ice, and serum was separated by centrifugation (7000 rpm for 9 minutes at 4°C) and stored at -20° C until assays are conducted. GLP-1, GIP, CCK and PYY levels in circulation are yet to be measured using ELISA and RIA as described in section 2.2.4.1 owing to the low volumes of blood plasma samples obtained.

2.2.8. Statistical Analysis

Analysis of the quantified RT-qPCR and immunoassays data were conducted using One-way ANOVA followed by Tukey's multiple comparison test. IBM SPSS™ version 21 (IBM., USA) as used for statistical analysis and GraphPad Prism version 5 (GraphPad Inc., USA) was used for generation of graphs. Significance was assigned when $p<0.05$. All data are expressed as mean ± standard error of mean (SEM).

2.3. RESULTS

2.3.1. *NUCB2* mRNA Expression in STC-1 Cells

A band of approximately 200 bp of *NUCB2* mRNA was detected (**Figure 2.2**) in STC-1 cells. The no template negative control was also incorporated in the gel.

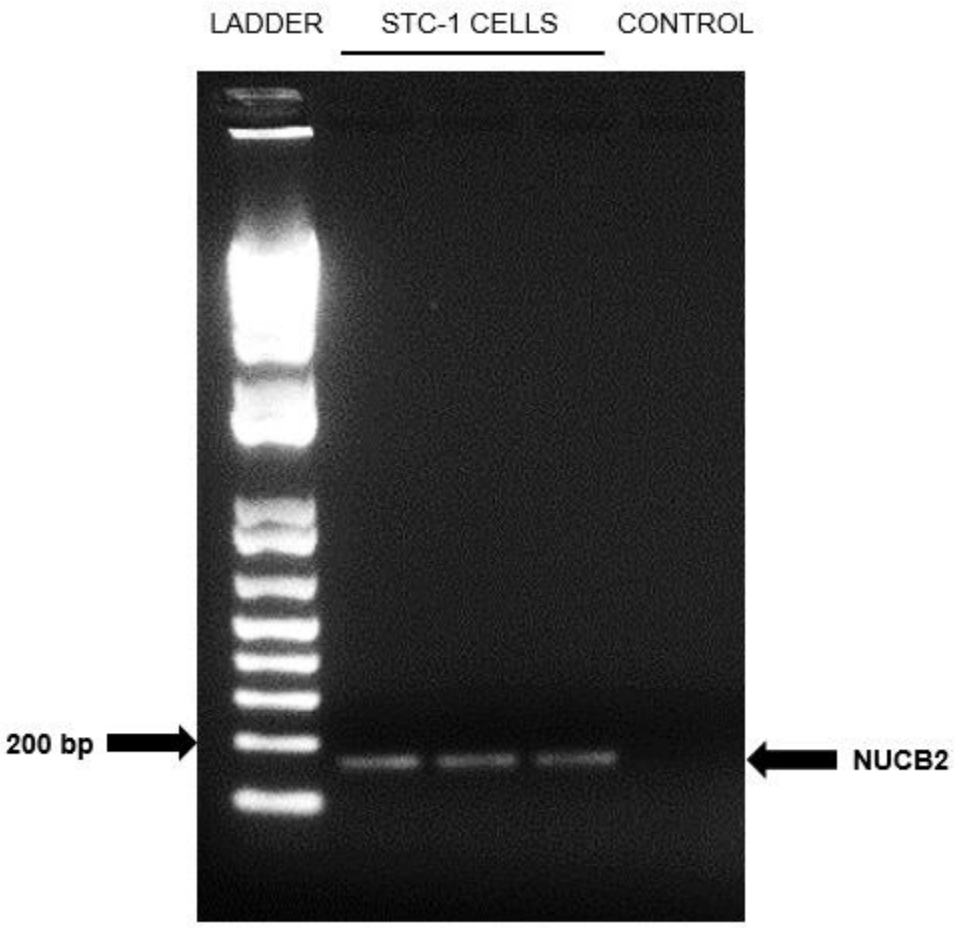

Figure 2.2. Gel electrophoresis image showing the presence of *NUCB2* mRNA in STC-1 cells. The amplicon size of the band was approximately 200 base pairs. The presence of a band provides evidence that *NUCB2* mRNA is expressed in STC-1 cells and it is indicative that enteroendocrine cells could be a source of NUCB2/nesfatin-1 *in vivo*.

2.3.2. Nesfatin-1 Immunoreactivity in the Cytoplasm of STC-1 Cells

Fluorescence microscopy imaging at a magnification of 40X, showed the existence of NUCB2/nesfatin-1 immunopositive cells. The figure below shows images of the cells cultured, fixed and stained in chamber slides. Nuclei were stained with DAPI and the immunopositive cells are stained red. NUCB2/nesfatin-1 immunoreactivity was observed in the cytoplasm of STC-1 cells (**Figure 2.3 – (A – C)**). The preabsorption control and no primary antibody control showed no presence of NUCB2/nesfatin-1 immunoreactivity (**Figure 2.3 – (D – E)**).

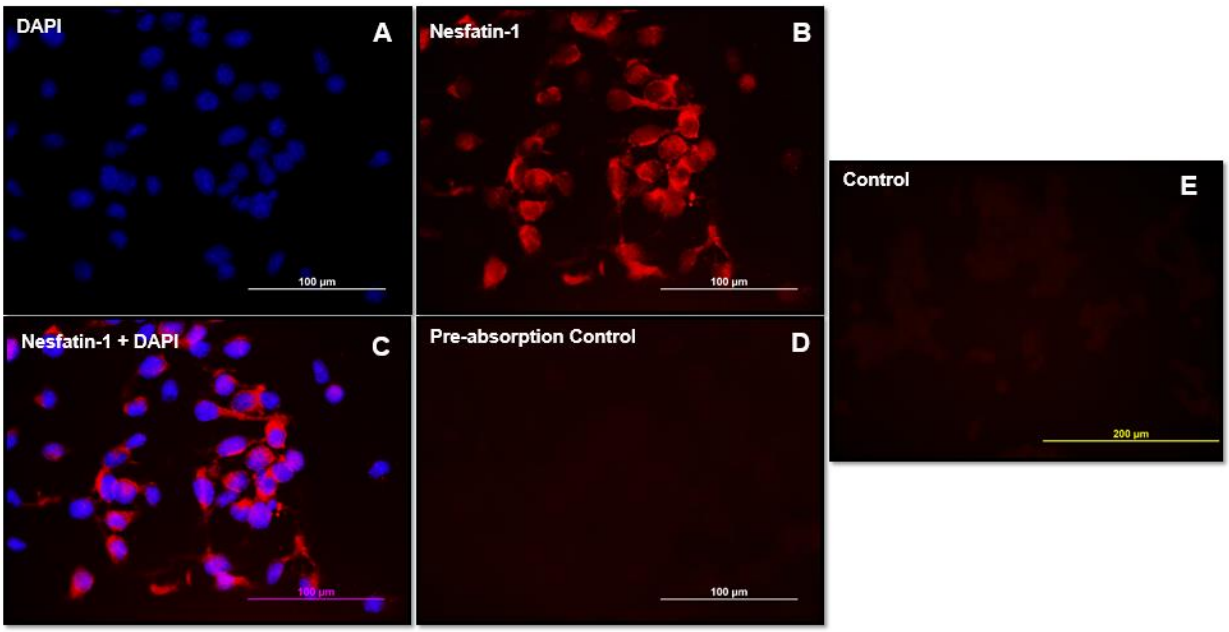

Figure 2.3. Characterization of NUCB2/nesfatin-1 immunoreactivity in STC-1 cells. **A** shows staining of nucleus by DAPI. Immunocytochemical staining of STC-1 cells for nesfatin-1 IR is depicted in **B**. Red staining of the cytoplasm shows the presence of nesfatin-1 IR. Merged images of nesfatin-1 and DAPI staining are shown in **C**. Primary antibody pre-absorbed in 10 µg of nesfatin-1 and labeled with secondary antibody is

shown in **D**. No-primary antibody negative control, labeled only with secondary antibody is shown in **E**.

2.3.3. Nesfatin-1 is Co-localized with GLP-1, GIP, CCK and PYY Immunoreactive Cells in the Intestine of Male C57BL/6J Mice

Fluorescence microscopy imaging at a magnification of 40X, showed the existence of NUCB2/nesfatin-1 (**B, E, G, J**) and the presence of GLP-1 (**A**), GIP (**D**), PYY (**H**) and CCK (**K**) immunopositive cells in duodenum. The figure below shows images of intestinal sections (small intestine – GLP-1, CCK and GIP; large intestine – PYY) collected from *ad libitum* fed male C57BL/6J mice. The tissues were incorporated in wax block, sectioned, fixed and stained over glass slides. Nuclei were stained blue with DAPI and immunopositive cells are stained in green and red, and co-localization is shown in yellow respectively (**Figure 2.4**).

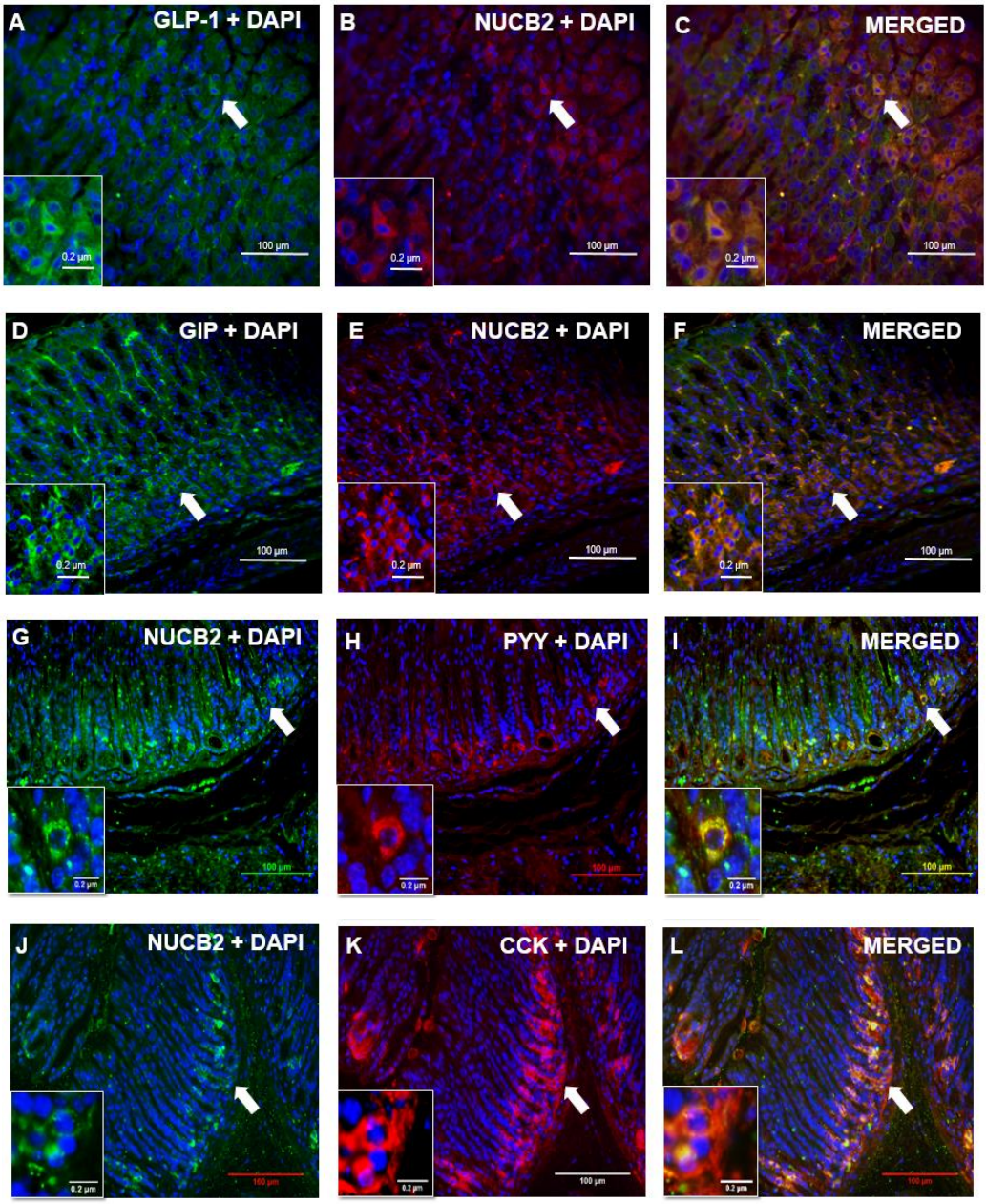

Figure 2.4. Nesfatin-1 is co-localized with GLP-1, GIP, CCK and PYY immunopositive cells in the intestine of male C57BL/6J mice. Immunohistochemical staining of intestinal sections for nesfatin-1 IR is depicted in **B, E, G** and **J**. Red (**B** and **E**) and green staining (**G** and **J**) shows the presence of nesfatin-1 IR in the cytoplasm of enteroendocrine cells. Staining for PYY, CCK, GLP-1 and GIP IR is depicted in **A, D**, **H** and **K**. Green (**A** and **D**) and red staining (**H** and **K**) shows the presence of respective hormone IR in the cell cytoplasm as well. Merged images of nesfatin-1 with enteric hormones and DAPI are

shown in yellow in **C, F, I** and **L**. No-primary antibody negative control, treated only with secondary antibody was tested for all sections above (data not shown).

2.3.4. Nesfatin-1 Upregulates Proglucagon mRNA and GLP-1 Secretion in STC-1 Cells

Nesfatin-1 significantly upregulated proglucagon mRNA expression and GLP-1 secretion into media in STC-1 cells (**Figure 2.5**).

