Nesfatin-1 Regulation of Cardiovascular Functions in Zebrafish and HL-1 Cardiomyocytes

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ABSTRACT

Nesfatin-1 is an eighty two amino acid long peptide cleaved from the N-terminal of its precursor protein, nucleobindin-2 (NUCB2). In addition to its metabolic actions, nesfatin-1 is also involved in modulating cardiovascular functions in rodents. Intracereberoventricular injection of nesfatin-1 increased mean arterial pressure in rats. In rats, nesfatin-1 acts as a post-conditioning agent and elicits cardioprotection against ischemia-reperfusion injury. It also affects the contraction and relaxation of the heart in rats in a dose dependent manner. Nesfatin-1 is emerging as a regulator of cardiovascular functions in rodents. However, whether nesfatin-1 regulates the cardiovascular system of non-mammals remain unknown. We hypothesized that nesfatin-1 is a modulator of cardiovascular functions in zebrafish. Here we characterized endogenous nesfatin-1 in zebrafish heart, and its effects on zebrafish cardiovascular physiology. We found that zebrafish cardiomyocytes express NUCB2 mRNA and nesfatin-1-like immunoreactivity. While NUCB2 mRNA was lower in unfed fish at 1 hour post-regular feeding time compared to the fish at 0 hour time point, it was observed that chronic food deprivation did not alter NUCB2 mRNA expression in zebrafish heart. Ultrasound imaging of zebrafish heart at 15 minutes post-intraperitoneal injection of nesfatin-1 (50 ng/g, 250 ng/g and 500 ng/g body weight) showed a dose-dependent inhibition of end-diastolic volume, but not end-systolic volume, while a significant increase in end-diastolic volume was found at the lowest dosage. However, these combined effects did not alter the stroke volume. A dose dependent decrease in heart rate and cardiac output was observed in zebrafish that received nesfatin-1. Nesfatin-1 caused a significant increase in the expression of Atp2a2a mRNA encoding the calcium-handling pump, SERCA2a, while it has no effects on the expression of calcium handling protein RyR1b encoding mRNA. NUCB2 mRNA and NUCB2/nesfatin-1 like immunoreactivity was detected in the cytoplasm of mouse HL-1 cardiomyocytes. High glucose increased NUCB2 mRNA expression in HL-1 cells. Genes involved in apoptosis, including Akt1, Caspases 1, 2, 3, and TNF were upregulated in the presence of 10 nM nesfatin-1. We also observed that NUCB2 mRNA expression was significantly increased in C57BL/6 mice heart in the presence of high glucose, whereas in diet induced obese C57BL/6 mice, NUCB2 mRNA expression was not altered. Together, our data supports the hypothesis that nesfatin-1 is expressed in the cardiovascular system of mouse and fish, and that nesfatin-1 modulates cardiovascular physiology in zebrafish.

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1. INTRODUCTION

1.1. INTRODUCTION

Cardiovascular (CV) diseases remain a major cause of mortality in the global population. Diseases including heart attack, stroke, peripheral vascular disease, hypertension and congenital heart defects account for many health problems and are leading causes of mortality. There is an urgent need to tackle the clinical and economic burden due to the diseases of the CV system (Smart et al., 2012). Several lines of research are in progress to understand the cellular and molecular processes involved in cardiovascular development and pathogenesis. However, strategies for the diagnosis, prevention and treatment of cardiovascular diseases require improvement. Research to enhance CV health and treatments for CV diseases consider naturally occurring regulators of heart function. For example, hormones are one of the key endogenous players in the maintenance of cardiovascular homeostasis. A number of peptidyl endocrine factors have multiple effects on the cardiovascular system. These hormones include ghrelin, glucagon-like peptide-1 (GLP-1), melatonin, apelin and peptide tyrosine tyrosine 3-36 (PYY3-36) (Kishimoto et al., 2012; Holst et al. 2007; Adrian et al., 1985). Hormones regulate the CV system by modulating various parameters including cardiac output, stroke volume, systemic arterial pressure and contractility. Another peptide emerging as a cardiovascular regulator is nesfatin-1, a recently identified appetite inhibitor (anorexigen) (Oh-I et al., 2006).

Nesfatin-1 (**N**EFA/nucleobindin **E**ncoded **S**atiety and **FAT I**nfluencing Protei**N-1**) is a naturally occurring 82 amino acid peptide that is cleaved from the N-terminal of the precursor protein, nucleobindin-2 (NUCB2) (Oh-I et al., 2006). It regulates a number of physiological processes including food intake (Oh-I et al., 2006), glucose homeostasis and insulin secretion (Gonzalez et al., 2011). Recently, nesfatin-1 has been identified in the heart (Mimee et al., 2012). It regulates cardiac functions by regulating the mean arterial, systolic and diastolic blood pressure (Mimee et al. 2012). Whether nesfatin-1 regulates fish CV system is currently unknown. At the commencement of this work, the nature of endogenous nesfatin-1 in the mouse CV system was also unknown. My thesis research focused to characterize nesfatin-1 in the CV system of mice and fish.

1.2.LITERATURE REVIEW

1.2.1. Heart and Cardiac Functions

Heart is the first organ to develop in vertebrates. It pumps blood to various tissues in the body to provide nutrients and oxygen, and to remove waste materials. The three layers that line the wall of the heart are epicardium, myocardium and endocardium. The human heart consists of left and right atria, a lower left and right ventricle, an aorta, superior and inferior vena cava, left and right pulmonary arteries and veins, pulmonary and aortic semilunar valves, mitral valve and a tricuspid valve (Taber et al., 2009). Most mammals and birds have the same anatomy with minor differences. For example, the mouse heart is a four-chambered heart with left and right atria and ventricles. However, the pericardium of the mouse heart is thinner than that compared to humans. In mouse, the veins merge together before continuing to the atria, whereas in humans, the veins do not merge and instead join the atria separately (Laflamme et al., 2012). Rats have a similar anatomy consisting of a thick pulmonary vein and a thin pulmonary artery (Heatley et al., 2009). Amphibians, on the other hand have one ventricle and two atria divided by a septum. However, in reptiles the septum running along the ventricle, divides it into two with sufficient gap between the pulmonary aorta and artery resulting in less or no mixing of oxygenated and deoxygenated blood (Romer et al., 1977).

Fish heart is considered to have a slightly different anatomy, where it is divided into four chambers: sinus venosus, atrium, ventricle and bulbus arteriosus. Fish heart is made of cardiac muscle and elastic tissue (Sherrill et al., 2009). The ventricle is a large single chambered composed of spongiosa (inner layer) and compacta (outer layer). The atrium is contractile and thin walled, whereas the bulbus arteriosus is made up of tissues that are non-contractile in nature. The fish heart consists of two main arteries known as afferent and efferent arteries. The afferent arteries are thick walled that helps in transport of blood from the heart to gills. The thin walled efferent arteries transport blood from the gills to various parts of the body (Sherrill et al., 2009).

The circulatory system of most vertebrates is closed. However, there is variation among species. In humans and few mammals, the circulatory system is closed, that is, the blood (deoxygenated) enters the right atrium (systemic circulation) from the superior and inferior vena cava. It then enters the right ventricle (through the tricuspid valve) where it is pumped out to the lungs (pulmonary circulation) through the pulmonary artery. In the lungs, the blood receives

oxygen and enters the left atrium through pulmonary veins. From the left atrium it enters left ventricle (via the mitral valve) and to the aorta (via semilumar aortic valve). The oxygenated blood then passes through the arteries, arterioles and capillaries to reach various cells. Deoxygenated blood returns to the right atrium via the venules, veins and finally the vena cava and the process is repeated. Reptiles and amphibians have a double circulatory system. This means that the blood leaving the heart through the capillaries does not reach the lungs, instead are drawn back to the capillaries. This type of circulation helps in maintaining the body temperature of cold-blooded animals. Amphibians also exhibit a double circulatory system. The circulatory pattern of fish is only single sided. Unlike many mammals, fish heart pumps deoxygenated blood to the gills, where gaseous and nutrient exchange takes place. Afferent arteries transport deoxygenated blood from the ventral aorta to the gills. After oxygen uptake in the gills, the efferent arteries transport oxygenated blood to the ventral aorta and further to different parts of the body (Romer et al., 1977).

The electrical conduction of the heart involves the SA node (sinoatrial node), AV node (atrioventricular node), bundle of His and purkinje fibers. Signals originating from the SA node stimulate the atria and thus results in atrial contraction. Next, the signals are conducted to the bundle of His following a delay by a millisecond. The signals are then conducted to the purkinje fibers present in the ventricular walls. This action potential causes the myocardium to contract thereby resulting in ventricular contraction (Betts et al., 2013). Systole is defined as the period when contraction occurs to help the heart to pump blood. Diastole is the period when the heart is in a relaxed state and allows filling of the heart chambers and thus the cycle of systole and diastole continues. Systole and diastole is the function of both atria and the ventricle. Stroke volume (SV) is defined as the amount of blood leaving the ventricle during the period of systole and diastole. Stroke volume is calculated by subtracting the end diastolic volume and end systolic volume (EDV – ESV). Heart rate (HR) is defined as number of heartbeats per minute. Cardiac output (CO) is the amount of blood that is pumped by the ventricle per minute. It is the product of stroke volume and heart rate (SV x HR). Excitation of the cardiomyocytes results in opening of many voltage-gated ion channels. For example, depolarization of the myocardial cell causes voltage-gated calcium ion channels to open, resulting in an influx of calcium ions from outside the cell, which in turn results in the release of Ca2+ ions by the sarco/endoplasmic Ca ATPase pump and thereby causing the muscles to contract. Following contraction, the calcium channel closes and potassium channels open, causing the muscles to relax. This suggests that calcium ion concentration plays an important

role in contraction and relaxation of the cardiac muscle. The following section describes two main calcium-handling proteins of the heart (Betts et al., 2013).