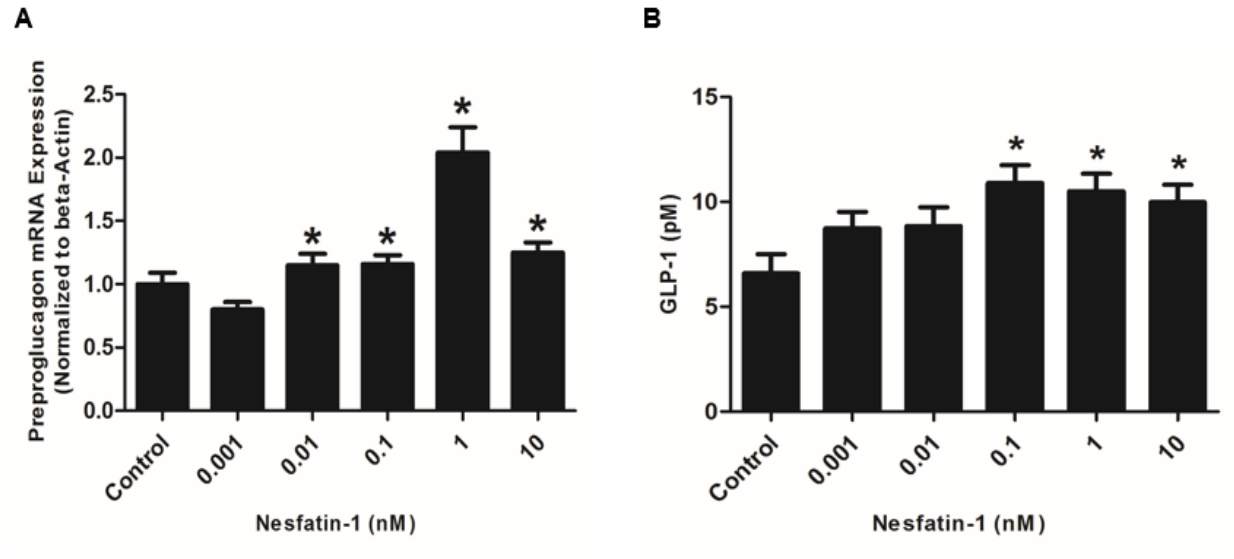

Figure 2.5. Treatment of STC-1 cells dose dependently with nesfatin-1 significantly upregulated proglucagon mRNA expression corresponding to 0.01, 0.1, 1 and 10 nM doses compared to control (**A**). Nesfatin-1 treatment also increased GLP-1 secretion into the media corresponding to 0.1, 1 and 10 nM doses in STC-1 cells (**B**). The mRNA expression data was normalized using β-Actin as the reference gene. Statistical analysis: One-way ANOVA with Tukey's multiple comparison test (n = 8 wells/ treatment). Data pooled from two independent studies.

2.3.5. Nesfatin-1 Upregulates GIP mRNA Expression and Secretion in STC-1 Cells

Nesfatin-1 significantly upregulated GIP mRNA expression and GIP secretion into media in STC-1 cells (**Figure 2.6**).

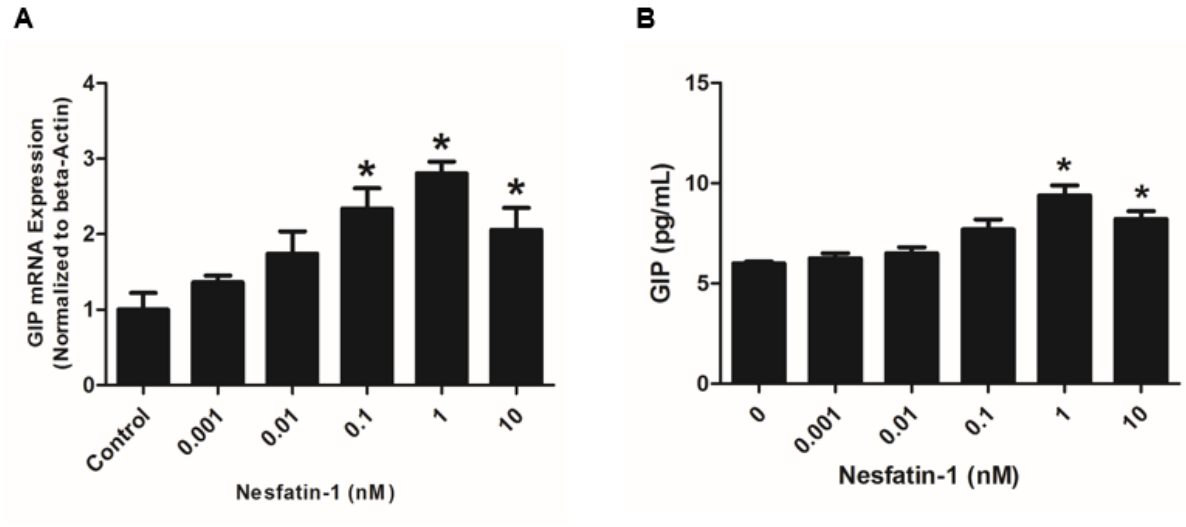

Figure 2.6. Treatment of STC-1 cells dose dependently with nesfatin-1 significantly upregulated GIP mRNA expression corresponding to 0.1, 1 and 10 nM doses compared to control (**A**). Subsequently, nesfatin-1 treatment increased GIP secretion into the media corresponding to 1 and 10 nM doses in STC-1 cells (**B**). The mRNA expression data was normalized using β-Actin as the reference gene. Statistical analysis: One-way ANOVA with Tukey's multiple comparison test (n = 8 wells/ treatment). Data pooled from two independent studies.

2.3.6. Nesfatin-1 Upregulates CCK mRNA Expression and Secretion in STC-1 Cells

Nesfatin-1 significantly upregulated CCK mRNA expression and CCK secretion into media in STC-1 cells (**Figure 2.7**).

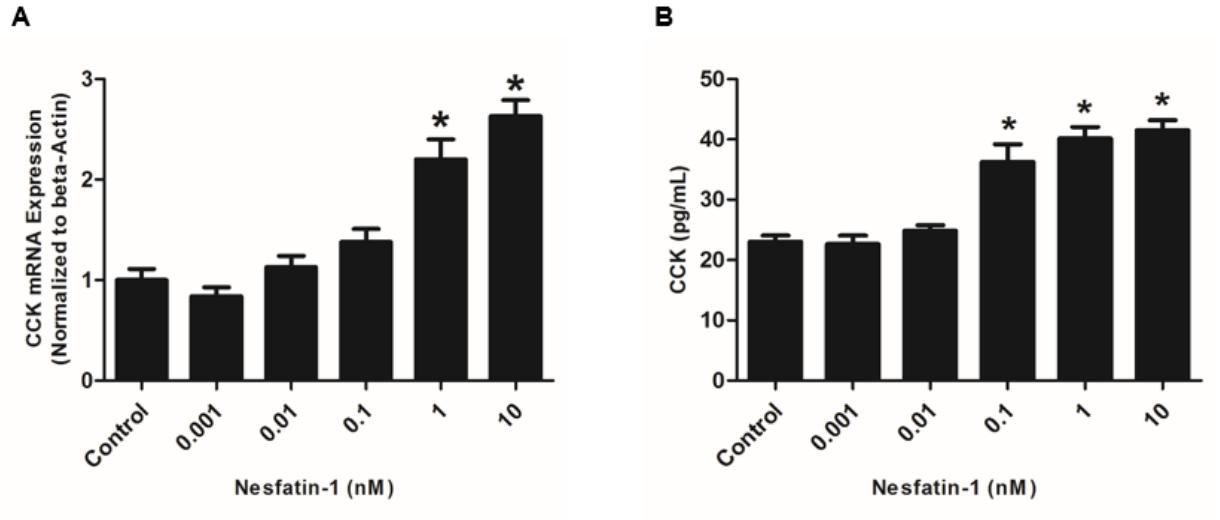

Figure 2.7. Treatment of STC-1 cells dose dependently with nesfatin-1 significantly upregulated CCK mRNA expression corresponding to 1 and 10 nM doses compared to control (**A**). Subsequently, nesfatin-1 treatment increased CCK secretion into the media corresponding to 0.1, 1 and 10 nM doses in STC-1 cells (**B**). The mRNA expression data was normalized using β-Actin as the reference gene. Statistical analysis: One-way ANOVA with Tukey's multiple comparison test (n = 8 wells/ treatment). Data pooled from two independent studies.

2.3.7. Nesfatin-1 Downregulates PYY mRNA Expression and Secretion in STC-1 Cells

Nesfatin-1 significantly downregulated PYY mRNA expression and PYY secretion into media in STC-1 cells (**Figure 2.8**).

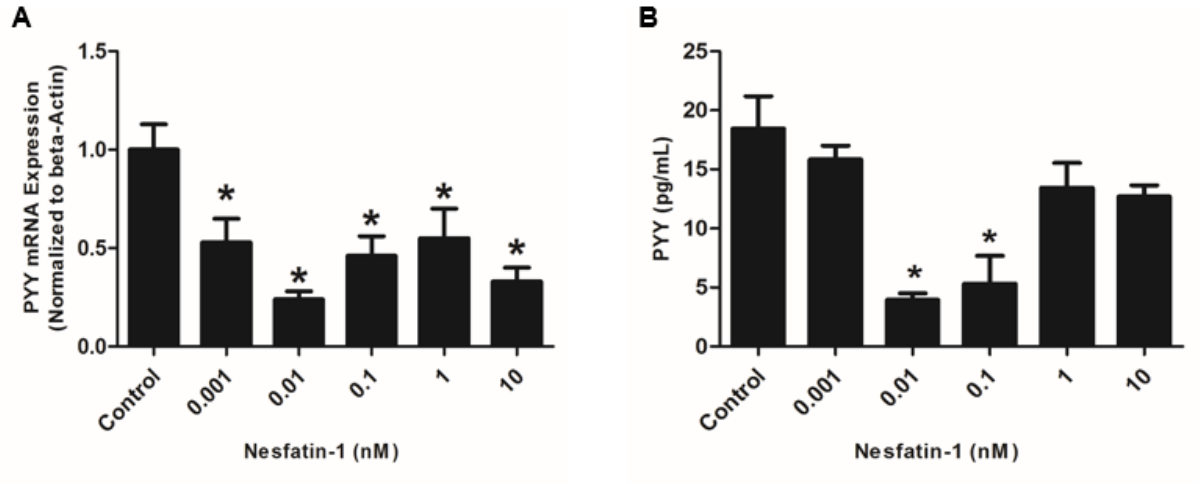

Figure 2.8. Treatment of STC-1 cells dose dependently with nesfatin-1 significantly downregulated PYY mRNA expression corresponding to all doses tested, compared to control (**A**). Subsequently, nesfatin-1 treatment decreased PYY secretion into the media corresponding to 0.01 and 0.1 nM doses in STC-1 cells (**B**). The mRNA expression data was normalized using β-Actin as the reference gene. Statistical analysis: One-way ANOVA with Tukey's multiple comparison test (n = 8 wells/ treatment). Data pooled from two independent studies.

2.3.8. Subcutaneous Infusion of Nesfatin-1 Does Not Alter Glucose Handling in Male C57BL/6J Mice

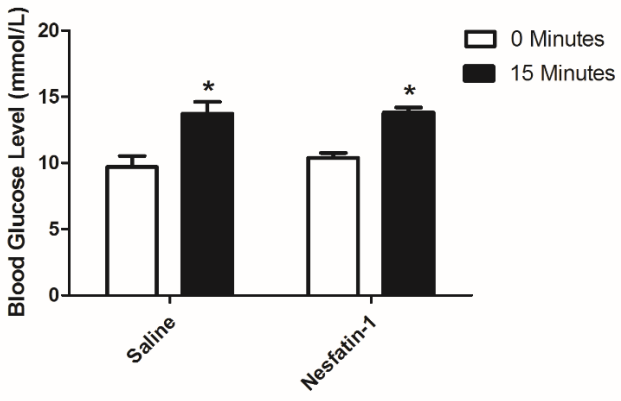

Figure 2.9. Administration of an oral load of 1 g/kg body weight D-glucose significantly increased blood glucose levels in both saline and in groups infused subcutaneously with

100 µg/kg body weight nesfatin-1. Statistical Analysis: Mann-Whitney U test (non-parametric) (n = 6 animals/group).

2.4. DISCUSSION

Nesfatin-1 is an anorexigenic peptide that was found in several tissues including the hypothalamic feeding centers, pancreas and stomach of mice [9, 12, 14]. Presence of *NUCB2* mRNA expression is also detected in non-mammalian species including goldfish [180]. Nesfatin-1 suppresses gut ghrelin in goldfish [184], the first report on the effects of nesfatin-1 on a gastric hormone. More recently, the presence of NUCB2/nesfatin-1 in mice intestine was characterized by Zhang *et al*., [193]. They showed that NUCB2/nesfatin-1 IR cells are localized in the lower third and middle portion of the gastric mucosal gland and the submucous layer in the duodenum of Sprague Dawley (SD) rats and institute of cancer research (ICR) mice. Also, Western blot analysis showed higher NUCB2 protein expression in pancreas, stomach and duodenum. However, this was contradictory to what was reported earlier by Stengel *et al*., [14] wherein they did not find nesfatin-1 immunopositive cells in the intestine. The research characterizes nesfatin-1 expression in the gastric mucosa of stomach and found that *NUCB2* mRNA expression is 10 fold higher in this region, compared to the total RNA extracts from rat brain. However, immunohistochemistry of intestinal sections showed absence of nesfatin-1 immunopositive cells in esophagus, colon and small intestine. Nonetheless, recent findings from our lab showed *NUCB2* mRNA and protein expression in large and small intestine of male C57BL/6J mice, contradicting the earlier observation by Stengel *et al*., [15]. This is in line and supportive of previous observations by Zhang *et al*., showing presence of NUCB2/nesfatin-1 in the intestine of both mice and rats. The findings in this chapter reiterate and confirm the consensus that NUCB2/nesfatin-1 is present in the intestine and that nesfatin-1 modulates enteric hormone secretion *in vitro*.

First, we showed that *NUCB2* mRNA is expressed in STC-1 cells and found nesfatin-1 immunoreactivity in their cytoplasm. These results are highly indicative that intestinal enteroendocrine cells could be a source of endogenous nesfatin-1 *in vivo*. Secondly, immunohistochemical staining of intestinal sections from male C57BL/6J mice showed NUCB2/nesfatin-1 immunoreactivity. Nesfatin-1 immunopositive cells,

specifically were observed in the crypts of the intestinal villi and in the submucosa being mainly concentrated around the Brunner's glands and in blood vessels surrounding the connective tissue. This is consistent with the observation by Zhang *et al.,*[193] wherein they found NUCB2/nesfatin-1 IR in the Brunner's glands of duodenum. These glands are primarily confined to the duodenal bulb. The main function of these glands are to produce an alkaline secretion containing bicarbonate and mucus to protect duodenum from the acidic chime, maintain an alkaline environment for proper action of intestinal enzymes and, to lubricate the intestinal walls. The presence of nesfatin-1 IR here highlights its potential role in enzyme activation, nutrient absorption and preservation of intestinal walls which warrants further consideration [193]. Besides enzymes, intestine is a major endocrine tissue, giving rise to a spectrum of hormones that has important functions in feed intake and energy metabolism as discussed earlier in section 1.2.2. Since we found nesfatin-1 IR in intestinal sections (both small and large intestine) and since our previous findings clearly shows its protein and mRNA expression in the same region [15] we hypothesized that nesfatin-1 could modulate intestinal enteric hormone secretion.

As a start point to address this hypothesis, immunohistochemical analysis of intestinal sections from *ad libitum* fed male C57BL/6J mice showed nesfatin-1 IR to be co-localized with GLP-1, GIP and CCK IR in small intestine and with PYY IR in large intestine. The co-localization was found distributed uniformly throughout the submucosa and in the crypts of villi. However, not all the cells observed under a given area of the section showed co-localization. There were some cells that were immunopositive for nesfatin-1 but not for the respective intestinal hormones. This is expected as enteroendocrine cells are capable of synthesizing and secreting all major enteric hormones tested in this chapter i.e. GLP-1, GIP, CCK and PYY depending on the nutritional status and nutrient availability of animal *in vivo*. Although, they are classified cytochemically as K, L and I cells, this classification is based on the major hormones they secrete and shouldn't be inferred that the cells secrete that specific hormone alone. Nonetheless, in all sections nesfatin-1 immunopositive cells were found in the aforesaid region consistently. The colocalization of nesfatin-1 with intestinal hormones suggests possible local (autocrine/paracrine) or endocrine actions of nesfatin-1 on these peptides.

Thirdly, *in vitro* studies involving treatment of STC-1 cells dose-dependently with nesfatin-1 showed increase in GLP-1, GIP and CCK mRNA expression and secretion into media. The study also showed that nesfatin-1 decreased PYY mRNA expression and secretion into media by RT-qPCR and immunoassays. From a food intake and appetite standpoint, previous research has classified GLP-1, GIP and CCK as anorexigenic peptides and PYY as orexigenic [93, 139, 147]. Our current findings show that nesfatin-1 is directly modulating, in that it increases the expression and secretion of anorexigenic intestinal hormones (GLP-1, GIP and CCK) and it suppresses the expression and secretion of orexigenic hormone PYY. Also, with regard to insulin secretion, previous research have shown incretins and CCK to be insulinotropic and PYY to be insulinostatic [6-8, 16]. Results from *in vitro* studies shows that nesfatin-1 increases insulinotropic and suppresses insulinostatic intestinal hormones. These novel results reveal yet another extra-pancreatic function of NUCB2/nesfatin-1 in peripheral tissues, besides the reported insulinotropic action [5]. The results also highlight an indirect mechanism of nesfatin-1 action on insulin secretion, by modulating the expression/secretion levels of insulinotropic/insulinostatic enteroendocrine hormones.

Analyzing the data points for the results from *in vitro* studies, the nesfatin-1 effect on GLP-1 expression and secretion is highly dose-dependent, with the upregulation quite apparent between 0.1 and 10 nM when compared to controls. One common concern regarding the RT-qPCR data on the relative mRNA expression of proglucagon is the specificity of the primer used, as proglucagon gene besides GLP-1, also encodes GLP-2, oxyntomodulin and glicentin in intestinal L cells *in vivo*. To negate this, the primer pairs used in the study is highly specific for the GLP-1 coding region of proglucagon gene and an upregulation in this region of the mRNA alone is the outcome of our dose-dependent nesfatin-1 treatment data. The dose-dependency on GIP expression and secretion is also clear, with nesfatin-1 having a stimulatory effect at higher doses of 1 and 10 nM tested. It will be interesting to see the extrapolation of this result in particular *in vivo*, as GIP secretion from K cells is modulated more by the rate of nutrient absorption in intestine rather than nutrient availability [113]. Therefore, nesfatin-1 modulation of GIP secretion *in vivo* is expected to be difficult to capture with studies on feed availability alone and might require a study testing various diets with differential rates of nutrient absorption.

Nesfatin-1 effect on CCK secretion also is dose-dependent with maximum expression and secretion being observed corresponding to the highest doses tested i.e. 1 and 10 nM. Though this effect is on total CCK in media, its effect on CCK-33 which is the predominant circulating form [134] or CCK-8 [139] that induces satiety upon infusion remains to be elucidated. Interestingly, the suppressive effect of nesfatin-1 on PYY expression and secretion is not dose-dependent at least at the level of translation/secretion. PYY is predominantly expressed in the large intestine, and in cell studies nesfatin-1 suppressed PYY mRNA at all doses tested. However, the downregulation was significant corresponding only to two doses at the level of secretion i.e. 0.01 and 0.1 nM. Our immunohistochemistry data shows co-localization of nesfatin-1 IR with PYY. On a comparative basis, the number of cells positive for co-localization is lesser than those compared to GLP-1 and CCK co-localization. As our *in vitro* data suggests opposite actions for nesfatin-1 and PYY, whether or not they are secreted in distinct cytoplasmic vesicles similar to nesfatin-1 and ghrelin in X/A like cells remains to be elucidated. Also considering the time food takes to reach large intestine *in vivo* and that intestinal PYY increases transit time of ingested food [237], the role of nesfatin-1 in these cells is likely to be paracrine wherein it stimulates other enteric hormone secretion upon secretion and autocrine wherein it inhibits PYY secretion at least immediately after an ingested meal.

Nesfatin-1 at an effective dose of 0.1 and 1 nM was reported to upregulate preproinsulin mRNA expression and glucose-dependent insulin release from MIN6 cells. It also stimulated insulin release from isolated mouse islets corresponding to 0.01, 0.1 and 1 nM doses [5]. These data are comparable with results from *in vitro* studies in STC-1 cells, wherein nesfatin-1 at similar doses of 0.1, 1 and 10 nM doses was effective in stimulating GLP-1, GIP, CCK secretion and inhibiting PYY secretion. In *in vivo* studies, the circulating glucose levels were significantly elevated in both saline and nesfatin-1 infused mice in response to an oral load of glucose, showing normal glucose handling in both groups. This result verifies that the glucose administration was effective, and there are no differences in glucose levels due to subcutaneous nesfatin-1 infusion alone. We originally planned and executed ELISAs for enteric hormones. However, due to low volumes of serum obtained and used, the ELISA results were not usable (most values

below low standard). Due to this setback in obtaining secreted levels of hormones, we are now planning intestinal hormone gene/protein expression in major peripheral tissues: stomach, pancreas, liver, and intestine, using the tissues collected at the end of the OGTT. This analysis is expected to partly provide the missing information on synthesis of intestinal hormones. Osmotic mini-pumps are reliable tools to infuse peptides of interest, and it was used previously for successfully administering and elevating nesfatin-1 levels in rats [13]. In order to ensure that circulating nesfatin-1 levels are indeed increased in response to continuous infusion using the mode selected, we will measure nesfatin-1 *in vivo* in control and peptide treated mice.

2.5. CONCLUSIONS

This research provides strong evidence for NUCB2/nesfatin-1 expression in intestine thus strengthening previous observations [15, 193]. It also for the first time reports the co-localization of nesfatin-1 with CCK, PYY, GLP-1 and GIP immunopositive cells in mouse intestine. The studies carried out characterizes STC-1 cells as NUCB2/nesfatin-1 expressing cells and that nesfatin-1 dose-dependently modulates hormone secretions from this cell line. This highlights STC-1 cells as useful *in vitro* models for studying nesfatin-1 biology, besides the secretion kinetics of intestinal hormones. The *in vivo* studies pertaining to this objective is currently in progress and will be completed soon. Results from them will provide a more complete picture whether or not nesfatin-1 modulates enteric hormone secretion *in vivo* via osmotic pump infusion in mice. Nesfatin-1 has been researched for its role on insulin secretion and satiety. It is becoming increasingly apparent that both central and peripheral nesfatin-1 have functions beyond these two, and this chapter is a major contribution in line with this notion. The results that nesfatin-1 is co-expressed with major intestinal hormones and that it modulates their secretion opens up a lot of possibility for testing new biological actions of this peptide.

TRANSITION

The following chapter focuses on objectives 3-4: Insulinotropic action of a NUCB1 encoded Nesfatin-1-Like Peptide (NLP). Endogenous *NUCB1* mRNA in pancreatic islets of C57BL/6J mice and in MIN6 cells by RT-qPCR and gel electrophoresis were detected. Subsequently, NUCB1 immunoreactivity was co-localized with insulin in islets and MIN6 cells and was detected by immunocytochemistry. This chapter will elaborate the studies carried out, major techniques used for answering specific research question mentioned above and the results obtained. This is followed by a discussion and conclusion of major findings pertaining to the research question. At the time of writing this chapter, nothing is known about a nucleobindin-1 (NUCB1) encoded bioactive peptide.

Contribution of Co-authors: Naresh Ramesh conducted the *in vitro* studies, analyzed the results and wrote the first draft of the manuscript. Ms. Haneesha Mohan assisted in carrying out the immunocytochemistry and qualitative analysis of *NUCB1* expression in mouse pancreas and MIN6 cells. Dr. Suraj Unniappan performed the *in silico* analysis of NUCB1 amino acid sequence, provided the infrastructure, funding, research ideas, assisted with experimental design, data analysis and manuscript preparation.

Publication: A part of this chapter has been published *(Article in Press)*.

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CHAPTER 3

INSULINOTROPIC ACTION OF A NUCB1 ENCODED NESFATIN-1-LIKE PEPTIDE (NLP)

3.1. INTRODUCTION

Nesfatin-1 (NEFA/nucleobindin-2-encoded satiety and fat-influencing protein-1) is an 82 amino acid peptide encoded in the N-terminal region of its precursor, nucleobindin-2 (NUCB2) [9, 15]. It was predicted that NUCB2 is processed by prohormone convertases (PC) to form nesfatin-1 [9]. Administration of the full-length nesfatin-1 or its 30 amino acid mid-segment (M30), considered its bioactive core, reduces food intake and fat mass in rodents [9, 13, 182], and food intake in pigs [238] and fish [180, 184]. We, and others have identified nesfatin-1 immunoreactivity in the pancreatic islet beta cells of rats, mice [10, 12, 13] and humans [10] and found an insulinotropic effect for this peptide *in vitro* [5, 10] and *in vivo* [13]. Nesfatin-1 increases glucose-stimulated insulin secretion from β cells by direct action involving Ca^{2+} influx through L-type calcium channels [239]. Nesfatin-1 is emerging as NUCB2-derived multifunctional peptide affecting major organ systems in vertebrates [240]. When originally discovered, nucleobindin-2 was given its name due to its very high sequence similarity with another secreted protein, nucleobindin-1 (NUCB1). Both NUCB1 and NUCB2 are homologous multi-domain Ca^{2+} and DNA binding proteins encoded in two unlinked genes. Human NUCB1 and NUCB2 exhibit 62% amino acid sequence identity and are remarkably conserved within the nesfatin-1 region between humans and zebrafish [19, 240, 241]. NUCB1 has been widely reported within the nucleus, endoplasmic reticulum and cytoplasm of stomach, intestine, adrenal glands, pituitary, ovary and testis [219].The N-terminal golgi retention domain allows NUCB1 to be targeted to various cellular components and its deletion renders the localization of NUCB1 to the cytoplasm [10, 230, 231]. The endogenous expression profile of NUCB1 was characterized recently by immunofluorescence staining, showing NUCB1 localization in the endocrine pancreas along with insulin, glucagon, somatostatin, ghrelin and pancreatic polypeptide immunopositive cells [208]. NUCB1 has been shown to play a crucial role in the maintenance of Ca^{2+} homeostasis, and it interacts with G proteins and

cyclooxygenases [20, 217]. In addition, oh-I and colleagues [9] indicated that both NUCB1 and NUCB2 are secreted proteins arising from two homologous genes arising from a single EF-hand ancestor. Therefore, whether NUCB1 encodes a biologically active nesfatin-1-like peptide remains unknown.

Is there a nesfatin-1-like peptide encoded in NUCB1? Does it possess PC cleavage sites? Is this peptide biologically active? *In silico* analysis found that a nesfatin-1-like peptide (NLP) is indeed present in the NUCB1. This research, for the first time, reports an insulinotropic activity for synthetic NLP. The endogenous NUCB1 protein expression in major peripheral tissues in response to feed availability was also determined. These findings provide important information on a new, naturally occurring insulinotropic peptide.

3.2. MATERIALS AND METHODS

3.2.1. *In Silico* Analysis

NUCB1 and NUCB2 sequences were obtained from the GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and aligned using ClustalW (http://www.clustal.org/clustal2/) and eBioX (http://www.ebioinformatics.org/ebiox/) software. Percentage identity between sequences were ascertained using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was used for predicting the signal peptide region in NUCB1. PC cleavage sites and putative peptides encoded within NUCB1 were predicted by the Prop 1.0 Server (http://www.cbs.dtu.dk/services/ProP/) and NeuroPred™ (http://neuroproteomics.scs.illinois.edu/neuropred.html).