1.2.2. Expression of Calcium Handling Proteins of the Heart

Calcium handling is important for good functioning of the heart. In my research, the two candidates of interest were SERCA2a and RYR1b. Sarco/Endoplasmic Reticulum Ca2+ ATPase pump (SERCA pump) is a calcium handling pump that is involved in muscle contraction and relaxation by release and uptake of calcium. Contraction is caused by the release of calcium with the help of ryanodine receptors and relaxation is mediated by the uptake of calcium by the SERCA pump. This suggests that SERCA has the ability to restore and lower the calcium levels in order to maintain contraction-relaxation cycles. This transmembrane protein has a molecular mass of 110 kDa. Three different genes encoding SERCA (SERCA1, SERCA2 and SERCA3) have been identified in vertebrates (Brandl et al., 1987; Brandl et al., 1986; Bastie et al., 1990; Guntenski et al., 1988; Lytton et al., 1988; MacLennan et al., 1985; Zarain et al., 1990; Burk et al., 1989). SERCA1 is expressed in fast-twitching skeletal muscle. SERCA2 encodes for SERCA2a (cardiac and slow-twitching skeletal muscle) and SERCA2b (found in many tissues at low levels). SERCA3, the third isoform is expressed in non-muscle tissues (Wuytack et al., 1994; Ver Heyen et al., 2001). Studies using SERCA2a$^{-/-}$ mice showed an increase in embryonic and neonatal mortality with evidence of cardiac malformations. Calcium uptake was reduced by 50% in the SERCA2a$^{-/-}$ cardiac myocytes. Also, in isolated cardiomyocytes, relaxation and calcium was significantly reduced. Importance of SERCA pump in heart failure has been studied extensively in both rodents and humans. It was observed that SR calcium is decreased in both models (Arai et al., 1992). SERCA2a mRNA and protein levels were decreased in failing hearts (Brandl et al., 1986; Hasenfuss et al., 1994; Mercadier et al., 1990; Sutko et al., 1996). Heart failure is found to be associated with decreased SR calcium uptake and content, leading to an altered Ca^{2+} transport. However, restoring the Ca^{2+} transport by increasing the SERCA level is critical for maintaining cardiac functions (Brandl et al., 1986). These data indicate that muscle specific SERCA is essential for normal cardiac development and for cardiac contraction-relaxation cycle.

Another protein of interest in our research to study the mechanistic action of nesfatin-1 in zebrafish was Ryanodine receptor 1b (RyR1b). They are a family of intracellular Ca^{2+} release channels that regulate the entry of calcium into the cytosol from the intracellular organelles. There

are three distinct RyR genes (RyR1, RyR2 and RyR3). RyR1 and RyR2 are expressed in skeletal and cardiac muscle, mutations of which causes skeletal myopathies and cardiac diseases. RyR3 is expressed in a number of other tissues including spleen, lung and kidney to name a few (Brennan et al., 2005). The primary role of RyR is to increase the Ca^{2+} during excitation and contraction coupling. Several studies in zebrafish have shown that inhibition of RyR function leads to impaired excitation contraction coupling and morphological defects in skeletal muscle (Hirata et al., 2007; Jurynec et al., 2008; Baicu et al., 2012).

Being the most important part of the cardiovascular system, heart exerts an endocrine function and this way it helps in the regulation of cardiovascular and renal function. Heart is no longer considered only as a pump that circulates blood and facilitates transport of oxygen and nutrients to tissues. Heart is involved in the regulation of various cardiovascular functions, circulation and hydroelectric homeostasis. The heart is also an organ involved in transferring information to other organs and systems. The endocrine function of heart is integrated with cardiomyocyte properties including contractility and excitability (Samantha et al., 2011). CV system is regulated by several hormones produced within itself and from other parts of the body.

1.2.3. Hormonal Regulation of Cardiovascular Functions

Hormones have an influence on a number of organs including the CV system. Heart is also a source of several endogenous hormones that can in turn affect CV tissues in a positive or negative manner. Hormones from other parts of the body can also regulate heart functions. For example, hormones of the adrenal medulla stimulate heart electrical activity. Heart secretes various hormones including atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). ANP acts to reduce blood pressure by reducing systemic vascular resistance, plasma volume, sodium and fat. BNP also acts by reducing blood pressure but to a lower extent to ANP (Clerico et al., 2006). Some hormones act by increasing the blood pressure, for example PYY3-36, while some, such as ghrelin and GLP-1, act by decreasing the blood pressure. Ghrelin, the first appetite-stimulatory peptide isolated from the stomach (Toshinai et al., 2001), is also the first known peptide hormone with an acyl modification (Kishimoto et al. 2012). Exogenous administration of ghrelin stimulates appetite in humans and rodents (Kishimoto et al. 2012; Nakazato et al. 2001; Horvath et al. 2001). Ghrelin also has important roles in the cardiovascular, skeletal and immune systems (Kojima et al., 2006). Ghrelin receptors were detected in lungs, liver, kidney, pancreas and the gastrointestinal

(GI) tract of rats and humans. They were widely expressed in the cardiovascular system of humans, mainly in the heart and aorta (Gnanapavan et al., 2002). Intravenous administration of ghrelin increased the plasma growth-hormone levels thereby affecting the cardiovascular system by improving left ventricular dysfunction and attenuating cardiac cachexia in rats and humans (Kojima et al., 2006). Inhibition of apoptosis of cultured cardiomyocytes and endothelial cells by ghrelin suggests that it can have direct effects on the cardiovascular system. It was observed that ghrelin treatment in a chronic manner improved the cardiac systolic dysfunction in rats with heart failure (Nagaya et al., 2001). Intravenous administration of ghrelin for three weeks improved the left ventricular ejection fraction in human patients suffering from congestive heart failure (Nagaya et al., 2004). Ghrelin elicits cardioprotection against ischemia reperfusion injury and this effect could be a result of ghrelin binding to its receptors in the heart and independent of GH release (Chang et al., 2004). Ghrelin also plays an important role in patients with cachexia by improving cardiac function and increasing appetite. Long-term administration of ghrelin enhanced cardiac performance by increasing cardiac output and stroke volume (Nagaya et al., 2004). Ghrelin decreases levels of norepinephrine and epinephrine thereby increasing the efficiency of the heart (Matsumura et al., 2002). These physiological properties make ghrelin a major modulator of cardiovascular system in mammals.

Pineal gland is an endocrine organ, which releases the hormone melatonin. Melatonin plays an important role in regulating cardiovascular functions and its receptors are found in the arteries and heart of rats. Melatonin is mainly released in the body during night. In humans suffering from cardiovascular diseases including hypertension (Jonas et al., 2003), ischemia reperfusion injury (Brugger et al., 1995), heart failure (Girotti et al., 2003) and myocardial infarction (Dominguez-Rodriguez et al., 2002) had decreased level of melatonin. Administration of melatonin decreased norepinephrine levels and blood pressure (K-Laflamme et al., 1998). Melatonin administration caused an increase in NO production. It decreased peripheral resistance and modified electrical stability (Paulis et al 2007). Another hormone known to affect the cardiovascular function is apelin. Apelin is a catalytic substrate of angiotensin converting enzyme 2 (ACE2). Apelin mediates its action via a G-protein coupled receptor, known as the apelin receptor (APJ). Apelin is known to regulate several cardiovascular functions including blood pressure, heart contractility and fluid balance in rats. Exogenous apelin upregulates contractility of the heart, and a reduction in cardiac

contractility was observed in apelin knockout mice. This suggests a possible role for apelin in regulating cardiovascular functions (Sato et al. 2013).

Glucagon like peptide-1 (GLP-1) is another GI hormone and is expressed in the cardiovascular system including endothelium, cardiomyocytes and coronary smooth muscle (Holst et al. 2007). GLP-1 is released by L cells of the intestine in response to nutrient ingestion. It promotes insulin secretion and enhances insulin sensitivity of the peripheral tissues. GLP-1 has a number of physiological roles including stimulation of glucose- dependent insulin secretion, promoting satiety and delaying gastric emptying. Currently two GLP-1 receptor agonists, marketed as Byetta™ and Victoza™, are available for the treatment of type 2 diabetes (Jespersen et al., 2013). Despite its anti-diabetic effects, GLP-1 analogues also have cardiac and vascular actions in both rodents and humans. Some of these effects include reduction of blood pressure, blood glucose, body weight and improvements in left ventricular ejection fraction. It is also expressed throughout the mouse cardiovascular system. It has been shown that administration of GLP-1 causes a range of effects including reduction of ischemia reperfusion injury and in improvement of the contractile function (Holst et al. 2007; Jespersen et al. 2013; Sun et al 2012; Sokos et al 2006; Nikoladis et al 2004). The above examples give us an overview of hormones that act by reducing blood pressure. However, few hormones act by increasing the blood pressure such as PYY3-36.

PYY3-36 is a 34 amino acid GI hormone released primarily from the ileum and colon into circulation after ingestion of food. A low dose of PYY3-36 caused a significant increase in the arterial blood pressure. It was also found to have potent vasoconstrictor effect in the cat submandibular salivary gland and intestine. It also increased the systemic arterial pressure without increasing the heart rate, suggesting a vasoconstrictor action (Adrian et al., 1985, 1986). Thyroid hormone is another hormone that regulates cardiovascular functions. Hyperthyroidism (increase in thyroid hormone) augments cardiac output and heart rate in, whereas hypothyroidism (decrease in thyroid hormone) causes attenuation of heart rate (Moolman et al., 2002; Mittang et al., 2013). The list of endocrine factors regulating cardiovascular physiology is growing. A recent addition to this list is nesfatin-1.

1.2.4. Nesfatin-1 – Tissue Distribution

Nesfatin-1, is a recently identified, naturally occurring anorectic protein encoded in NUCB2. NUCB2 is a protein consisting of 396 amino acids and a signal peptide of 24 amino acids at the N-terminal end. Post-translational processing of NUCB2 by prohormone convertases (PCs), is predicted to produce three fragments. These fragments were named nesfatin-1 (1-82 amino acids), nesfatin-2 (85-163 amino acids) and nesfatin-3 (166-396 amino acids). Nesfatin-1 was purified from the rat cerebrospinal fluid (Oh-I et al., 2006). Oh-I et al. (2006) determined the presence of a 9.7 kDa nesfatin-1 peptide using High Performance Liquid Chromatography (HPLC) and Enzyme Linked Immunosorbent Assay (ELISA) in rat hypothalamic extracts and the cerebrospinal fluid. It was observed that when rats were starved for 24 hours, NUCB2 gene expression was reduced in the paraventricular nucleus (PVN). A decrease in nesfatin-1 concentration in the PVN was also observed in these rats. While nesfatin-1 inhibited feeding, injections of nesfatin-2 and nesfatin-3 did not affect appetite. Mid-segment of nesfatin-1 (24-53 amino acids) is the active peptide inhibitory segment as the injections of C-terminal (1-23) and N-terminal (54-82) regions had no anorectic effects in mammals (Oh-I et al., 2006; Myers et al., 2006).

Oh-I et al., (2006) found the presence of NUCB2 in the rat hypothalamus by immunohistochemical analysis using a NUCB2 antibody named NUCB2 Ab-l, which identifies the full length NUCB2. The team found that NUCB2 is expressed in the arcuate nucleus, nucleus tract solitarius and also the lateral hypothalamic areas. Nesfatin-1 was co-localized with PC 1/3 and PC 2 in the neurons, suggesting the processing of NUCB2 by these enzymes (Kerbel et al., 2012). Nesfatin-1 is found in peripheral tissues including stomach, pancreas, testis, gut and the cardiac muscle (Stengel et al. 2009; Gonzalez et al. 2009; Li et al., 2010; Mimee et al. 2012). ELISA and HPLC confirmed that nesfatin-1 is a secreted protein and is present in the cerebrospinal fluid and in circulation. NUCB2/nesfatin-1 like immunoreactivity was found to be co-localized with several peptides and neurotransmitters involved in feeding regulation. It was co-localized with melanocyte stimulating hormone (MSH), pro-opiomelanocortin (POMC), neuropeptide Y (NPY), growth-hormone releasing hormone (GhRH), oxytocin and tyrotropin releasing hormone (TRH) (Foo et al., 2008; Brailoiu et al., 2007; Kohno et al., 2008; Fort et al., 2008; Inhoff et al., 2010; Maejima et al., 2009; Okere et al., 2010). Nesfatin-1 co-localizes with insulin in beta cells

in the pancreatic islets of both rats and mice (Gonzalez et al., 2009). This co-localization was also observed in high-fat diet induced obese mice (Gonzalez et al., 2009). The co-localization was age-dependent suggesting that nesfatin-1 is involved in glucose metabolism during growth. The blood-brain barrier is an important passage between central nervous system (CNS) and the peripheral tissues that helps in the transport of feeding related peptides and regulatory proteins (Banks et al., 1990). It was observed that nesfatin-1 crosses the blood brain barrier in both directions in mice, which is blood to brain and brain to blood. Nesfatin-1 mRNA is expressed in the stomach of mice and this expression was 10 fold higher than that in the brain suggesting that stomach is a source of circulating nesfatin-1. The ability of nesfatin-1 to cross the blood brain barrier via a non-saturable mechanism results in possibility of nesfatin-1 release from the stomach that act centrally (Price et al., 2007). The half-life of nesfatin-1 in circulation is approximately 6-7 minutes (Price et al., 2007). These studies suggest that nesfatin-1 is widely expressed in a number of tissues endogenously. Heart also expresses nesfatin-1 (Angelone et al., 2012). The following section describes nesfatin-1 in the heart and how it is involved in the regulation of cardiovascular system in mammals.

Nesfatin-1 in the Cardiovascular System

In a recent study by Angelone et al., (2012), it was observed that the rat heart expresses nesfatin-1 and NUCB2 mRNA. This observation was similar to that observed in the brain and stomach indicating cardiac tissue to be a source of nesfatin-1. Recently, it was observed that nesfatin-1 is secreted and synthesized by both human and murine cardiomyocytes. A 9.5 kDa band representing nesfatin-1 was detected using Western blot analysis in the primary cardiomyocyte culture lysates of rats, mice and human (Feijóo-Bandín et al., 2013). Immunohistochemistry analysis also revealed the presence of nesfatin-1 like immunoreactivity in perinuclear location, around the nuclear envelope in cardiomyocytes. Iwasaki et al., (2009) provided evidence suggesting that stimulation of Ca2+ through N-type channels can help nesfatin-1 to activate the vagal afferent neurons, through which nesfatin-1 signals the brain and results in suppression of food intake. Thus nesfatin-1 exerts its anorexigenic effect by acting through various neurotransmitters in the brain. Nesfain-1 regulates cardiac functions in mammals (Angelone et al., 2012). A dose dependent effect of negative inotropism (weak muscle contractions) and lusitropism (slowed relaxation of heart) was observed post exposure to nesfatin-1 in Langerdoff-perfused heart

of Wistar rats. It was also determined that nesfatin-1 can act as a cardioinhibitory peptide. Studies conducted on isolated Langerdoff-perfused rat heart showed that the contractility and relaxation was decreased by nesfatin-1 in a dose-dependent manner. These effects suggest that nesfatin-1 can modify the performance of mammalian heart (Angelone et al. 2012).

Nitric oxide (NO) is an important modulator of vascular performance. Nesfatin-1 counteracts NO-dependent vasodilation of the arteries, suggesting a possible hypertensive role. Hypothalamic melanocortin-3/4 receptors act via the neuronal circuits responsible for hypertension (Oh-I et al., 2006). It was found that melanocortin-3 receptors are present in the peripheral tissues including gut, heart, ovary, skeletal muscles and most recently in murine mesenteric vascular bed. Thus, there is a possibility that nesfatin-1 affects the peripheral arterial contractility through the melanocortin-3 receptor (Yamawaki et al., 2012). Nesfatin-1 was also found to elicit hypertensive role by activating these neurons (Yamawaki et al., 2012). The hypertensive effect of nesfatin-1 was also found to mediate via the hypothalamus oxytocin receptor, which is further downstream of the melanocortin receptor (Yosten et al., 2010). Nesfatin-1 modulated peripheral arterial contractility and blood pressure in rats. Yamawaki et al. (2012), observed that nesfatin-1 affects contractility of rat mesenteric artery. Nesfatin-1 impaired the cGMP production and thereby impairing sodium nitroprusside (SNP) induced relaxation of the smooth muscle. SNP induces smooth muscle relaxation by activating guanylate cyclase and in turn by producing cGMP (Rapoport et al., 1983). cGMP decreases the cytosolic calcium concentration by activating cGMP-dependent protein kinase (Karaki et al., 1988). Thus it is presumed that nesfatin-1 exerts its inhibitory action by inhibiting guanylate cyclase activity. Yamawaki et al. (2012) determined that nesfatin-1 regulates blood pressure by modulating the contractility of blood vessel. Intravenous administration of nesfatin-1 increased the systolic, mean and diastolic blood pressure. However, the SNP-induced decrease in blood pressure was also impaired by nesfatin-1 administration in rats (Yamawaki et al., 2012). These results suggest that hypertensive role of nesfatin-1 is mediated possibly via both its actions on the central nervous system and on the peripheral blood vessels.

It was observed that nesfatin-1 elicits cardioprotection (Yosten et al. 2009). Yosten et al. 2009, determined that central administration of nesfatin-1 caused a significant increase in mean arterial pressure in conscious rats. Rats injected with 60 and 180 pmol of nesfatin-1 had increased

mean arterial pressure significantly (Yosten et al., 2009). As previously discussed, the melanocortin antagonist, SHU9119, abolished satiety induced by nesfatin-1 (Oh I et al., 2006). Pretreatment with SHU9119 blocked the increase in mean arterial pressure by nesfatin-1. Nesfatin-1 also helps in cardioprotection. It induced a significant protective effect against myocardial I/R injury (Angelone et al., 2012). Nesfatin-1 also altered the distribution of GLUT4, glucose transporter of the heart, by translocating it from the cytoplasm to cell periphery in cultured cardiomyocytes (Feijóo-Bandín et al., 2013). This suggests that nesfatin-1 is involved in the regulation of glucose metabolism in cardiomyocytes. Glucose uptake by HL-1 cardiomyocytes and rat cardiomyocytes in culture was increased in the presence of nesfatin-1 (Feijóo-Bandín et al., 2013)

Dose and time dependent increase in phosphorylation of Akt and ERK 1/2 w0as observed in the presence of nesfatin-1. Akt is phosphorylated by nesfatin-1 in the hypothalamic nuclei and is involved in the mediating glucose homeostasis (Yang et al., 2012). However, ERK 1/2 is activated by nesfatin-1 in perfused rat hearts (Angelone et al., 2013). Both Akt and ERK1/2 promotes GLUT4 translocation and glucose uptake in muscle and cardiac cells (Peng et al., 2011; Steinbusch et al., 2011). AS160 is a distal insulin signaling protein that is linked to GLUT4 translocation (Cartee et al., 2007). It is an Akt substrate, which is phosphorylated by nesfatin-1 in cardiomyocytes (Feijóo-Bandín et al., 2013).