3.2.2. Cell Culture

MIN6 cells used in the study were a kind gift from Dr. Robert Tsushima (York University, Toronto). MIN6 cells are derived from transgenic founder mice that developed pancreatic insulinoma [242]. These cells have the morphological characteristics of β cells and exhibit glucose-inducible insulin secretion, comparable to normal cultured mouse islets cells. MIN6 expresses high levels of liver type glucose transporter (GT) mRNA,

suggesting the importance of transporter in glucose-stimulated insulin secretion. Treatment of MIN6 with Interferon-γ induces high levels of major histocompatibility complex (MHC) class I antigen on the cellular surface, emphasizing that the cells retain the physiological characteristics of β cells [242]. MIN6 also have been reported to have the innate ability to form cell clusters [243]. Given the study that monolayer cultures of cells have less GSIS, and that cell to cell contact is highly important for GSIS, MIN6 are suitable immortalized cell lines to analyze the molecular mechanisms by which β cells regulate insulin secretion in response to varying concentrations of extracellular glucose.

Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen, Catalog #11995-040) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Catalog# 12484), penicillin (100 U/mL), streptomycin (100 µg/mL) (Invitrogen, Catalog# 15140-122) and 2-Mercaptoethanol (Life Technologies, Catalog# 21985-023). Cells were incubated at 37°C and 5% CO_2 culture conditions in a humidified incubator to promote growth. Cell culture media was changes every 48 hours after washing twice with Dulbecco's Phosphate-Buffered Saline (DPBS, Life Technologies, and Catalog # 14190-250). The cells were sub-cultured once they reached 85-90% confluency using 0.25% trypsin-EDTA (Life Technologies, Catalog# 25200-056) as described earlier [53].

3.2.3. Detection of Endogenous NUCB1 Protein Expression in MIN6 cells and in Mouse Pancreatic Islets

MIN6 cells were cultured in a Labtek^{TM} Chamber Slide System^{TM} (Nalge Nunc, New York). Cells were washed with 1X phosphate buffer saline (PBS), fixed in 4% paraformaldehyde, permeabilized using 0.3% Triton-X (Bioshop, Catalog# TRX777), and incubated in blocking buffer containing 10% goat serum. For immunohistochemical studies, pancreas was collected from male C57BL/6J mice (Charles River, Quebec, Canada) cared under the Canadian Council of Animal Care guidelines, as approved by the University of Saskatchewan Animal Care Committee. Mice were euthanized under deep carbon dioxide inhalation followed by cardiac puncture, pancreas collected and fixed in 4% formaldehyde overnight at 4^{o} C, processed, embedded in paraffin sections of 4 µm thickness were prepared. Cells and slides were then incubated with primary antibody at 4^{o} C overnight, followed by secondary antibody incubation for 4 hours at room

temperature. Primary antibodies used were: rabbit anti-nesfatin-1 (Phoenix Pharmaceuticals, 1:500 dilution, Catalog# H-003-22), rabbit anti-nucleobindin 1 (Abcam, 1:500 dilution, Catalog# ab23387), guinea pig anti-insulin (Abcam, 1:100 dilution, Catalog# ab 7842) or mouse anti-glucagon (Abcam, 1:200 dilution, Catalog# K79bB10). The respective secondary antibodies were goat anti-rabbit Texas Red® IgG (Vector Laboratories, Red-Nesfatin-1, 1:100 dilution, Catalog# TI-1000), goat anti-guinea pig FITC IgG (Abcam, Green-Insulin, 1:200 dilution, Catalog# ab6904) and goat anti-mouse FITC IgG (Abcam, Green-Glucagon, 1:200 dilution, Catalog# ab6785). The slides were washed in 1X PBS and mounted using Vectashield® mounting medium containing the nuclear dye DAPI (Blue; Vector Laboratories). Tissue and cells were analyzed under a BX51 microscope (Olympus, Ontario, Canada), images were captured using an Olympus DP70 camera and were assessed using the DP controller program.

3.2.4 Qualitative Analysis – Expression of *NUCB1* mRNA in MIN6 Cells and in Mouse Pancreas

Cells were grown as described in section 3.2.3.1. Upon confluence the total RNA was extracted as described in section 2.2.4.1. Pancreas was collected and processed from *ad libitum* fed male C57BL/6J mice by euthanizing the animals using cervical dislocation. The collected tissue was dissected and stored in RNALater™ (Life Technologies, Catalog# AM7020). The tissues were then thawed for 10 minutes at room temperature and transferred to new tubes containing TRIzol® regent. This was followed by total RNA extraction using TRIzol method as described in section 2.2.4.1. Synthesis of cDNA was conducted using iScript™ Reverse Transcription Supermix as described in section 2.2.4.2. The cDNA synthesized was used as template to determine the qualitative expression of *NUCB1* in MIN6 cells and mouse pancreas. The primer sequences were obtained from National Center for Biotechnology Information Gene Bank (NCBI) and the primers were synthesized using Primer-BLAST™, primer designing tool from NCBI (www.ncbi.nlm.nih.gov/tools/primer-blast/). The GenBank accession number is given under each gene. The forward and reverse primers are provided in **Table 3.1**.

Table 3.1. Primer Pairs for mouse *NUCB1* and β-Actin with Respective Annealing Temperatures

Gene	Forward (5' – 3')	Reverse (3' – 5')	Amplicon Size (bp)	Annealing Temperature
Mouse NUCB1 (NM_001163662.1)	ggacctcagctagggg gtgta	Agcctaccctaactccca gg	96	60°C
Mouse β-Actin (NM_007393.3)	ccactgccgcatcctctt cc	ctcgttgccaatagtgatg ac	77	60°C

Primers were validated and optimized for high primer efficiency and annealing temperatures. The primers were then used for conducting qualitative PCR. The PCR was conducted on a thermo cycler (Bio-Rad, Canada) for 35 cycles. The samples were stored at -20° C until gel electrophoresis. The components of the mastermix are provided in **Table 2.4**. The components for the qualitative PCR are provided in **Table 2.5**. The reaction protocol used is described in **Table 3.2** below.

Table 3.2. Optimized PCR conditions with respective annealing temperatures for *NUCB1* and β-Actin. (Step 1: Initial Activation; Step 2: Denaturation; Step 3: Annealing; Step 4: Elongation; Step 5: Infinite Hold)

	Step 1	Step 2	Step 3	Step 4	Step 5
Temperature	95°C	95°C	60°C	73°C	4°C
Time	5 minutes	30 seconds	30 seconds	30 seconds	Infinite Hold

Following PCR, the samples were run on 1% agarose gel as described in section 2.2.4.2. The images were captured using GelDoc™ EZ system (Bio-Rad, Canada).

3.2.5. Nesfatin-1-Like Peptide Effects on Preproinsulin mRNA Expression and Insulin Secretion

Static incubation study was conducted to determine the effects of dose-dependent synthetic NLP treatment on preproinsulin mRNA expression in MIN6 cells. Cells were cultured as described in section 3.2.2. Cells at 2 x 10^5 cells per well density were seeded with 1 mL DMEM (25 mM glucose) in 24-well plates. On the day of study, medium was removed and cells were washed twice with PBS. The cells were then treated with 1 mL of DMEM containing 0 (control), 0.001, 0.01, 0.1, 10, 100 or 1000 nM rat synthetic NLP (VPVDRAAPHQEDNQATETPDTGLYYHRYLQEVINVLETDGHFREKLQAANAEDIKSG KLSQELDFVSHNVRTKLDEL; Abgent Technologies, California) based on a 77 amino acid predicted sequence within the rat nucleobindin 1 (GenBank # AAI00644.1). The peptide is >95% pure and the mass and purity were confirmed by LC-MS. The culture media was isolated to evaluate the insulin content by insulin ELISA. Total RNA extraction and preproinsulin relative mRNA expression was determined as described below. The studies were repeated twice and data from these independent studies were pooled. Cells were collected from the study to assess the relative mRNA expression of preproinsulin. Total RNA extraction using the TRIzol method was carried as described in section 2.2.4.1. The purity of RNA was determined using Nandrop™ 2000C. The RNA sample is stored in -80° C until further required. cDNA was synthesized using iScript cDNA synthesis kit as described in section 2.2.4.2. The samples were stored at -20°C until used as a template for RT-qPCR. The relative mRNA expression of preproinsulin was conducted using iQ™ SYBR® green supermix for 35 cycles as described in section 2.2.3.3. Forward and reverse primers for preproinsulin were synthesized as described in section 3.2.4.3 and are provided in **Table 3.3** below. β-Actin was used as internal control and the details are described in **Table 3.1**.

Table 3.3. Primer Pair of Mouse Preproinsulin with Respective Annealing Temperature

Gene	Forward (5' – 3')	Reverse (3' – 5')	Amplicon Size (bp)	Annealing Temperature
Mouse Preproinsulin (NM_008386.3)	ggcttcttctacacaccca	cagtagttctccagctggta	182	59°C

The relative gene expression data were obtained after normalizing the data to β-Actin using Pfaffl method [155].

The insulin secretion into the media was measured using mouse ultrasensitive insulin ELISA kit (ALPCO, Catalog# 80-INSMS-E01) and Insulin-^{125}I RIA kit (MP Biomedicals™, Catalog# 07RK547) according to the manufacturer's instructions. The limit of assay sensitivity was 0.06 ng/mL (ELISA) and 1 ng/mL (RIA). The detectable range for the kits were from 0.188 – 6.9 ng/mL and 0.1 – 25 ng/mL respectively. The plates were read using SoftMAX® 190 microplate reader equipped to read absorbance at 450nm (Molecular Devices, USA). The amount of immunoreactive material was determined using non-linear regression curve-fit, which was used to quantify and compare the concentration of insulin secretion in media samples.

3.2.6. Is a Specific Amino Acid Organization Critical for the Biological Activity of Nesfatin-1-Like Peptide?

There are two reasons for this study. The first objective was to determine whether the NLP sequence is critical for its insulinotropic action. The second aim was to test whether any peptide that shares the same amino acids and length, but highly dissimilar amino acid arrangement could also elicit the same response. In order to test these, we decided to use a scrambled peptide that has 77 amino acids that constitute the NLP, but

not in the order seen in NLP. A 79 amino acid (including the two amino acids constituting the cleavage site) scrambled peptide was designed using the Sequence Manipulation Suite™ (www.bioinformatics.org/sms2/) and PepControls™ (bioware.ucd.ie/~cyclops/.../PepControls.../1.3/.../control_peptides.html) (University of California, Davis) online tool. The peptide scramble (PDSRSDDGSPSVQLQDYALIADAEVTLTHIELFGSPQNATKLLNKTERLRFLKVVRGK HRENVVATEHYQAQKYPEEDE) with the lowest similarity to the NLP amino acid sequence and with low synthesis issues was selected. The peptide synthesized (Pacific Immunology, California, USA) was >95% pure and the mass and purity was confirmed by LC-MS.

For static incubation studies, MIN6 cells at 2 x 10^5 cells per well density were seeded in 1 mL DMEM (25 mM glucose) in 24-well plates. On the day of study, medium was removed and cells were washed twice with PBS. The cells were then treated with 1 mL of DMEM containing 10 and 100 nM rat synthetic nesfatin-1-like peptide, 10 and 100 nM of scrambled peptide and 1 nM synthetic rat nesfatin-1 as a positive control. After 1 hour incubation, media samples were collected and insulin content was measured as described in section 3.2.5.4. The cells were collected and total RNA extracted, cDNA synthesis followed by RT-qPCR for preproinsulin mRNA expression was carried out as described under 3.2.5. The studies were repeated twice and data from these independent studies were pooled.

3.2.7. Quantitative Analysis of Endogenous NUCB1 Protein Expression in *ad libitum* Fed and Food Deprived Mice

3.2.7.1. Animals

Age matched 4 months old C57BL/6J mice (Charles River Laboratories, Quebec, Canada) were housed in a temperature-controlled vivarium with 12h/12h dark and light cycles. Animals had *ad libitum* access to standard mouse chow and water. All protocols strictly adhered to the guidelines of the Canadian Council for Animal Care, and were approved by the University of Saskatchewan Animal Research Ethics Board.

3.2.7.2. Western Blot Analysis

For characterizing the endogenous levels of nucleobindin-1 (NUCB1), 4 months old C57BL/6J male mice (Charles River Laboratories, Canada) were divided into two groups; fed (n = 6) and unfed (n = 6) group respectively. The unfed group were fasted for 24 hours, while the fed group had ad libitum access to standard mouse chow and water during the same time. After 24 hour differential feed availability, all animals were euthanized by cervical dislocation. Dissected whole stomach, pancreas, liver, small and large intestine were homogenized in T-PER® tissue protein extraction reagent (Thermoscientific, Catalog# 78510) followed my measurement of protein concentration by Bradford assay. The samples were prepared in 1X laemmli buffer containing 0.2% 2-mercaptoethanol (Bio-Rad, Catalog# 161-0737 and -0710) and subsequently were boiled at 95°C for 5min followed by vortexing. The whole sample volume (20 µL) each containing 50 µg protein was loaded and run in a Mini-PROTEAN® TGX™ 8-16% gradient gel (Bio-Rad, Catalog# 456-1104). After separation the proteins were transferred to a 0.2 µm BioTrace™ nitrocellulose membrane (PALL Life Sciences, Catalog# 27377-000) and then membrane was blocked in 1X RapidBlock™ solution (aMReSCO, Catalog# M325). NUCB1 protein detection was performed using rabbit antiserum directed against mouse NUCB1 (Custom Antibody, Pacific Immunology, Catalog# 1312-PAC-02) diluted 1:1000 and β tubulin protein was detected by use of rabbit antiserum directed against mouse β tubulin (Cell Signaling Technology®, Catalog# 2146) diluted 1:2000. As secondary antibody, goat anti-rabbit IgG (H+L) HRP conjugate (Bio-Rad, Catalog# 170-6515) diluted 1:3000 was used. For protein visualization the membrane was incubated for 5min in Clarity™ Western ECL substrate (Bio-Rad, Catalog# 170-5061) and imaged using ChemiDoc™ MP imaging system (Bio-Rad, Catalog# 170-8280) with chemiluminescence detection. Membrane stripping in between protein detection was conducted using Restore™ PLUS Western blot stripping buffer (Thermoscientific, Catalog# 46430). Precision plus protein™ Dual Xtra standards (Bio-Rad, Catalog# 161-0377) were used as molecular weight markers.