Obese patients have high serum levels of nesfatin-1 and cardiovascular diseases are often seen in obese patients (Garcia-Galiano et al., 2010). Increased serum nesfatin-1 levels mimic the mechanism of action of leptin and also have protective roles against dysrhythmias (Price et al., 2008). Nesfatin-1 protects against dysrhythmias by blocking NPY (Price et al., 2008). NPY is involved in vagal inhibition. In patients with hypertension, nesfatin-1 level was increased and this could be an effort to block the inhibition of vagal activity and hypertension (Price et al., 2008). Kemp et al., (2012) showed that oxytocin increases heart rate variability in resting humans. These receptors may also help in protecting autonomic system of the heart that may result in an up-regulation of parasympathetic response and a down-regulation of sympathetic responses. Nesfatin-1 mechanism of action can also be mediated through oxytocin in supraventricular tachycardia (SVT) condition. SVT is a dysrhythmic condition in humans, where the heart rate exceeds 100 beats per minute. In patients with SVT, it was observed that nesfatin-1 levels increased and this in

turn activated the oxytocin to increase the autonomic cardiac control mechanism (Celik Ahmet et al., 2013).

Preeclampsia is a metabolic syndrome associated with several cardiovascular diseases with obesity and inflammation being two of the possible mechanisms of preeclampsia. Nesfatin-1 plays an important role in both obesity (Oh-I et al., 2006) and inflammation (Bonnet et al., 2009; Tang et al., 2012) It is likely that nesfatin-1 plays a role in preeclampsia as well. In patients with severe preeclampsia, nesfatin-1 levels were decreased compared to control group (healthy subjects) (Zhang et al., 2014). All these results provide evidence in support of nesfatin-1/NUCB2 involvement in the regulation of cardiovascular functions in mammals.

Nesfatin-1 in Metabolism

Oh-I et al., (2006) found a reduction in the NUCB2 mRNA gene expression in the paraventricular nucleus after starving the rats for 24 hours. Intracereberoventricular injections of nesfatin-1 caused a decrease in food intake and suppressed weight gain. Feeding is regulated by the hypothalamic nuclei and the hormones that are anorectic in nature include leptin and POMC. It was observed that nesfatin-1 induced satiety had occurred in Zucker obese rats with leptin receptor mutation. It was observed that rats receiving intracereberoventricular injections of nesfatin-1 had decreased food intake and suppressed body weight gain. It also decreased the weight of subcutaneous, epididymal and mesenteric fats (Oh-I et al. 2006). The wide expression of nesfatin-1 and its changes in response to the nutrient status suggest possible metabolic actions for this protein in the brain (Oh-I et al., 2006). The expression of NUCB2 in PVN was stimulated after a central injection of α-MSH. Injection of SHU9119, a MSH antagonist, abolished nesfatin-1 induced satiety (Oh-I et al., 2006). These studies suggest that nesfatin-1 effects on feeding might involve a leptin-independent melanocortin signaling system (Oh-I et al., 2006). Dark phase food intake in rats was decreased when nesfatin-1 was injected into the third brain ventricle in a dose-dependent manner. A low dose of nesfatin-1 injection (5 pmol) suppressed the food intake for up to 6 hours. However, with a higher dose (25 pmol) the suppressive effects lasted for 48 hours. A similar result was seen in mice following intracereberoventricular injections of nesfatin-1 (Atsuchi et al., 2010; Konczol et al., 2012).

Intracereberoventricular injections of nesfatin-1 affect several functions including food intake and body temperature for 48 hours (Konczol et al., 2012). It also modified the circadian

rhythm. Earlier reports show the effect of nesfatin-1 on food intake and body temperature for 24 hours (Oh-I et al., 2006). However, a study conducted by Konczol et al., (2012) showed that nesfatin-1 induced reduction in food intake and body weight lasted for 48 hours. Nesfatin-1 injected during the light phase showed significant reduction in food intake in male wistar rats. This suggests that nesfatin-1 may be involved in circadian rhythm interaction. Nesfatin-1 also affected the body temperature. In rats injected with lower dose of nesfatin-1, an increase in body temperature by 1% was observed during the light phase (Konczol et al., 2012). This effect lasted until the second day, where the increase in body temperature did not return to normal. During dark phase, lower dose of nesfatin-1 elevated body temperature, which failed to come back to normal levels during the day, suggesting a possible interaction with the sympathetic activity. They also observed an increase in heart rate with increase in nesfatin-1 concentration especially during the light phase when compared to dark phase (Konzcol et al., 2012). Melanin concentrating hormone (MCH) has been identified to regulate sleep (Verret et al., 2003). Disturbance of sleep is a major cause for the development of obesity and it can affect food intake. NUCB2/nesfatin-1 co-localizes with MCH. This association of nesfatin-1 with MCH, suggests that nesfatin-1 is involved in regulation of sleep. However the mechanism is yet to be elucidated (Jego et al., 2012). Nesfatin-1 is also involved in gastric emptying (Xia et al., 2012). Nesfatin-1 injected into the brain fourth ventricle inhibited the stimulation of gastric acid secretion. This was observed in mice and rats. It also acts in the brain to influence gastric secretion (Xia et al., 2012). Nesfatin-1 also regulates drinking behavior. Injection of nesfatin-1 at 160-540 pmol reduces consumption of water. Microinjection of low doses of nesfatin-1 into the subfornical organ elicits a dipsogenic effect, suggesting a possible role of nesfatin-1 in maintaining fluid balance (Yosten et al., 2012).

Nesfatin-1 stimulates the hypothalamo-pituitary-adrenal axis by releasing corticotropin releasing factor (CRF) from the PVN (Price et al., 2008). CRF receptor signaling system is involved in the regulation of food intake. When nesfatin-1 is injected into the brain third ventricle of rats, release of CRF from PVN was increased. Whereas, nesfatin-1 injected into the PVN directly also caused an increase in the excitability of CRF neurons. It also increases the plasma concentration of ACTH and corticosterone (Konczol et al., 2010). These results provide evidence that nesfatin-1 stimulates the HPA axis. Neuropeptide Y is a potent orexigenic peptide. Nesfatin-1 elicits its anorexigenic property by acting on NPY. Nesfatin-1 injection into the arcuate nucleus

caused hyperpolarization of NPY neurons, thereby promoting an anorexigenic effect (Price et al., 2008).

Nesfatin-1 is involved in the regulation of other aspects of metabolism as well. A four-fold increase in nesfatin-1 level was observed in MIN6 cells after they were incubated with high concentration (16.7 mM) of glucose (Gonzalez et al., 2011). Nesfatin-1 stimulates glucose stimulated insulin secretion from rat and mouse pancreatic islets in a dose dependent manner. In hyperglycemic db/db mice, nesfatin-1 had an antihyperglycemic effect by lowering blood glucose level (Su et al., 2010). In vivo studies using rats, it was observed that nesfatin-1 stimulates insulin secretion. This insulinotropic effect is mediated by Ca2+ stimulation through the L-type calcium channel. Nesfatin-1 stimulated the expression of preproinsulin mRNA in MIN6 cells (Gonzalez et al., 2011). Goebel et al. (2011) showed that fasting decreases nesfatin-1 levels and re-feeding normalizes this decrease. Circulating nesfatin-1 in humans was increased after an oral ingestion of glucose (Tsuchiya et al., 2010). These studies suggest that nesfatin-1 is regulated by nutritional status and starvation in circulation and its involvement in regulating whole body glucose and energy metabolism (Tsuchiya et al., 2010).

Nesfatin-1 also has anti-hyperglycemic properties, where it was found to act on hyperglycemic db/db mice (Su et al., 2010). Intraventricular injections of nesfatin-1 caused a decrease in blood glucose levels in these mice for approximately six hours, suggesting a possible role of nesfatin-1 as an anti-hyperglycemic agent (Su et al., 2010). In patients suffering from type 2 diabetes, blood nesfatin-1 was lower compared to healthy control subjects and patients with type 1 diabetes (Su et al., 2010). However, in type 1 diabetes nesfatin-1 levels were higher compared to healthy controls (Su et al., 2010). Low level of nesfatin-1 in type 2 diabetic patients could be due to insulin sensitivity impairment, which requires further analysis (Su et al., 2010). Li et al., (2013) determined that peripheral administration of nesfatin-1 affected glucose metabolism by acting on skeletal muscle, adipose tissue and liver. Improvement to glucose tolerance and insulin sensitivity was observed in mice fed with normal or a high fat diet following continuous infusion of nesfaitn-1 (Li et al., 2013). Akt phosphorylation was also up-regulated by nesfatin-1 in pancreas and islet cells of MIN6 (Miki et al., 2001). The GLUT4 expression was also increased with nesfatin-1 (Stengel et al., 2010). These results suggest that peripheral nesfatin-1 increases glucose

metabolism and insulin sensitivity by altering the Akt phosphorylation and GLUT4 membrane translocation (Xu et al., 2009)

Nesfatin-1 is expressed in the PVN. Nesfatin-1, when injected centrally evoked anorexia and activation of oxytocin producing neurons in the PVN. Several factors including insulin and glucose regulate nesfatin-1 neurons in the PVN (Gantulga et al., 2012). Activation of neurons usually occurs in the presence of activated Ca^{2+} concentrations. Presence of insulin and high glucose enhances calcium concentration and thereby influences nesfatin-1 neurons. The study revealed that neurons responsive to glucose also showed response to insulin. However, some nesfatin-1 neurons failed to show responsiveness to glucose, insulin and a combination of both, suggesting that these nesfatin-1 neurons could be regulated by other factors responsible for drinking behavior, stress, cardiovascular and sympathetic nerve activity (Gantulga et al., 2012).