3.2.8. *In Vivo* Effects of NLP on Dark and Light Phase Feed Intake

Animals were housed as described in section 3.2.7.1. Twenty four hours prior to the study, animals were grouped into two: saline-treated groups (n = 6) and NLP-treated groups (n = 6). On the day of the experiment, osmotic pumps (ALZET®, Catalog# 2001D) were filled either with saline or 100 µg/kg body weight NLP dissolved in saline, as per manufacturer's instruction. Subsequently, the pumps were implanted subcutaneously into animals as described in section 2.2.5 earlier. The feed intake was monitored by measuring the weight of food for all animals at four time point i.e. 1 AM (+6 hour dark phase), 7 AM (+12 hour dark phase), 1 PM (+6 hour light phase) and 7 PM (+12 hours light phase) respectively. To maintain consistency the timing and duration of experiment, and sample measurement were kept constant for all animals.

3.2.9. Statistical Analysis

Analysis of the quantified RT-qPCR and immunoassays data were conducted using One-way ANOVA followed by Tukey's multiple comparison test. IBM SPSS™ version 21 (IBM., USA) as used for statistical analysis and GraphPad Prism version 5 (GraphPad Inc., USA) was used for generation of graphs. Significance was assigned when $p<0.05$. All data are expressed as mean ± standard error of mean (SEM).

3.3. RESULTS

3.3.1. *In Silico* Analysis of NUCB1

A nesfatin-1-like region is present within the NUCB1. The nesfatin-1-like peptide, especially its putative bioactive core (**Figure 3.1 - A**) exhibited very high similarity with nesfatin-1 encoded in NUCB2 (**Figure 3.1 - B**). The bioactive cores of 82 amino acid nesfatin-1 and 77 amino acid NLP have 76.6% amino acid sequence identity in mouse. The bioactive core region (M30) within the NUCB1 presented a greater degree of similarity. A signal peptide cleavage site is present at position 25 (Arginine) and 26 (Valine) in the NUCB1 sequence. Analysis of NUCB1 using the ProP 1.0 Server and NeuroPred™ tools revealed potential proprotein convertase cleavage site at Lys-Arg

(KR), forming a 77 amino acid nesfatin-1-like peptide. Due to its high similarity to nesfatin-1 from NUCB2, we named this protein nesfatin-1-like peptide (NLP).

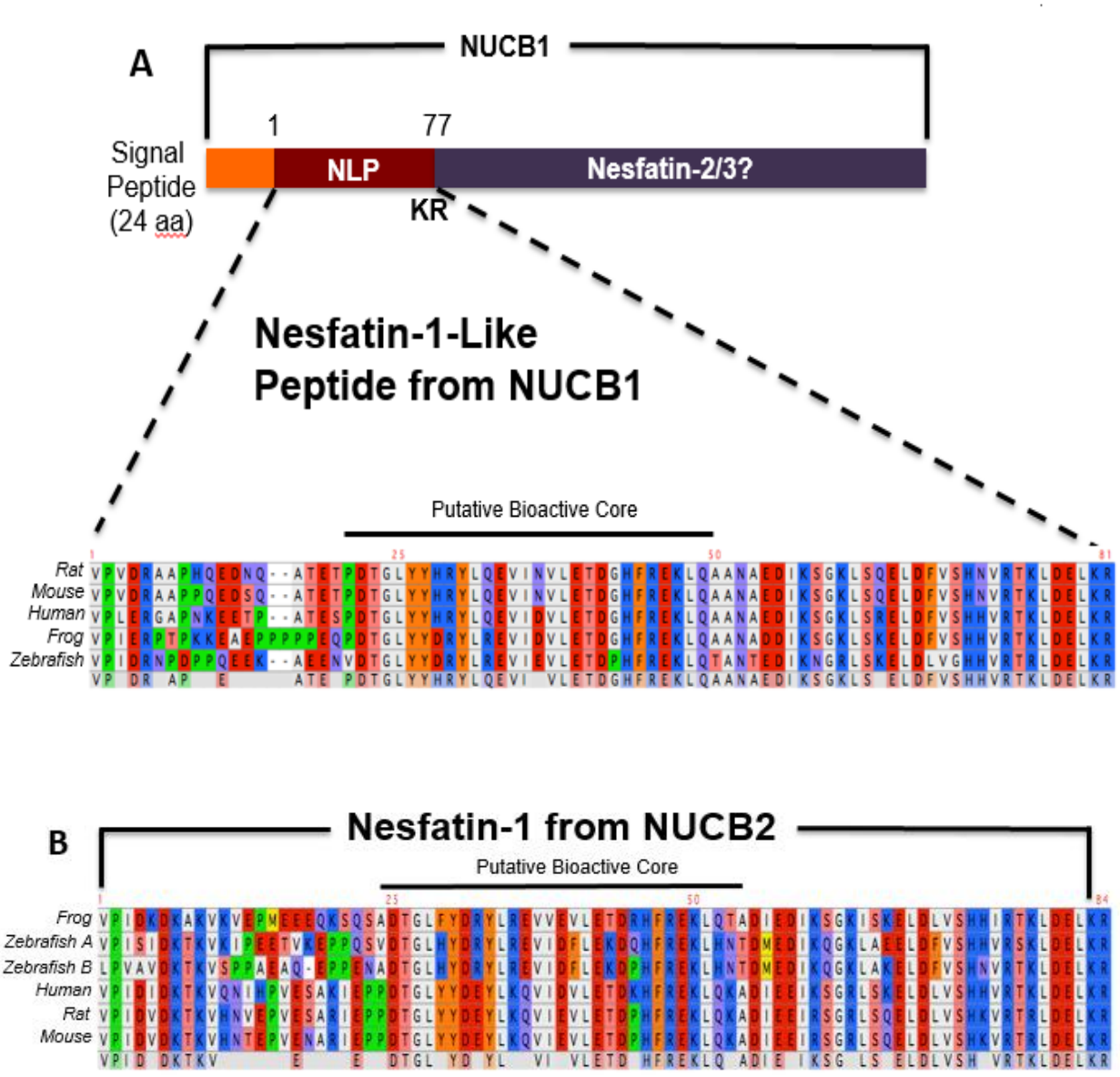

Figure 3.1. A. Scheme showing the signal peptide, nesfatin-1-like peptide region (1-77), and the putative processing sites (KR) in mouse NUCB1 sequence. NUCB1 encoded NLP sequences from various species. The putative bioactive core region is marked. **B.** Sequence comparison of NUCB2 encoded nesfatin-1 showing the bioactive core. GenBank Accession numbers of sequences used: *Rattus norvegicus* (NUCB1, AAI00644.1; NUCB2, AAH61778.1), *Mus musculus* (NUCB1, AAH72554.1; NUCB2,

AAH10459.1), *Homo sapiens* (NUCB1, NP_006175.2; NUCB2, NP_005004.1), *Danio rerio* (NUCB1, NP_001038928.1; NUCB2A, NP_958901.1; NUCB2B, NP_958887.1), and *Xenopus tropicalis*, (NUCB1, AAH67991.1; NUCB2, AAH90107.1).

3.3.2. *NUCB1* mRNA Expression in MIN6 cells and Mouse Pancreas

A band of approximately 96 bp of *NUCB1* mRNA was detected (**Figure 3.2**) in MIN6 cells and mouse pancreas. The negative control reaction resulted in no bands.

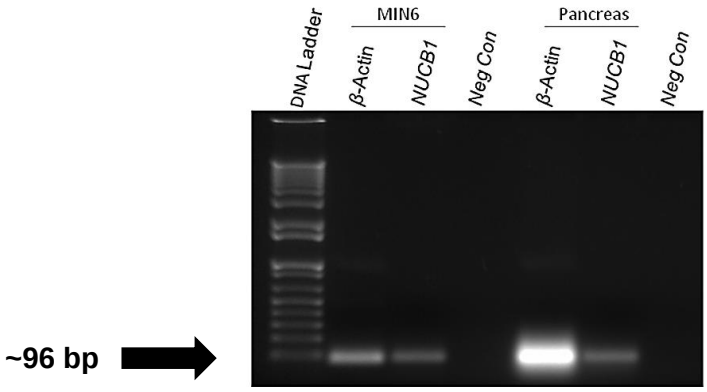

Figure 3.2. Gel electrophoresis image showing the presence of *NUCB1* mRNA in MIN6 cells and mouse pancreas. The amplicon size of the band was approximately 96 base pairs.

3.3.3. NUCB1 and NUCB2 Immunoreactivity in Pancreatic Islets and MIN6 Cells

NUCB1 immunoreactivity was detected in MIN6 cells **(Figure 3.3 - C)** and mouse pancreatic islet beta cells **(Figure 3.4; C-D)**. Co-localization **(Figures 3.3; E-F, 3.4 - E)** of NUCB1 **(Figures 3.3 C, 3.4; C-D)** and insulin **(Figures 3.3; A-B, 3.4 - E)** was observed in MIN6 cells and pancreatic islets. No co-localization of NUCB1 and glucagon was found in alpha cells **(Figure 3.4 - F)**.

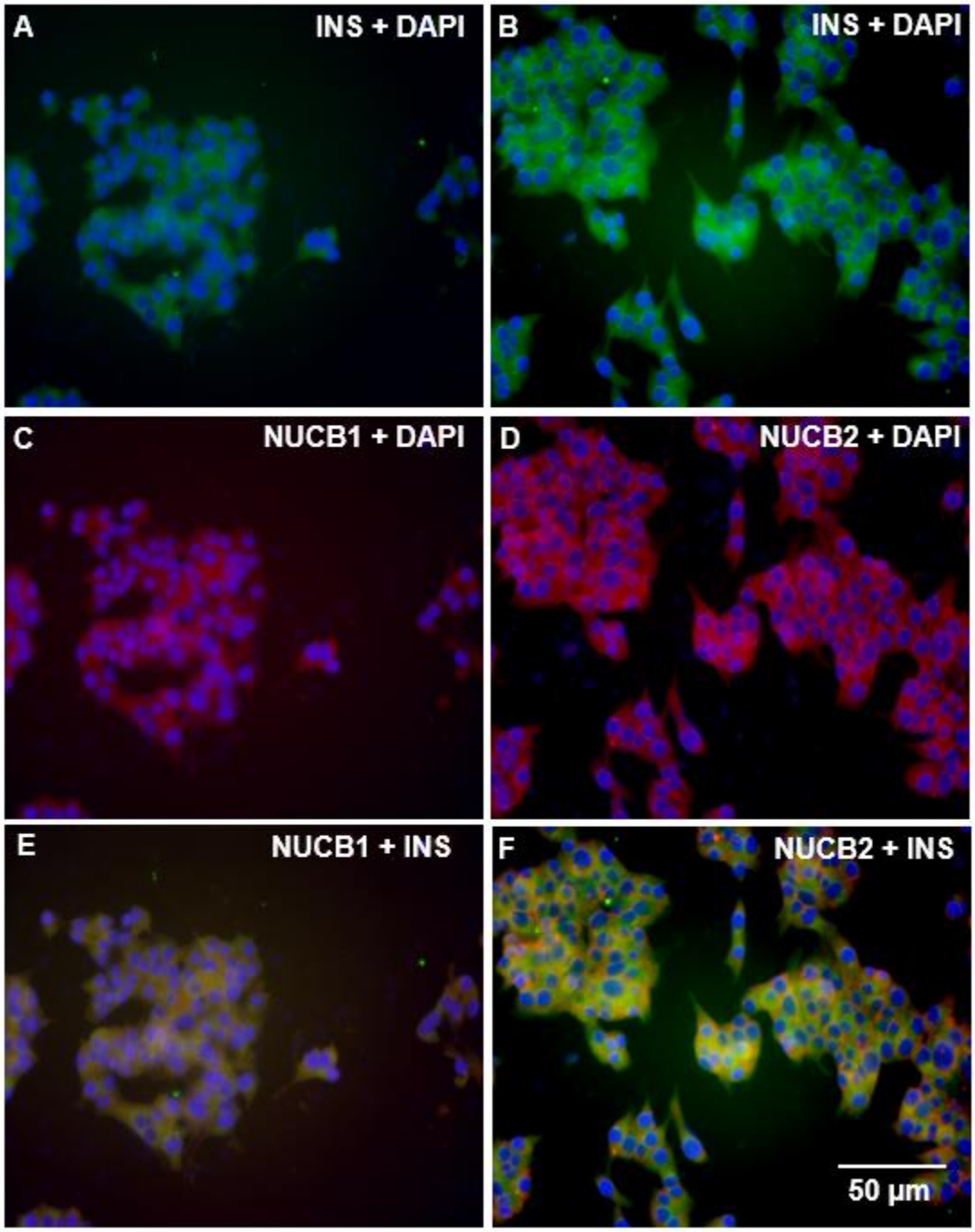

Figure 3.3. Photomicrographs showing insulin (**A-B**; green), NUCB1 (**C**; red), NUCB2 (**D**; red), and co-localization (yellow) of NUCB1 (**E**) or NUCB2 (**F**) with insulin in MIN6 cells. Scale bar = 50 µm.