Fasting and obesity causes alteration of several pathways and expression of genes responsible for causing diseases. For example, mTOR, a mammalian signaling molecule target of rapamycin, is detected in the gastric endocrine X/A cells. mTOR alteration causes alteration in ghrelin production, where inhibition of mTOR signaling molecule caused increase in ghrelin release. Li et al., (2012) found that mTOR is also responsible for expression changes of NUCB2/nesfatin-1 in the gastric endocrine X/A cells. Immunohistochemistry analysis showed co-localization of mTOR molecule (pS6K1) with NUCB2/nesfatin-1 in the gastric mucosa. To determine the effect the mTOR signaling on NUCB2/nesfatin-1 mRNA and protein levels, three different groups of C57BL/6 mice used, a control group (ad libitum food), fasted group (fasted for 24h) and re-fed group (re-fed after 24h fasting for 2h). Fasting caused a decrease in pS6K1 phosphorylation, a decrease in both NUCB2/nesfatin-1 mRNA and protein levels. However, in high-fat diet induced obese mice there was an increase in mTOR signaling and NUCB2/nesfatin-1 levels. Inhibition of pS6K1 by intraperitoneal injection of rapamycin decreased the expression of NUCB2 in both lean and obese mice. This was in contrast to ghrelin levels, where an increase in expression was observed. Therefore, mTOR signaling in the gastric mucosa might be of interest in developing therapies related to obesity in the future (Li et al. 2012).

Nesfatin-1 is proposed to act on a membrane bound G-protein coupled receptor in the neurons of cultured rat hypothalamus. Activation of this receptor led to an increase in the calcium concentration, which is in turn linked to activation of protein kinase A. It was observed that in a

calcium free medium, nesfatin-1 induced increase in calcium concentration was abolished, suggesting a possible role of nesfatin-1 in increasing the calcium influx (Brailoiu et al., 2007). Overall, there is enough evidence available now to indicate that nesfatin-1 is a multifunctional protein. The mechanism of tissue specific actions of nesfatin-1 is currently an emerging area of research.

As discussed above, nesfatin-1 is negatively correlated to the body mass index (BMI) and blood glucose during fasting. In patients with chronic liver disease including non-alcoholic fatty liver disease (NAFLD), there was a decrease in serum nesfatin-1 level. This decrease could cause an increase in appetite and thereby over nutrition. This in turn leads to obesity and diabetes mellitus and hence causing NAFLD. This indicates that an increase in nesfatin-1 levels can help reduce obesity and NAFLD (Basar et al., 2012). Patients suffering from gestational diabetes mellitus (GDM) also showed lower levels of nesfatin-1. Type 2 diabetic rats showed decreased level of nesfatin-1, however in mice with diet induced obesity NUCB2 mRNA expression was increased (Aslan et al., 2012). A reduction in nesfatin-1 causes dysregulation in insulin secretion in patients with gestational diabetes mellitus. Therefore, nesfatin-1 could increase insulin level and hence act as an anti-diabetic factor (Aslan et al., 2012).

Zegers et al., (2012) showed that NUCB2 gene mutations cause severe obesity. Two heterogeneous mutations on the NUCB2 gene, one at the missense (L125H) and another at nonsense (K178X), were observed in obese girls. In patients with the K178X mutation, Western blot analysis showed the presence of 42kDa band of NUCB2, whereas nesfatin-1 peptide band was not observed. This was similar to the results shown by Stengel et al, 2009 and Gonzalez et al, 2009, where the presence of only pronesfatin-1 and not nesfatin-1 was detected in pancreas, pituitary and gastric mucosa of rodents. L125H mutation is associated with the leucine rich N-terminal of NUCB2 gene which is involved in the release of the protein from the Golgi complex. A mutation at this position results in failure of the protein release from the Golgi bodies. K178X mutation is associated with DNA binding region of NUCB2 gene, where a stop codon is present and hence destroys the DNA binding property of the molecule. However the mechanism causing obesity in these patients is not yet known (Zegers et al., 2012).

Obese patients have high levels of ghrelin secreted from the gastric endocrine X/A cells (Ariyasu et al., 2001). Ghrelin stimulates food intake (Tschop et al., 2000) and are high in patients

with anorexia (Janas-Kozik et al., 2007). NUCB2/nesfatin-1 was also detected in ghrelin secreting endocrine X/A cells and was decreased in patients with anorexia. Therefore the gastric endocrine cells are able to produce two different peptides causing a decrease or increase in food intake depending on the peptides released from them. Previously, co-localization of ghrelin and nesfatin-1 like immunoreactive cells were observed in the rat stomach (Stengel et al., 2009). With this study, expression of both ghrelin and nesfatin-1 was observed in human stomach similar to that in rats. Body Mass Index (BMI) also plays an important role in the release and regulation of these peptides. Higher BMI subjects had decreased levels of ghrelin immunoreactive cells and increased nesfatin-1 immunoreactive cells compared to the lower BMI group (Stengel et al., 2013). The enzyme that is responsible for the acylation of ghrelin, known as Ghrelin O-acyltransferase (GOAT), is expressed in mice higher than in rats. The expression of this enzyme was detected in the same endocrine X/A like cells. However, in human stomach samples, GOAT and ghrelin were found in the same vesicles, though all GOAT expressing cells also expressed ghrelin. GOAT expression decreased in subjects with higher BMI, an effect similar to ghrelin (Stengel et al., 2013).

Patients suffering from type 2 diabetes mellitus have decreased plasma nesfatin-1 levels compared to normal subjects and in individuals with impaired glucose regulation. Decreased levels of nesfatin-1 may suggest a possible role of nesfatin-1 in thyroid dysfunction (Liu et al. 2014). Also, in patients with metabolic syndrome, nesfatin-1 plasma levels were increased. Ari et al., (2011) determined that nesfatin-1 levels increased in patients suffering from major depressive disorders whereas in patients suffering from epilepsy (Aydin et al., 2011), a neurological disorder, nesfatin-1 level was decreased. There is enough evidence that nesfatin-1 is an emerging multifunctional protein. Apart from playing an important role in metabolism, it also elicits important roles in other functions including reproduction both in mammals and non-mammals.

Nesfatin-1 in Reproduction

Nesfatin-1 like immunoreactivity was observed in Leydig cells of humans and rodents. Leydig cells are the cells of the testes that produce the hormone testosterone (Garcia Galiano et al., 2012). Nesfatin-1 is expressed in testis and is involved in controlling several reproductive functions of the body including the onset of puberty and gonadotropin release in male wistar rats (Garcia Galiano et al. 2012). The presence of NUCB2 mRNA was detected using reverse transcriptase PCR and a western blot analysis showed the 42kDa band of NUCB2. NUCB2 mRNA

expression in the testes was up-regulated significantly during development. A significant increase, approximately 10 fold, in the levels of prepronesfatin during the adult stage when compared to the pubertal stage was observed (Garcia Galiano et al., 2012).

Nesfatin-1 in Non-Mammals

While the physiological role of nesfatin-1 has been studied extensively in mammals, its existence and functions in non-mammalian vertebrates is only emerging. Nesfatin-1 is also present in non-mammals, including goldfish (Gonzalez et al., 2010), zebrafish (Hatef et al., 2014) and trout (Caldwell et al., 2014). Recently, Gonzalez et al., (2010) characterized nesfatin-1 in goldfish. Goldfish nesfatin-1 was expressed in various tissues including liver, pituitary, olfactory bulbs, hypothalamus and adipose tissue. A studied conducted in our lab, also shows expression of NUCB2 mRNA in tissues including heart, liver, muscle, gut, ovary, brain and testis of zebrafish (Hatef et al., 2014). A 50 kDa band was observed recently in brain samples of a frog. *M. ornata*. Nesfatin-1 immunoreactivity was also observed in the olfactory regions, pineal gland, ventromedial and posterior thalamic nucleus and the pituitary gland of *M. ornata* (Senejani et al., 2014). Immunohistochemical analysis showed nesfatin-1 like immunoreactivity in the hypothalamus of goldfish brain. Enteroendocrine cells lining the J-loop also showed nesfatin-1 like immunoreactivity. It was observed that NUCB2 mRNA expression in goldfish hypothalamus did not change until after feeding (Gonzalez et al. 2010). A peri-prandial study conducted by the team showed a 2 fold increase in the hypothalamus NUCB2 mRNA at 1 and 3 hours post feeding. It was observed that, in fasted goldfish, NUCB2 mRNA level in the hypothalamus was significantly lower compared to fed group (Gonzalez et al., 2010). Re-feeding after 7 days of food deprivation also increased NUCB2 mRNA in the hypothalamus of goldfish. During food deprivation, liver gluconeogenesis acts as a primary source of glucose to maintain normoglycemia. However, food deprivation in goldfish elevated NUCB2 mRNA expression in liver. This increase in nesfatin-1 could indicate a direct role of the peptide in liver physiology during food deprivation. Intraperitoneal injection of synthetic native nesfatin-1 at 50 ng/g body weight (BW), resulted in a reduction of food intake for one hour by 20% in goldfish. This result was in consistent with that observed in mammals. When nesfatin-1 is injected centrally at 0.5 ng/g BW, there was a 43% decrease in food intake for one hour (Gonzalez et al., 2010). The effect of nesfatin-1 on food intake was more readily observed through central injections as compared to peripheral administration.