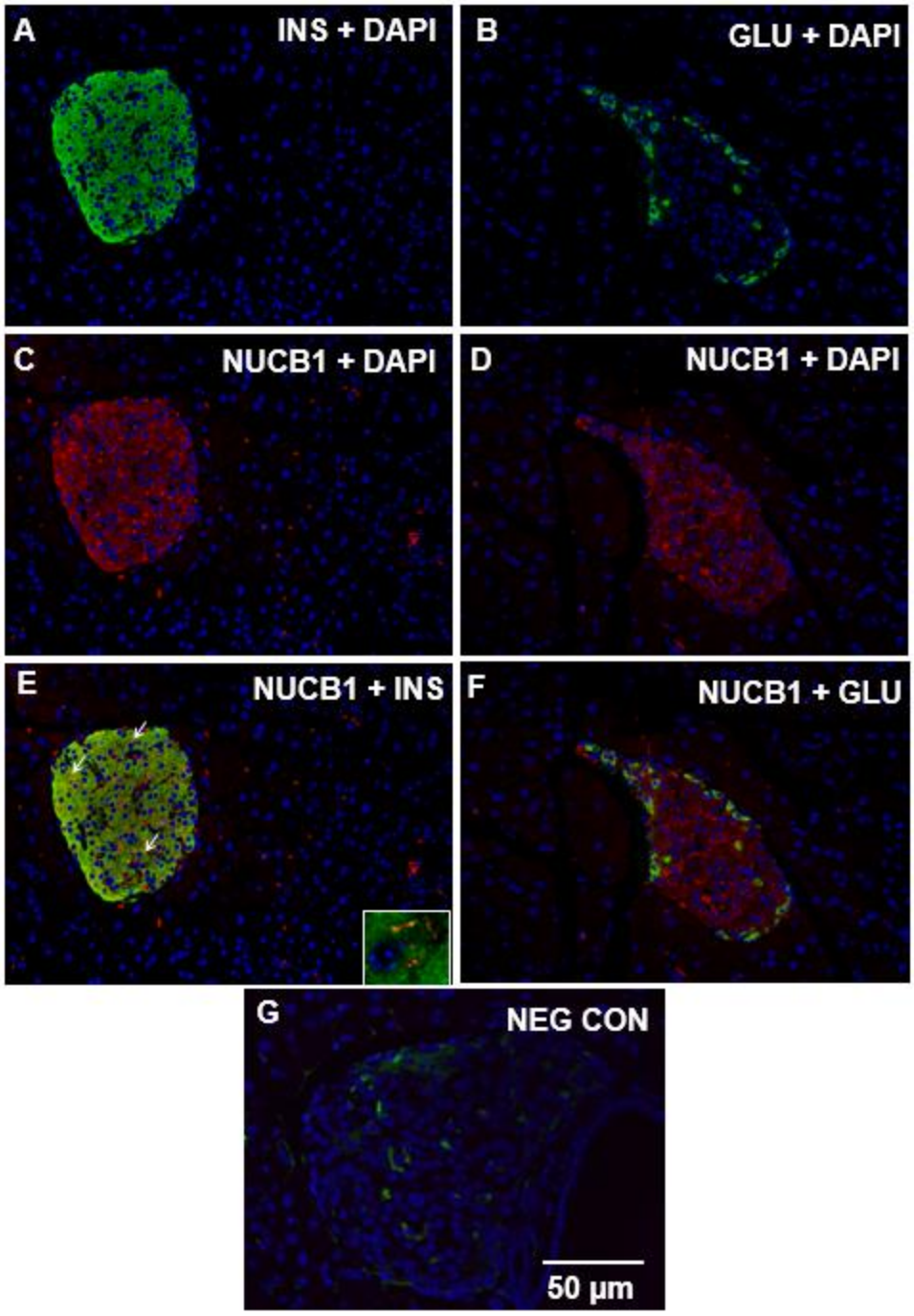

Figure 3.4. Photomicrographs showing insulin (A; green), glucagon (B; green), NUCB1 (C-D), co-localization of NUCB1 with insulin (E; yellow; cells pointed by arrows), and lack of co-localization of NUCB1 and glucagon (F) in the pancreatic islets of mice. No NUCB1 staining was detected in secondary antibody alone controls (G). Scale bar = 50 µm.

3.3.4. NLP Stimulates Insulin Release from MIN6 Cells

NLP (10 and 100 nM) stimulated preproinsulin mRNA expression (**Figure 3.5 - A**) and insulin release (**Figure 3.5 - B**) from MIN6 cells at 1 hour post-incubation. No effects were detected for other doses of NLP tested (**Figures 3.5; A-B**).

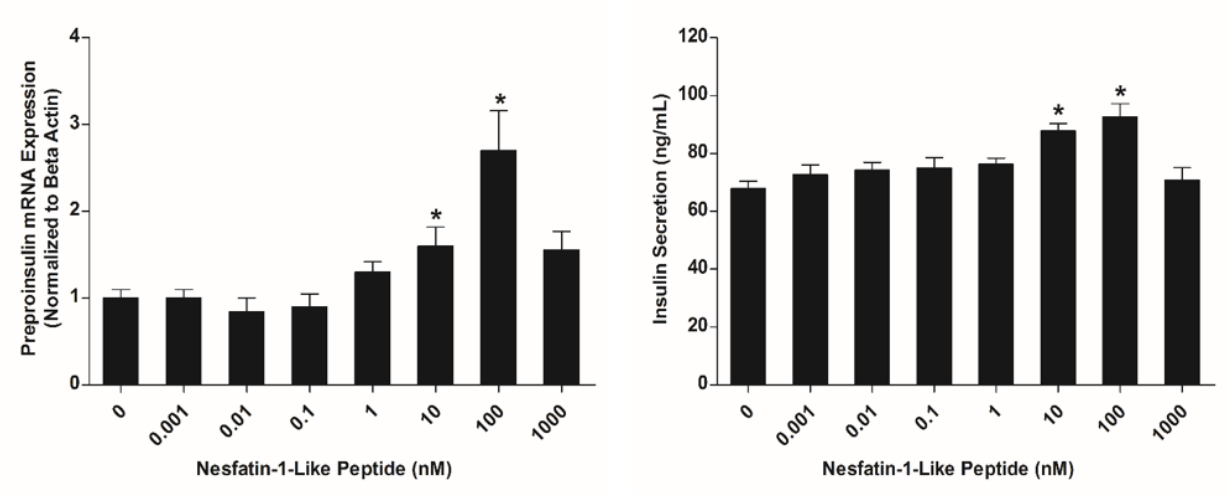

Figure 3.5. Nesfatin-1-like peptide (NLP) enhances preproinsulin mRNA expression (**A**), and stimulates insulin secretion (**B**) from MIN6 cells at 10 and 100 nM (significance denoted by *) compared to no treatment controls and other doses tested. Data are presented as mean + SEM. n = 8-12 wells pooled from three different studies.

3.3.5. A Scrambled Peptide Based on the NLP Sequence had no Effect on Preproinsulin mRNA Expression and Insulin Secretion in MIN6 Cells

NLP (100 nM) stimulated preproinsulin mRNA expression (**Figure 3.6 - A**) and insulin release (10 and 100 nM) (**Figure 3.6 - B**) from MIN6 cells at 1 hour post incubation. No effects were detected for similar doses of scrambled peptide. Nesfatin-1 (1 nM) stimulated both preproinsulin mRNA expression and insulin secretion into media in MIN6 cells.

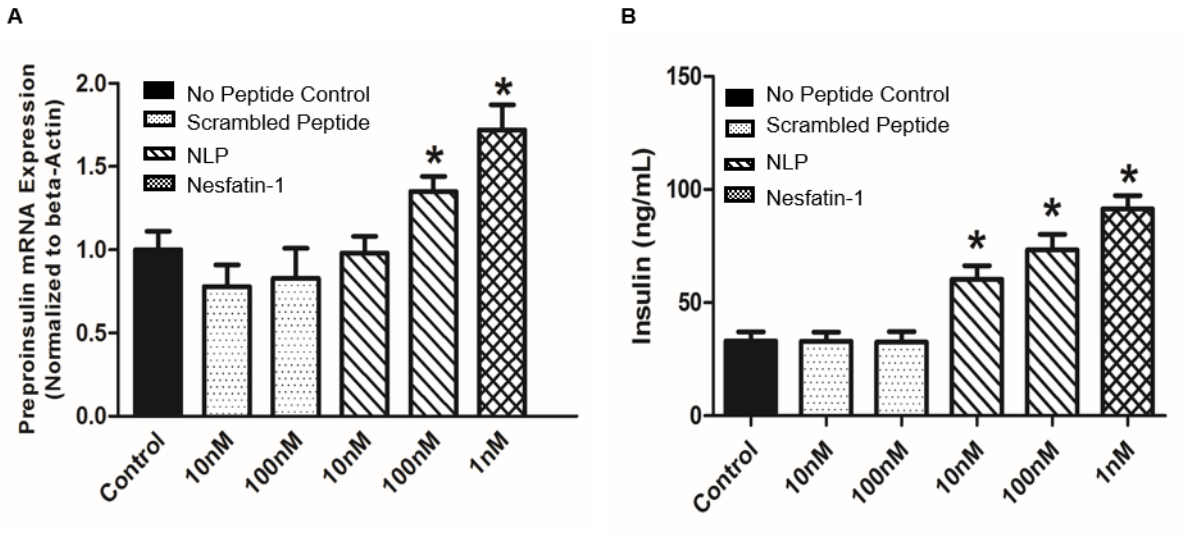

Figure 3.6. Nesfatin-1-Like peptide (NLP) and nesfatin-1 enhances preproinsulin mRNA expression. However, a scrambled peptide to NLP did not elicit any effects on preproinsulin mRNA (**A**). NLP and nesfatin-1 stimulates insulin secretion (**B**) from MIN6 cells at 10, 100 nM and 1 nM respectively (significance denoted by *) compared to no treatment control. Data are presented as mean + SEM. n = 8 wells/ pooled from two different studies.

3.3.6. Fasting increases NUCB1 Protein Expression in Mouse Liver

Mice fasted for 24 hours had a significant increase in NUCB1 protein expression in liver when compared to controls (**Figure 3.7 – A**). However, no changes in expression were observed in pancreas (**Figure 3.7 – B**) and stomach (**Figure 3.7 – C**).

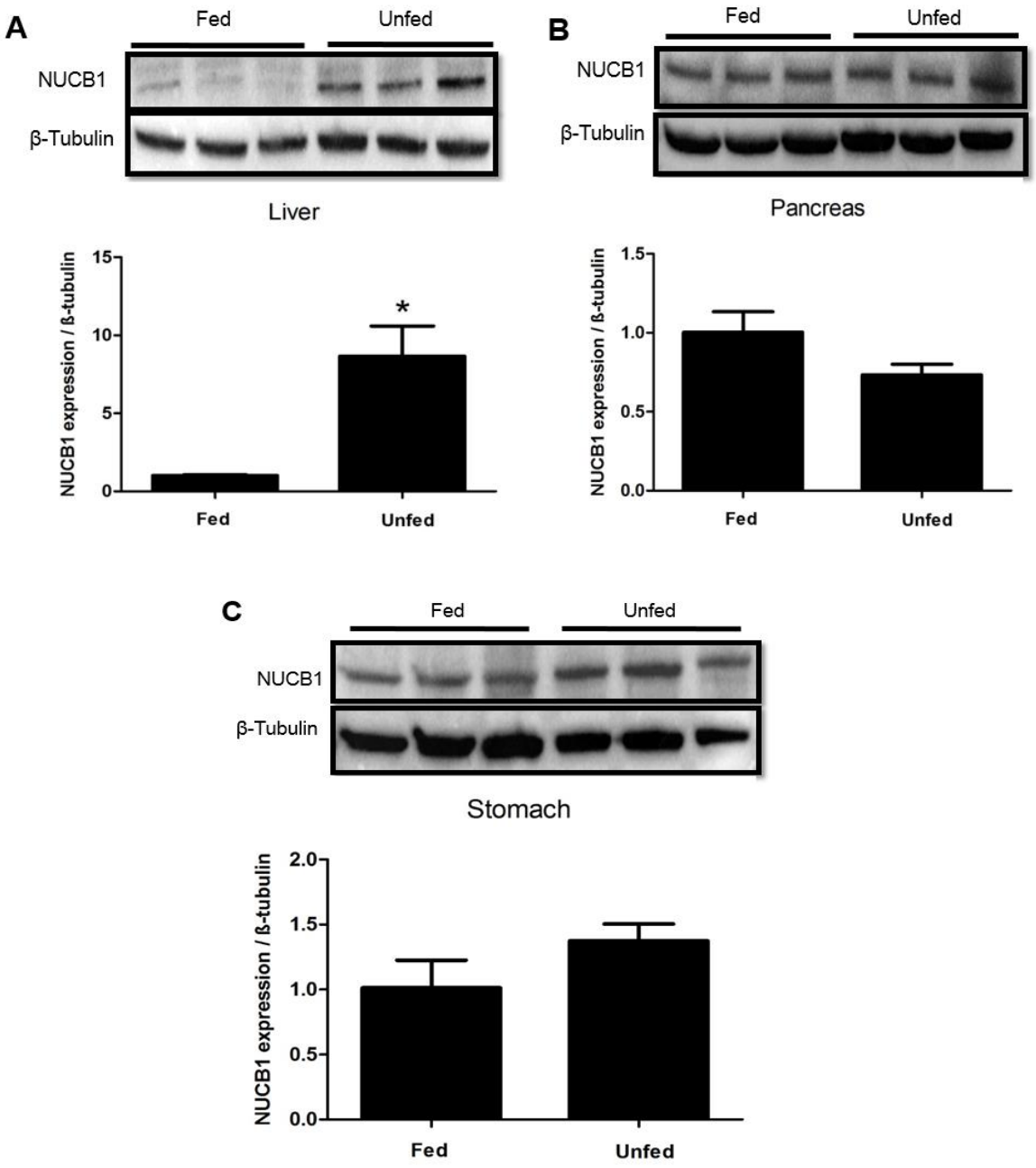

Figure 3.7. Food deprivation for 24 hours results in a significant increase in NUCB1 protein expression in liver of C57BL/6J mice when compared to controls (**A**). No changes were observed in pancreas (**B**) and stomach (**C**). The band intensities were quantified using ImageJ™ software and were normalized to β-Tubulin. Data represented as mean + SEM. n = 6 animals/group.