Further studies are required to determine the mechanism of action of nesfatin-1 in the goldfish brain that causes an anorexigenic effect. These results provide the first molecular and functional evidence for the wide presence and distribution of NUCB2 and anorectic effects of nesfatin-1 in non-mammalian vertebrates.

Nesfatin-1 in the goldfish also co-localizes with various appetite regulatory peptides. In a recent study conducted by Kerbel et al. (2011), it was observed that nesfatin-1 co-localizes with ghrelin in the hypothalamus. Stengel et al. (2009) showed nesfatin-1 like immunoreactivity in the X/A cells in the stomach, where it was found to co-localize with ghrelin. Ghrelin like immunoreactivity has been seen in fish by using mammalian antibodies. As described previously, nesfatin-1 like immunoreactivity in the J-loop of goldfish was co-localized with ghrelin like immunoreactivity (Gonzalez et al., 2010). This suggests a possible interaction between nesfatin-1 and ghrelin. Ghrelin is an orexigenic peptide in goldfish and is expressed in the telencephalon and hypothalamus. To study the possible interaction of nesfatin-1 and ghrelin in goldfish, intracereberoventricular injections of *gf*nesfatin-1 and *gf*ghrelin was employed (Kerbel et al., 2011). *Gf*ghrelin stimulated food intake in goldfish, whereas *gf*nesfatin-1 inhibited food intake in goldfish. Ghrelin administration also reduced NUCB2 mRNA expression in the forebrain, suggesting that ghrelin has a suppressive role on nesfatin-1. The opposite effect was observed when nesfatin-1 injection resulted in a significant reduction of ghrelin mRNA expression in the forebrain.

Expression of cholecystokinin (CCK) was increased significantly at 30 minutes post-nesfatin-1 administration in goldfish (Kerbel et al., 2011). However, orexin expression was increased after 1 hour of nesfatin-1 injection. The interaction between nesfatin-1 and ghrelin as seen in the forebrain was not observed in the gut. NUCB2 expression increased post-*gf*ghrelin administration and vice versa. Injection of ghrelin increases food intake beyond the normal level. The body then triggers NUCB2 mRNA expression to increase nesfatin-1 in circulation and activates the satiety response. Nesfatin-1 administration caused a reduction in ghrelin receptor mRNA expression in brain by 29%. These results provide evidence of direct actions of nesfatin-1 and ghrelin on each other in the forebrain. Collectively these results show that nesfatin-1 interacts with ghrelin, orexin and CCK to regulate feeding in goldfish (Kerbel et al., 2011).

Nesfatin-1 attenuates the stimulatory effects of maturation-inducing hormone on germinal vesicle breakdown of incubated zebrafish follicles (Gonzalez et al., 2012). It was found that nefatin-1 modulates hypothalamic and pituitary hormones. A single intraperitoneal injection of nesfatin-1 produced a significant and acute decrease in the expression of salmon gonadotropin releasing hormone (sGnRH) and chicken GnRH –II (cGnRH), two GnRH isoforms in the forebrain of goldfish, at 15 min post injection. Both sGnRH and cGnRH-II mRNA expression returned to control values at 30 min post injection. This shows an inhibitory role for nesfatin-1 on the gonadotropin releasing hormone system in the goldfish forebrain. Similarly, the expression of pituitary luteinizing hormone and follicle stimulating hormone mRNAs were also found significantly suppressed after nesfatin-1 injection. It was also shown that nesfatin-1 co-localizes GnRH immunoreactivity in the goldfish brain. NUCB2 mRNA was also determined in goldfish gonads. Serum LH levels were also found to be lower at 60 minutes post-nesfatin-1 injection, suggesting a possible role of nesfain-1 in fish reproduction (Gonzalez et al., 2012). These studies show that nesfatin-1 plays a major role in fish reproduction. However, this area requires further research as there is paucity of information.

In a more recent study conducted on Ya-fish (*Schizothorax prenanti*) showed high similarity of the NUCB2A sequence with other vertebrates, including goldfish and zebrafish (Lin et al., 2014). NUCB2A mRNA was detected in several tissues including hypothalamus, gonads, ovary, hepatopancreas, pituitary and kidney. Presence of NUCB2 mRNA in these tissues suggests a possible of nesfatin-1 in maintaining energy homeostasis, metabolism and reproduction. As observed in goldfish (Gonzalez et al., 2010), feeding in Ya-fish also increased NUCB2A mRNA levels in the hypothalamus. Food deprivation decreased NUCB2A levels and this was restored to normal with re-feeding (Lin et al., 2014). Another study conducted using rainbow trout, saw no difference in nesfatin-1 levels in restricted feeding groups as compared to control group which were fed ad libitum. The trouts were female and post-spawned (Caldwell et al., 2014). Together these data and studies from our lab have shown that nesfatin-1 is a novel physiological regulator of feeding and reproduction in non-mammalian species as well. However, nothing is known about nesfatin-1 and its effect on cardiac functions in zebrafish. My research will primarily focus on nesfatin-1 effects on zebrafish cardiovascular system as well as a murine cardiomyocyte cell line.

1.2.5. Zebrafish – A suitable model organism

Zebrafish (*Danio rerio*) is a vertebrate, which is extensively used in metabolism research. Its small size and high fertility makes it cost effective and convenient for use. The whole genome information of zebrafish is available, which allows for both forward and reverse genetic screens (Bakkers. 2011). Zebrafish are similar to higher vertebrates including rodents and humans on evolutionary basis. High throughput screening of zebrafish fish makes it a suitable model for studying gene expression analysis, drug discovery and pharmacology. Live imaging of zebrafish embroys is possible due to its optical transparency (Sun et al., 2008). Zebrafish produce high number of offspring that are able to develop rapidly. Zebrafish allows for studying chronic diseases due to its short generation time. Due to similarity to higher vertebrates, they are useful in studying diseases of the immune system, glucose metabolism disorders and cancer. It is also an excellent model to study toxicity and effects of various chemicals and endocrine disruptors on growth and development, survival rates and reproduction. The ability to regenerate, for example fins, heart, spinal cord and nerves makes it an excellent model in the field of biology. Zebrafish embryos have the ability to grow and develop outside the mother and thus suitable for studying bone formations, vasculature and blood vessel development (Segner et al., 2009).

Apart from these, zebrafish anatomy and physiology also make it suitable in studying the cardiovascular system. Zebrafish heart is similar in structure to the human heart. It consists of atria, ventricles, valves and a conduction system. With the help of zebrafish, identification and characterization of novel genes for cardiovascular development is made easier. Zebrafish does not depend on a complete cardiovascular system for its development, as a result of which it is extensively used as a study model in the field of cardiovascular physiology. It is used in studies related to heart failure, congenital heart diseases (CHD), blood vessels development and several other diseases related to the heart (Bakkers et al., 2011). It has emerged as an excellent genetic model organism for studies related to heart. Zebrafish allows for visual analysis of the early development processes. Optical methods, however are not suitable for visual analysis of adult zebrafish as they are not transparent in nature. Sun et al., (2006) however, used high frequency ultrasonic imaging on adult zebrafish to study cardiac functions. This technique consumed a lot of time and real time visualization was impossible. High frequency ultrasound monitoring made it

possible to visualize and produce high-resolution images which can further help in studying the cardiac functions in zebrafish (Sun et al., 2008).

1.3. Rationale

Nesfatin-1 is emerging as a regulator of cardiovascular functions in rodents. Nesfatin-1 is known to have feeding and reproductive roles in fish. However, if it is a modulator of the cardiovascular system in fish is yet to be known. Is nesfatin-1 involved in the acute regulation of cardiac functions in fish? Using zebrafish, my MSc thesis will address this area of research. A very preliminary ultrasound analysis of nesfatin-1 global knockout mice (Source: Mouse Genome Informatics; these mice are currently bred for our research) found putative developmental or structural defects of the heart that were reflected in altered cardiac functions. This effect suggests that endogenous nesfatin-1 might have some roles in cardiac and vascular tissues in mice. My research will use HL-1 cardiomyocytes to address the effects of nesfatin-1 in mammals. Nesfatin-1 is also an emerging multifunctional protein in fish. It is expected that the results of my research will provide significant new information on our fundamental understanding of nesfatin-1 and the cardiovascular system.

1.3.1. Hypothesis

I hypothesize that nesfatin-1 acutely modulates the cardiovascular physiology of adult fish, and it also regulates growth and apoptosis of mouse cardiomyocytes.

1.3.2. Objectives

The overall objective of my research is to study NUCB2/nesfatin-1 in the CV system of fish and the effects of nesfatin-1 in murine cardiomyocytes. The specific objectives of my MSc thesis research are:

(1) To study the direct effects of nesfatin-1 on cardiovascular functions in zebrafish.

(2) To determine the effects of nesfatin-1 on mouse heart and murine cardiomyocytes.

I will investigate the effects of nesfatin-1 on the cardiac functions of fish, and on the growth and proliferation of mouse cardiomyocytes using techniques including high frequency ultrasound monitoring, molecular biology and cell culture.

TRANSITION

The following chapter focuses on my first objective: nesfatin-1 in the zebrafish cardiovascular system. As discussed earlier, nesfatin-1 has been studied only in the mammalian cardiovascular system and not much is known about its effect on non-mammals, including zebrafish. We found that zebrafish heart is a source of endogenous nesfatin-1 and chronic food deprivation did not alter the mRNA expression of NUCB2 in zebrafish heart. We also conducted an ultrasound study to determine the role of exogenous nesfatin-1 in modulating cardiac functions. This part of my thesis was conducted in collaboration with Dr. Lynn Weber and Courtney Gerger (MSc Student in Dr. Lynn Weber's research lab).

Contributions of co-authors: Dr. Weber and Courtney Gerger taught me how to use the ultrasound machine, ultrasound data analyses, and helped with the writing of methods, results and discussion pertaining to the ultrasound data. I conducted the studies, analyzed the results and wrote the first draft of the manuscript. Dr. Suraj Unniappan provided the infrastructure, funding, research ideas, helped with experimental design and data analyses and manuscript writing.

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Chapter 2: Nesfatin-1 Regulation of Cardiac Functions in Zebrafish

2.1. Introduction

Hormones play a key role in the maintenance of cardiovascular physiology. A number of peptidyl endocrine factors have multiple effects on cardiovascular functions (Kishimoto et al., 2012; Holst. 2007; Adrian et al., 1985) and one among them is nesfatin-1 (Oh-I et al., 2006). NUCB2 is cleaved by prohormone convertases (PCs) into three fragments named nesfatin-1 (1-82 amino acids), nesfatin-2 (85-163 amino acids) and nesfatin-3 (166-396 amino acids). Mid-segment of nesfatin-1 (24-53 amino acids) is the active peptide inhibitory segment as the injections of C (1-23) and N (54-82) of nesfatin-1 had no anorectic effects in mammals (Oh-I et al., 2006). Nesfatin-1 has an extensive distribution in tissues including stomach (Stengel et al., 2009), pancreas (Stengel et al., 2009; Gonzalez et al., 2009), testis, gut and the cardiac muscle (Mimee et al., 2012). Intracereberoventricular injections of nesfatin-1 led to loss of appetite, and caused less frequent hunger and a sense of fullness (Oh-I et al., 2006). Acute administration of nesfatin-1 by peripheral and central injections has shown to reduce food intake in rats for up to six hours (Oh-I et al., 2006; Shimizu et al., 2009; Stengel et al., 2009) post-administration. These studies provided evidence for nesfatin-1 as a metabolic regulator with anorectic and weight reducing effects.

Recently, there has been a large amount of data available to attribute a role for nesfatin-1 in regulating cardiac functions. Rat heart expresses nesfatin-1 (protein) and NUCB2 mRNA (Mimee et al., 2012). Nesfatin-1 modulated blood pressure in rats by directly modulating the peripheral arterial resistance (Yamawaki et al., 2012). Intracereberoventricular injections of nesfatin-1 significantly increased the mean arterial pressure in conscious rats (Yosten et al., 2009). Western blot analysis showed the presence of 9 kDa nesfatin-1 band in the lysates of mice, rat and human cardiomyocytes (Feijóo-Bandín et al., 2014). Nesfatin-1 acts on several calcium channels. L-type Ca^{+} channels are activated by nesfatin-1 to elicit its insulinotropic effects in rats (Gonzalez et al., 2011). It also acts via N-type Ca^{+} channels, which can in turn activate the vagal afferent neurons, signaling the brain resulting in suppression of food intake (Iwasaki et al., 2009). These studies suggest that nesfatin-1 acts on several calcium channels to elicit its anorexigenic actions. Overall these studies indicate the role for NUCB2/nesfatin-1 in the regulation of cardiovascular functions. Nesfatin-1 is also shown to regulate multiple physiological processes in fish (Gonzalez et al., 2010; Kerbel et al., 2012; Gonzalez et al., 2012). However, whether nesfatin-1 regulates

cardiovascular functions in fish remain unknown. Using zebrafish (*Danio rerio)*, I studied the effects of nesfatin-1 in regulating cardiac functions employing techniques including ultrasound bio-microscopy (UBM). Zebrafish has been used to study cardiovascular biology including the study of blood clotting, blood vessel development, heart failure and congenital heart diseases (Bakkers, 2011). One major advantage is that zebrafish are vertebrates that allow the identification of novel genes that are required for cardiovascular development (Sun et al., 2008). They emerged as an excellent model organism for studies related to heart. We hypothesized that NUCB2 is expressed in the heart and regulates the cardiac physiology of zebrafish. In this research using adult zebrafish, it was determined that zebrafish heart expresses NUCB2 mRNA and also NUCB2/nesfatin-1 like immunoreactivity. Chronic food deprivation however did not alter NUCB2 mRNA expression in the heart. I also found that various doses of nesfatin-1 modulates cardiac functions including end diastolic (EDV) and systolic volumes (ESV), heart rate (HR) and cardiac output (CO). Expression of calcium handling proteins of the heart, SERCA2a and RYR1b were determined post- nesfatin-1 injection and ultrasound.

2.2. Materials and Methods

2.2.1. Animals

Zebrafish (*Danio rerio; 1-2 months old*) were purchased from Aquatic Imports (Calgary, Canada) and housed in a recirculating aquatic system at 28°C held at 12 h light:12 h dark cycle (lights on at 7 am and off at 7 pm) and humidity. Fish had *ad libitum* access to food (Nutrafin flakes). All protocols strictly adhered to the guidelines of the Canadian Council of Animal Care, and were approved by the University of Saskatchewan Animal Research Ethics Board (AREB protocol number 2012-0082).

2.2.2. Detection of NUCB2/nesfatin-1 like Immunoreactivity in Zebrafish Heart.

2.2.2.1. Immunohistochemistry

Male and female zebrafish were anesthetized with 20 mg/L of Aquacalm (Syndel Laboratories, Canada). Following this, hearts were dissected and collected in 4% paraformaldehyde. After an incubation period of 24 h, the fixed tissues were washed with 70% ethanol, embedded in paraffin and sectioned (3 µm in thickness). Sections were deparaffinized with xylene (3 x 10 minutes, 25° C) and rehydrated in graded ethanol series (100%, 95%, 70% and 50%). DAKO-serum free protein block reagent (DAKO Corporation, CA) was used to block the section for 10 minutes prior to incubation with rabbit anti-nesfatin-1 primary antibody (Phoenix Pharmaceuticals, Catalog # H-003-22), dilution 1:100. This antibody was previously validated for use in goldfish (Gonzalez et al., 2010; Kerbel et al., 2012) and zebrafish (Gonzalez et al., 2012; Hatef et al., 2014), and pre-absorption of the antibody with synthetic goldfish nesfatin-1 resulted in the absence of staining in sections. The sections were incubated for 24 h at room temperature. Slides were washed with 1X PBS (3 x 10 minutes) and incubated with 1:100 dilution ratio of goat anti-rabbit Texas Red IgG (Vector Laboratories, CA). The slides were incubated for 1 h at 37° C in a humidified incubator. Lastly, the slides were washed with 1X PBS (3 x 10 minutes) and mounted using Vectashield mounting medium containing DAPI (blue nuclear stain, Vector Laboratories). Negative controls were treated with secondary antibody alone. Slides were assessed using a confocal microscope.

2.2.2.2. Confocal Microscopy

Slides were stained for NUCB2/nesfatin-1 and viewed using a Leica SP5 Laser-Scanning Confocal Microscope. A 63X oil immersion objective lens was used to determine NUCB2/nesfatin-1 like immunoreactivity in zebrafish cardiomyocytes. Images were obtained using LAS AF software and modified using the Image J software (http://imagej.nih.gov/ij/). Deconvolution of the images was achieved using the AutoQuantX3 software (http://www.mediacy.com/index.aspx?page=AutoQuant_X3), and 3D images were interpreted using the Imaris 3D software (www.bitplane.com/imaris/imaris).

2.2.3. Characterization of NUCB2 mRNA in Zebrafish Heart

2.2.3.1. Total RNA Extraction

Zebrafish (n = 6) was anaesthetized using Aquacalm (Syndel Laboratories, Canada). The hearts were dissected and stored in RNALater™ (Source) at -80°C. The hearts were then thawed for 10 minutes at room temperature and transferred to new tubes containing TRIzol® Reagent (Life Technologies, Canada). The tissues were homogenized using a needle and syringe. Approximately 200 µl of chloroform was added and centrifuged at 13000 rpm (10,000 xg) for 15 minutes. The supernatant containing RNA was separated from DNA and protein layers. Isopropanol was added in order to precipitate RNA in solution. 70% ethanol is added to remove any phenol impurities from the pellet. Purified RNA was isolated using RNase free water (20µl). The quality and quantity of the extracted mRNA was determined using a NanoDrop 2000C (Thermo Vanta, Finland). Total RNA was quantified by measuring the optical density (OD) and absorption ratio (A260nm/A280nm) of diluted RNA. Pure RNA has a ratio of ~2.0. The ratio of samples used for my study ranged from 1.9 – 2.0. All RNA samples used for cDNA synthesis and qRT-PCR had high quality. Isolated RNA was stored at -80°C until further analysis.

2.2.3.2. cDNA Synthesis

Synthesis of cDNA was conducted uszing iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Canada) as directed by the manufacturer. The reaction set up for cDNA synthesis is described in **Table 2.1**, and the conditions used in the 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 the manufacturer; BioRad).