3.3.7. Osmotic Pump Infusion of NLP had no Effect on Dark and Light Phase Feed Intake in Mice

No changes in dark and light phase feed intake were observed in C57BL/6J mice when 100 µg/kg body weight NLP was infused using osmotic pumps (**Figure 3.8**).

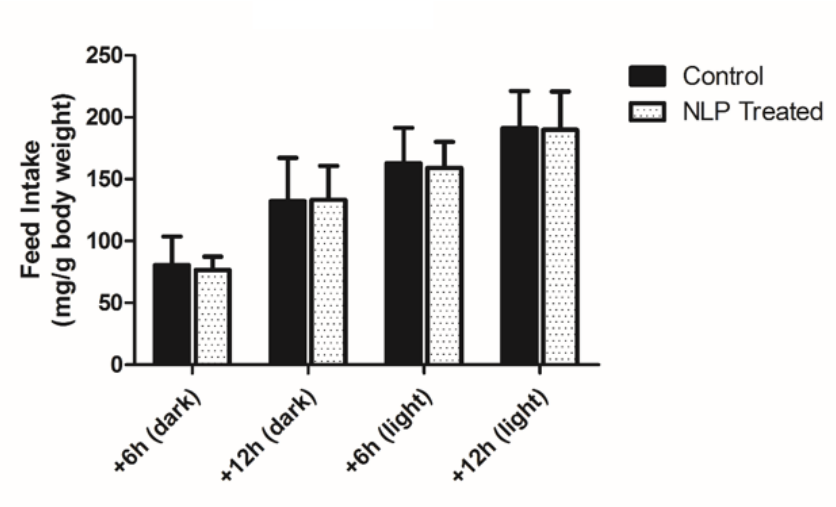

Figure 3.8. Subcutaneous infusion of 100 µg/kg body weight NLP using osmotic pump in male C57BL/6J mice had no effect on feed intake over a period of 24 hours, when compared to saline treated group. Data are represented as mean + SEM. n = 6 mice/group.

3.4. DISCUSSION

In the original article that reported nesfatin-1, Oh-I and colleagues [9] indicated that both NUCB1 and NUCB2 are secreted proteins arising from two homologous genes arising from a single EF-hand ancestor. Whether NUCB1 encodes a biologically active nesfatin-1-like peptide remains unknown. Here, we report the first detailed sequence analysis showing the presence of a nesfatin-1-like peptide in NUCB1, its presence within the endocrine pancreas, and an insulinotropic role for NLP. Our analysis of the NUCB1 sequence using the same tools that were employed by Oh-I and colleagues [2] found a signal peptide in the N-terminal, followed by the NLP region flanked by signature sequences representing proprotein convertase cleavage sites. The presence of these key

processing sites, which were used to discover nesfatin-1, suggests that there is a very strong possibility of NLP being processed from NUCB1. The mid-segment composed of 30 amino acids is designated as the bioactive core of nesfatin-1 [176]. We found that the NLP is highly similar to nesfatin-1, and there is a greater degree of identity within the M30 region of nesfatin-1 and NLP (76.6%). Twenty three amino acids in the M30 region are conserved (identical) between mouse nesfatin-1 and NLP. Only 7 dissimilar amino acids (Asp-Glu-Lys-Gln-Glu-Pro-Lys at positions 50, 51, 54, 55, 58, 64 and 72 of nesfatin-1 replaced by His-Arg-Gln-Glu-Asn-Gly-Ala in the M30 region of NLP) were found. These similarities suggest that NLP, like nesfatin-1, is a biologically active peptide.

Our *in vitro* studies using synthetic NLP is in agreement with this notion. Short-term exposure of MIN6 cells cultured in 25 mM glucose to synthetic NLP at 10 and 100 nM resulted in an increase in preproinsulin mRNA expression and insulin release into the media. These results indicate that NLP is insulinotropic. The stimulatory effect was absent at a higher concentration (1000 nM) of NLP. This could be due to the desensitization or downregulation of the receptor(s) through which nesfatin-1 and NLP elicits its effects. There are some suggestions that nesfatin-1 effects are mediated via one or more G-protein coupled receptors (GPCRs) [180], but the identity of nesfatin-1 receptors remains unknown. Recently, GPCR12 family was proposed as nesfatin-1 receptors [182]. It is possible that NLP also mediates its insulinotropic effects through the same or related nesfatin-1 receptors. In a separate study, we did not find any insulinotropic effects for NLP on MIN6 cells cultured at 2 mM or 5.6 mM glucose media (data not shown). This result shows that NLP stimulates insulin only at high glucose concentrations, but not at hypo- or normoglycemic conditions. Also, *in vitro* studies using the scrambled peptide to NLP suggests that the specific sequence of 77 amino acid is critical for the insulinotropic action of NLP. The data clearly shows that both 10 and 100 nM of scrambled peptide to NLP could not elicit the same response in preproinsulin expression and insulin secretion when compared to similar doses of NLP. When comparing the data from the scrambled peptide and NLP, the basal insulin from controls in scrambled peptide study were lower (25-30 ng/mL) than in the initial NLP study. This can be attributed to the difference in the MIN6 confluency between both independent studies.

This research also indicates that the endocrine pancreas is an abundant source of NUCB1. The expression of NUCB1 mRNA was detected in both mice pancreas and MIN6 cells. Specifically, NUCB1 was found to be co-localized with insulin immunopositive cells in pancreatic islets. This is in line with the previous immunohistochemical studies by Williams *et* al., [219] which shows NUCB1-IR to be distributed with insulin immunopositive cells. They also found NUCB1 to be co-localized with golgi apparatus protein, giatin, shedding light on its subcellular localization in pancreas. This indicates that the pancreas, especially the endocrine pancreas is a source of NUCB1 and NLP. As a confirmation to this at the protein level, immunocytochemical studies show clear co-localization of NUCB1 with insulin in cultured MIN6 cells and islet beta cells of mice. Preliminary results evaluating the endogenous NUCB1 protein expression in major peripheral tissues in response to feed availability shows a significant increase in liver NUCB1 expression under fasted state. Previous results have shown a similar 5 to 6 fold increase in liver *NUCB2* mRNA expression in fasted goldfish [180]. Also, nesfatin-1 treatment in *ad* libitum fed rats increased phosphoenolpyruvate carboxykinase 1 (PEPCK1) and gluose-6-phosphatase (G6P) mRNA expression in liver [13]. Both PEPCK1 and G6P plays crucial role in hepatic gluconeogenesis. The increase can be attributed to the inhibition of caspase mediated cleavage of NUCB1 [244]. The liver is actively involved in gluconeogenesis under fasted state compensating for the lack of glucose. Glyceraldehyde-3-phosphate (G3P) which is an important intermediate in the pathway is expected to be higher during gluconeogenesis. Previous studies have also established a direct inhibitory role of G3P on caspases [245]. This is physiologically relevant as cleavage site for caspases is highly conserved between both NUCB1 and NUCB2 [244]. Whether or not NUCB1 or NLP regulates the expression of enzymes involved in hepatic gluconeogenesis, be it G6P, PEPCK1 or G3P requires further assessment. Contrastingly, no changes in response to feed availability were observed in stomach and pancreas. Western blots were unable to capture the presence of a processed peptide i.e. NLP. This can be attributed to the specificity of the antibody used. Immunoprecipitation of protein antigen from media and plasma samples using a highly specific antibody targeting the NLP region in NUCB1 should provide a clearer picture on NLP secretion. Also,

assessment of the levels of NLP in conditioned culture media *in* vitro and its levels in circulation *in vivo* using a custom ELISA warrants consideration.

Subcutaneous infusion of 100 µg/kg body weight NLP in mice had no effect on feed intake. This study needs repetition to test additional doses of NLP, and to determine whole body energy homeostasis using a metabolic cage system. The bioactive core of nesfatin-1 containing 30 amino acids was attributed to its anorexigenic action as neither the N-terminal segment nor the C-terminal segment increased central POMC and c-Fos expression upon peripheral or I.C.V administration in mice [9]. Interestingly, the mid segment of nesfatin-1 has high homology to the active site of AgRP and α-MSH. Subsequent studies on the activities of mutant molecules by replacing mid-segment sites with alanine clearly showed that the similarity to AgRP active site is critical for a peptide to be anorexigenic [182]. Considering that NUCB1 is a secreted protein, NLP presumably could elicit its biological functions in an endocrine manner. The presence of nesfatin-1 and NUCB1 in pancreatic islet beta cells suggests possible local effects, in addition to the endocrine effects for NLP in islet hormone secretion. In conclusion, these results provide the first set of information on NLP as an endogenous insulinotropic peptide encoded in NUCB1. Future studies, examining NLP under normal and atypical physiological conditions *in vivo*, especially in diabetes and obesity, and mechanism of action of NLP warrant consideration.

3.5. CONCLUSIONS

This research provides the first set of evidence for a NUCB1 encoded biologically active nesfatin-1-like peptide (NLP). We characterized the expression of *NUCB1* mRNA and NUCB1 immunoreactivity in MIN6 cells and mouse pancreas. We found NUCB1 to be co-localized with insulin immunopositive cells in pancreas and MIN6 cells. *In silico* analysis of NUCB1 amino acid sequence detected a potential prohormone convertase cleavage site. Given the fact that both MIN6 cells and pancreatic islets have the appropriate enzyme machinery (prohormone convertases) indicates that NUCB1-encoded NLP could be secreted. Since both nesfatin-1 and NLP were highly similar we tested insulinotropic action of NLP by treatment of MIN6 cells dose-dependently with exogenous NLP. NLP upregulated preproinsulin mRNA expression and insulin secretion.

Although, MIN6 cells are highly representative of pancreatic β cells, the consistency of the current results needs to be tested in the primary cells of pancreatic islets by islet isolation. By static incubation studies of cells with a peptide scramble of NLP, we showed that the predicted 77 amino acid sequence arrangement of NLP might be critical for its insulinotropic action *in vitro*. Also since nesfatin-1 was reported to be an anorexigenic peptide capable of inducing satiety and reducing dark phase feed intake and since NLP was highly similar to nesfatin-1, we tested the potential appetite regulatory function of NLP on C57BL/6J mice and found that subcutaneous infusion of 100 µg/Kg body weight NLP had no effect on both dark and light phase feed intake. A future study evaluating the effect of I.C.V and I.P injection of NLP on feed intake will provide a more complete picture. We also performed studies to evaluate the changes in the endogenous expression levels of NUCB1 with respect to feed availability. Preliminary results from Western blots show a significant increase in NUCB1 protein expression in liver under fasted state. However, no changes in the protein expression in stomach and pancreas were observed. The Western blot analysis for the current chapter is currently under progress and will be completed soon. Overall, findings in the current chapter shed light on a NUCB1 encoded bioactive NLP. The results in this chapter iterate the insulinotropic action of NLP, attributing its biological action to its specific 77 amino acid sequence. This project has also opened a variety of avenues to explore to reconfirm and evaluate the current hypothesis, and some future extensions are discussed in chapter 4.

CHAPTER 4

INTEGRATION OF FINDINGS AND FUTURE DIRECTIONS

4.1. INTEGRATION OF FINDINGS

This thesis research is novel and highly original, and was aimed to extend our current knowledge on nesfatin-1 biology. It resulted in two major discoveries: 1. nesatin-1 presence in enteroendocrine cells, and its modulation of intestinal hormones, and 2. identification of an insulinotropic, nesfatin-1 like peptide encoded in NUCB1. This section of the thesis will address some of the implications of the results obtained, limitations of the research and future directions.

The focus of chapter 2 in this thesis was to evaluate the presence of endogenous NUCB2/nesfatin-1 in mouse intestine and to study the effects of nesfatin-1 administration *in vitro* in mouse intestinal cell line, STC-1 and *in vivo* in mouse intestine. *NUCB2* mRNA was present in STC-1 cells. The result was highly indicative that intestinal enteroendocrine cells could be a source of endogenous nesfatin-1 *in vivo,* possibly contributing to the post-meal increase reported previously [11]. This also highlights the utility of STC-1 cells for studying nesfatin-1 biology in addition to study the secretion kinetics of major enteric hormones GLP-1, GIP, PYY and CCK. This research also considered the expression of NUCB2 in *ad libitum* fed mouse intestine. Supporting the previous evidences [15, 193, 246], NUCB2/nesfatin-1 immunoreactive cells were found in mouse intestine and were predominantly localized in the crypts of the intestinal villi, Brunner's glands and in the blood vessels surrounding the connective tissue. Besides being a crucial site for nutrient absorption, intestine is a major endocrine organ secreting hormones that modulate energy metabolism and glucose homeostasis. Nesfatin-1 was found colocalized with a number of enteric hormones including GLP-1, GIP, PYY and CCK. The co-presence of these hormones in the same cells that secrete important metabolic hormones implies its significance in physiological roles, especially the regulation of whole body energy homeostasis and insulin secretion by nesfatin-1. It also raises the possibility that nesfatin-1 might have local autocrine or paracrine effects in

regulating the secretion of intestinal hormones. Whether nesfatin-1 is stored in the same secretory vesicles, and is secreted with other hormones remain unknown.

The presence of nesfatin-1 immunoreactivity in intestine raised an important question: Does nesfatin-1 modulate intestinal hormone secretion? Immunohistochemical staining of C57BL/6J small and large intestinal sections showed NUCB2/nesfatin-1 to be co-localized with GLP-1, GIP, PYY and CCK immunopositive cells. Subsequent *in vitro* studies on STC-1 cells showed a significant dose-dependent upregulation of GLP-1, GIP, CCK, and downregulation of PYY mRNA expression and secretion. These results are highly suggestive that nesfatin-1 has the potential to activate insulinotropic intestinal hormones and suppresses insulinostatic hormones of intestinal origin, to modulate insulin secretion. A major limitation of this approach is that the studies were conducted only *in vitro*, and that again used immortalized cell lines. While the results obtained are conclusive, it is important to determine whether biological actions of nesfatin-1 found *in vitro* also exist *in vivo*. The *in vivo* studies evaluating the relative levels of intestinal hormones in circulation in response to subcutaneous osmotic pump infusion of 100 µg/kg body weight nesfatin-1 in mice was indeed conducted. However, some technical challenges prevented us from obtaining conclusive data on enteric hormone secretion in this study. The tissues collected will now be evaluated for relative mRNA and protein expression of enteric hormones using RT-qPCR and Western blotting. This work also requires further enquiries on the mechanistic aspects of nesfatin-1's action in intestine including the nature of its receptors, chapter 2 details its presence in the intestine, establishing enteroendocrine cells as a source for endogenous nesfatin-1. It also affirmed that nesfatin-1 indeed modulates secretion of intestinal hormones *in vitro,* paving way for a thought that an indirect route of its insulinotropic action is possibly by stimulating other enteric insulinotropins and inhibiting enteric insulinostatins. Additional studies using enteric hormone knockout mice will be a great tool to determine whether nesfatin-1 action on other insulin regulatory hormones is critical for its insulinotropic actions. To the best of our knowledge, this remains the first report on direct actions of nesfatin-1 in modulating enteric hormones **(Figure 4.1)**. This finding adds an additional function, intestinal hormone regulation, to the growing list of biological actions of nesfatin-1 **(Figure 4.1)**.

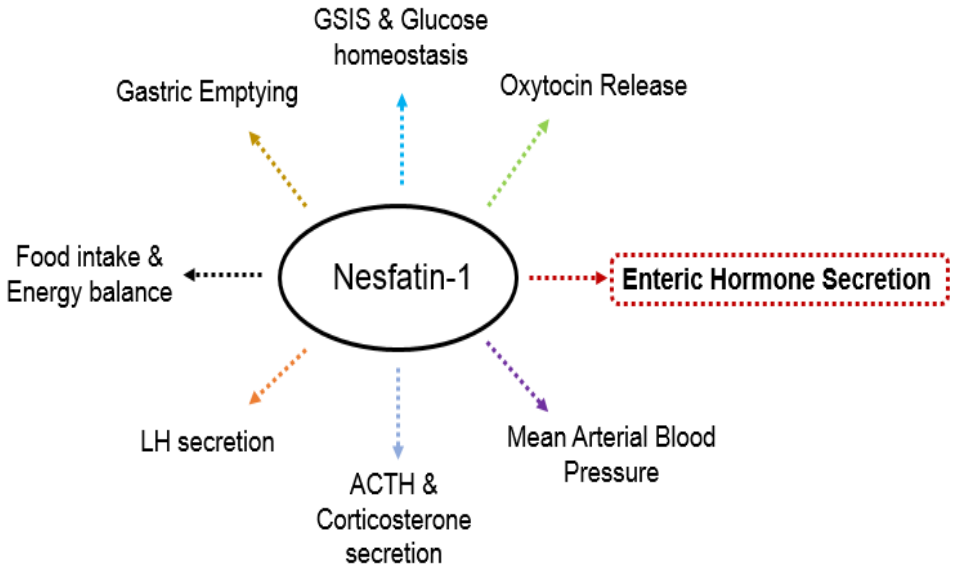

Figure 4.1. A summary figure depicting the multi-functional stimulatory and inhibitory roles of nesfatin-1 reported. Chapter 2 extends this by the identification of a direct role for nesfatin-1 in regulating intestinal hormone secretion.

The findings elaborated in chapter 2 are in line with our hypothesis. Further studies are required to reconfirm and clarify the role of nesfatin-1 in regulating intestinal hormone secretion. Specifically, the immunoassays took into account total GLP-1 and GIP content. Therefore, a separate assay evaluating the secretion of levels of active forms of both the peptides (GLP-1 (7-37) and GIP (1-42) is warranted along with the use of DDP-IV inhibitors. Similarly, whether or not nesfatin-1 modulates the secretion of CCK-8 and CCK-33, which are two bioactive forms of CCK in circulation also requires clarification. With respect to the mRNA expression data on GLP-1, the proglucagon gene besides GLP-1 also encodes GLP-2 and oxyntomodulin. Although the primer used for the study targets the GLP-1 encoding region of the proglucagon gene, yet a Western blot evaluating the changes in the GLP-1 protein expression in response to dose-dependent treatment of nesfatin-1 is necessary for research rigor. Results from the *in vivo* studies including the changes in circulating levels of intestinal hormones in response to subcutaneous, I.P or I.C.V administration of nesfatin-1 will provide a complete picture on the nature of nesfatin-1 modulation of enteric hormones. Some of the possible future directions for this chapter are outlined later in this chapter.

At the time when we initiated research in chapter 3, no information existed about a NUCB1 encoded bioactive peptide. We detected both *NUCB1* mRNA expression and immunoreactivity in pancreatic islets of C57BL/6J mice and in mouse insulinoma cells MIN6. This reaffirms that endocrine pancreas is an abundant source of endogenous NUCB1. Also, NUCB1 immunoreactivity was co-localized with insulin immunopositive cells in islets, suggesting that NUCB1 or an encoded peptide could modulate insulin secretion. Similar to the characterization of nesfatin-1 from NUCB2 [9], we hypothesized that NUCB1 encodes a bioactive nesfatin-1-like peptide (NLP) that could be secreted. *In silico* analysis of NUCB1 amino acid sequence convincingly showed the presence of a 77 amino acid NLP. We have previously demonstrated that both islets and MIN6 cells express NUCB2/nesfatin-1 and that nesfatin-1 dose-dependently stimulates GSIS [5]. Owing to its high similarity to nesfatin-1, especially high identity (76.6%) in the 30 amino acid mid segment region (the bioactive core of nesfatin-1), we hypothesized that NLP could have an insulinotropic action similar to nesfatin-1. Testing this hypothesis via *in vitro* studies on MIN6 cells cultured under hyperglycemic conditions (25 mM glucose), we demonstrated that short-term exposure to exogenous NLP stimulates preproinsulin mRNA expression and insulin secretion at 10 and 100 nM doses. To confirm the specific 77 amino acid sequence of NLP is critical for its bioactivity and to test whether any peptide that shares the same amino acid length but dissimilar amino acids could also elicit the same response as NLP, we treated MIN6 cells with a scrambled peptide to NLP at doses that were found effective with NLP (10 and 100 nM). The results from the scrambled peptide study clearly demonstrated that the specific sequence of 77 amino acids is required for NLP's action on insulin secretion *in vitro*. We then quantified the endogenous levels of NUCB1 expression in response to feed availability in mice. We found a significant increase in NUCB1 expression in fasted mice liver and found no changes in pancreas and stomach. Further, we tested whether NLP modulates appetite similar to nesfatin-1, by subcutaneously infusing 100 µg/kg body weight NLP in C57BL/6J mice. Results from this study demonstrated that NLP, at the dose tested and mode of administration has no effect on both light and dark phase feed intake.

The findings described in chapter 3 are supportive of our hypothesis. However, whether an endogenous NLP exists still remains inconclusive. Future efforts should focus

on addressing this critical question, and identify the processed endogenous peptide from *NUCB1*. NLP also requires rigorous set of studies designed to evaluate its insulinotropic action *in vivo*, especially under atypical physiological conditions like T2D, obesity and in islets isolated from perfused mice pancreas (primary cell line studies). This is necessary because when compared to nesfatin-1, NLP is insulinotropic at higher doses and the increase in insulin secretion is modest. Also, under both *in vitro* and *in vivo* conditions, the basal levels of NLP in culture media and in circulation remain to be elucidated by means of a dedicated immunoassay (ELISA or RIA). This will aid in better design for future studies evaluating the dose-dependency of NLP in any given biological system and also to affirm that NLP indeed is secreted *in vivo*. Similar to nesfatin-1, the N-, C- and mid-segment of NLP could be fragmented by incubation of peptide with prohormone convertases and caspases, with each fragment being tested for insulinotropic action and satiety. A few possible future directions for this chapter are described in section 4.2 below. A preliminary conclusion is that both NUCB1 encoded NLP, and NUCB2 encoded nesfatin-1 modulate insulin secretion in mammals. This insulinotropic effect is direct. Our results, especially from chapter 2, trigger a new possibility where nesfatin-1 and/or NLP act directly on intestinal hormones, and this modulation of intestinal hormones indirectly mediates its insulinotropic action (**Figure 4.2**).

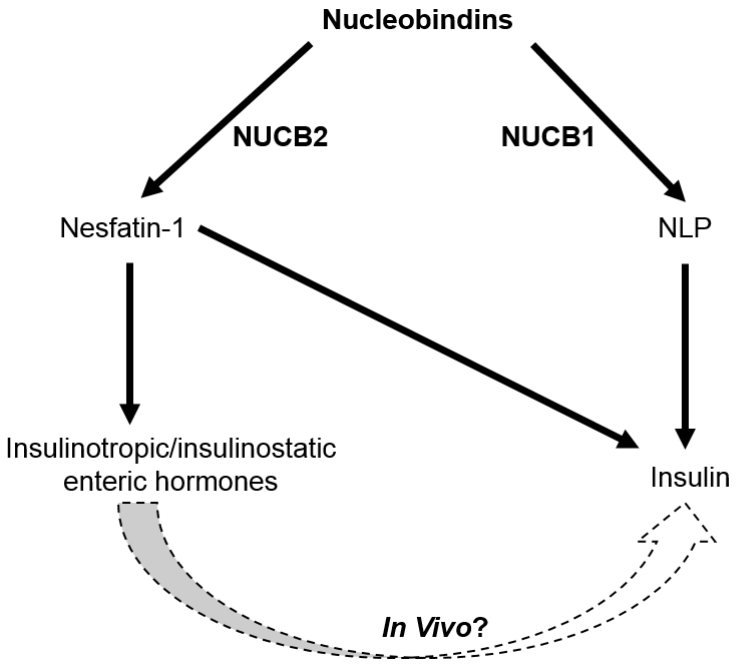

Figure 4.2. A schematic representation depicting the overall conclusion of chapter 2 and 3. Nesfatin-1 encoded by *NUCB2* is present in intestine and modulates major intestinal hormones (GLP-1, GIP, CCK and PYY). A nesfatin-1-like peptide encoded by *NUCB1* stimulates insulin secretion *in vitro* in MIN6 cells and the same response is absent upon administration of an amino acid scramble to NLP. This diagram concludes that two known nucleobindins (NUCB1 and NUCB2) modulate insulin secretion. While *NUCB2* encoded nesfatin-1 stimulates insulin secretion via a direct action on pancreatic islets and indirectly via stimulation/inhibition of insulinotropic/insulinostatic intestinal hormones, *NUCB1* encoded NLP modulates insulin secretion by a direct action *in vitro*.

4.2. FUTURE DIRECTIONS

1. A time-dependent study of nesfatin-1 doses on enteric hormone secretion in STC-1 cells: One limitation of the current study is that it considered only a single time point i.e. 1 hour incubation. In a future study, a dose of nesfatin-1 effective in modulating enteric hormone secretion should be tested at 15, 30, 45 minutes and 1.5, 2 hours to determine whether the effects of exogenous nesfatin-1 reported in this chapter continues to exist for both longer and shorter durations. Also, new and previous data generated will

be normalized using two or more reference genes (β-Actin, GAPDH and α-tubulin) for consistency and rigor.

2. Determine the serum levels of enteric hormones post intraperitoneal injection of nesfatin-1: While the changes in the serum levels of enteric hormones in response to 24 hour osmotic pump infusion of nesfatin-1 are currently being tested, whether these effects are consistent in response to an I.P injection of nesfatin-1 remains to be seen. Immunoassays for circulating levels and Western blot for changes in the protein expression will be carried out in the tissues collected.

3. Absence of endogenous nesfatin-1: The studies explored the effects of exogenous nesfatin-1 on mice enteric hormone secretion *in vivo* and the role of nesfatin-1 treatment in STC-1 cells *in vitro*. However, these studies do not help understanding whether endogenous nesfatin-1 is critical for this action. Intestine specific *NUCB2* knockout and knockdown strategies should be used to delete or attenuate endogenous nesfatin-1, and determine how the secretion levels of intestinal hormones in the absence of this regulatory peptide are, with respect to feed availability. This will provide crucial insights and start points for studying the mechanism of nesfatin-1 action on intestinal hormones.

4. Endogenous Expression of NLP – Changes based on Feed Availability: While we are currently focusing on the endogenous *NUCB1* expression in peripheral tissues under feed availability, we so far have met with moderate success in capturing the band for the processed NLP peptide. To negate this, immunoprecipitation using a highly NLP specific antibody is helpful. The study will also ascertain whether or not endogenous NUCB1/NLP has variable tissue specific expression under feed deprivation.

5. Determine the levels of NLP in circulation: While the current studies tested the changes in insulin secretion upon dose-dependent treatment of NLP in MIN6 cells, evaluating its level in circulation and particularly under atypical physiological conditions like diabetes and obesity is necessary. Also, these studies will iterate that NLP is indeed a secreted protein *in vivo*.

6. Time-dependent Study of NLP Doses on Insulin Secretion: The current study design have considered only a single time point i.e. 1 hour. In a future study, an effective

dose of NLP capable of stimulating insulin secretion should be tested at different time points. This study is crucial when addressing a novel peptide like NLP, as it helps determining the efficacy of the peptide and its kinetics.

7. Biological Actions of NLP: The studies in the thesis elucidated NLP to be an insulinotropic peptide similar to nesfatin-1. However, whether NLP and nesfatin-1 share their biological actions pertaining to energy balance remains to be studied. Studies evaluating IPGTT, ITT, OGTT and metabolic parameters (using CLAMS) of NLP infused mice will provide insights on its regulation of glucose and energy homeostasis similar to nesfatin-1. Also, assessing whether or not NLP modulates intestinal hormone secretion similar to nesfatin-1, opens up new possibilities for its unknown biological actions.

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