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 a 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	Hold at infinity

2.2.3.3. Real Time Qualitative PCR

The complimentary DNA synthesized was used as a template to determine the qualitative expression of NUCB2A in zebrafish hearts. The gene sequences were obtained from National Center for Biotechnology Information Gene Bank (NCBI) and the primers were synthesized using OligoPerfect^{TM} Designer software (Life Technologies, Canada) (http://tools.lifetechnologies.com/content.cfm?pageid=9716). 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 with Respective Annealing Temperatures

Gene	Forward (5'-3')	Reverse (3'-5')	Annealing Temperature
Zf β Actin (AF057040.1)	ttcaaacgaacgaccaact	ttccgcatcctgagtcaatg	58°C
Zf NUCB2A (BC046077.1)	aggagcggcatgaagaattt	gatggttgactttggggtga	58°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). 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	100 µl
25nm $MgCl_2$	60 µl
dATP	5 µl
dTTP	5 µl
dCTP	5 µl
dGTP	5 µl
Sterile water	820 µl

Table 2.5. Reaction components for qualitative PCR

Components	Volume required
Master mix	20 µl
Forward Primer	1 µl
Reverse Primer	1 µl
cDNA template	1 µl
Taq Polymerase	0.5 µl
Total Volume	23.5 µl

Table 2.6. Optimized PCR conditions with respective annealing temperatures for NUCB2 and beta actin. (Step 1: Initial Activation; Step 2: Denaturation; Step 3: Annealing; Step 4: Elongation; Step 5: Hold at infinity)

	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	Hold at infinity

The samples were stored at -20° C until gel electrophoresis.

2.2.3.4. Gel Electrophoresis

Following PCR, the samples were run on 1% agarose gel. One gram agarose (BioShop, Catalog # AGA002.500) is mixed with 100 mL of 1X TAE buffer (Fisher Scientific, Catalog # BP1332-1 (50X)). 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 is placed. Once set, the comb is taken out and the electrophoresis chamber is filled with 1X TAE

buffer. This was followed by the addition of 3 µL of 10x loading buffer (Invitrogen, Canada) to the PCR samples and approximately 20µl of sample was loaded into each well. One Kb plus DNA ladder (5µL) (Invitrogen, Canada) was also loaded alongside the samples to detect the amplicon size of the gene. The gel is run from negative to positive, as the DNA is negatively charged, for one and a half hours at 120V. The image was obtained using GelDoc System (Bio-Rad, Canada) and the expression of genes of interest was qualitatively analyzed.

2.2.4. Effect of Chronic Food Deprivation on NUCB2 mRNA Expression in Zebrafish Heart

This study was conducted to determine NUCB2A mRNA expression in zebrafish heart after food deprivation for three or seven days. Fish were acclimated to tank conditions and a scheduled time (12 PM) until the study. At the commencement of the study, two groups were unfed from day 1, and two groups (n = 8 zebrafish/group) were fed *ad libitum*. At days 3 and 7, hearts were collected from one fed and one unfed group. Tissue extraction and relative gene expression was determined as described below.

2.2.4.1. Total RNA Extraction

Tissues collected from each group was thawed and homogenized using a needle and syringe. Extraction was carried out using TRIzol reagent procedure as described in section 2.2.3.1. The purity of RNA is determined using NanoDrop 2000C. The RNA is stored in -80°C until further required.

2.2.4.2. cDNA Synthesis and Quantitative PCR

cDNA was synthesized using iScript DNA synthesis kit as described in section 2.2.3.2. The samples were stored at -20° C until used as a template for quantitative PCR. The expression of NUCB2 mRNA was conducted using SsoAdvanced SYBR Green SuperMix kit (Bio-Rad, Canada). All reactions were performed with a final volume of 20µl as per **Table 2.7**. The reaction protocol was set up in the CFX Connect thermal cycler (Bio-Rad, Canada) as per **Table 2.8**. Forward and reverse primers for NUCB2 and Beta Actin were used (see **Table 2.3** for gene sequences and respective annealing temperatures).

Table 2.7. Components of PCR Reaction Set Up.

Components	Volume per reaction
SsoAdvanced SYBR Green Supermix	10 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Sterile water	8 µl
cDNA Template	1 µl
Total Volume	20 µl

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	73°C	4°C
Time	5 seconds	10 seconds	30 seconds	30 seconds	Hold at infinity

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 were obtained for both reference gene and gene of interest. Reference genes/housekeeping genes are important in order to help normalize the PCR data. It is also 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, β_2-microglobulin and elongation factor alpha (Livak et al., 2001). PCR data is generally analyzed using relative quantification method rather than absolute quantification method. In absolute quantification, the target gene is compared to a standard curve, where as in relative quantification analysis, the target gene is compared to a reference/housekeeping (HK) genes. The C_T values for both the gene of interest and HK genes

were determined for control group and the experimental group. The data were normalized to HK genes ($\Delta C_T = C_{T\,(exp)} - C_{T\,(control)}$). The reference gene used for my research was β-Actin (PCR Application Guide, Bio-Rad).

2.2.5. Pre and Post-prandial Expression of NUCB2 mRNA in Zebrafish Heart

The NUCB2 mRNA expression in zebrafish heart at pre and post-feeding hours were determined in this study. Zebrafish were divided into seven groups with n = 9 fish/group. They were acclimatized for two weeks and fed *ad libitum* at 12 pm (feeding time). On the day of the study, heart samples were collected at 3 h before feeding (-3), 1 h before feeding (-1), at the time of feeding (0), 1 h after feeding (+1) and 3 h after feeding (+3). Two groups of fish were left unfed at the regular feeding time. Samples were also collected from these two unfed groups: 1 h post feeding time and 3 h post feeding time. These were considered as unfed groups. Total RNA extraction, cDNA synthesis and quantitative PCR to determine the relative expression of NUCB2 mRNA were conducted as described in sections 2.2.4. The NUCB2 mRNA was normalized to the expression of NUCB2 at 0 h (at feeding).

2.2.6. Dose Dependent Effects of Nesfatin-1 on Cardiac Functions of Zebrafish

2.2.6.1. High Frequency Ultrasound Monitoring

Cardiac function measurements were carried out using a VEVO 660 high frequency ultrasound machine (Visual Sonics, Markham, ON) equipped with the B-mode imaging and pulsed-wave Doppler. Prior to ultrasound experiments, fish were anaesthetized with Aquacalm (Syndel Laboratories, Canada) at 20 mg/L in clean aerated water. Fish were injected intraperitoneally with synthetic goldfish nesfatin-1 (*gf*nesfatin-1; VPISIDKTKVKLPEETVKES PQNVDTGLHYDRYLREVIDFLEKDQHFREKLHNTDMEDIKQGKLAKELDFVSHHVRTK LDEL; GenScript, NJ) (Gonzalez et al., 2010) at doses of 50 ng/g, 250 ng/g and 500 ng/g body weight. Six microliters of nesfatin-1 dissolved in saline were injected. Each control group received 6 µL of saline (0.9% sodium chloride) with n = 8 zebrafish/treatment. Fish were allowed a recovery period of 15 minutes. Anesthetized fish were then transferred to a holding dish containing a thin layer of 3% agarose gel (Sigma-Aldrich, Oakville, ON) with a groove where fish were placed ventral side up with the aid of minutien pins (Fine Science Tools, Vancouver, BC). The holding dish was filled with aerated water containing 20 mg/L Aquacalm to maintain anesthesia throughout ultrasound testing. Temperature has been shown to have a significant effect on cardiac function

(Blank et al., 2002; 2004). Therefore, all ultrasound experiments were conducted at the temperature (28°C ± 0.5°C) used for zebrafish housing, achieved using a recirculating water bath (VWR International, Mississauga, ON).

A RMV 708B scan heads (22-83 MHz) was used to obtain short and long-axis views of the zebrafish ventricle in B-mode. The areas of three different short axis views along the ventricle were measured (A_1, A_2, A_3) while the ventricular length of one long axis view was measured and divided by three to give ventricular height (h). All of these values were measured at both systole and diastole using the Visual Sonics software (Markham, ON) for the ultrasound machine. Using these values, end systolic and diastolic volumes in units of mm^3 (equivalent to µl) were each calculated for the fish ventricle using:

(Equation 1) $$V = (A_1+A_2)h + ((A_3h)/2) + (\pi/6(h^3))$$

End systolic volume was subtracted from end diastolic volume to give stroke volume. Ventricular and atrial heart rates were calculated by counting the number of heart beats per 10 second ultrasound video loop and multiplying by six in order to convert to beats per minutes (bpm). Cardiac output (Q) was then calculated using ventricular heart rate (f_H) and stroke volume (V_S):

(Equation 2) $$Q = f_H * V_S$$

The body weight of the fish used was measured and all data were normalized to the body weight of the respective fish and analyzed further using statistical methods.

2.2.6.2. Expression of SERCA2a and RyR1b Post Nesfatin-1 Injection

Following ultrasound monitoring, heart tissues were collected from all groups. Sampling was done at approximately 15-20 minutes post ultrasound monitoring (that is, 20 minutes post-injection of nesfatin-1 or saline). Total RNA extraction and cDNA synthesis were achieved by methods explained earlier in section 2.2.3, and quantitative analysis of genes of interest were conducted as follows.

2.2.6.2.1. Quantitative PCR

The mRNA was reverse transcribed to its complementary DNA with iScript cDNA synthesis kit (Bio-Rad, Canada). The cDNA was stored at -20° C until PCR analysis. The primer sequences of zebrafish Atp2a2a (gene encoding for SERCA2a) and RyR1b used for quantitative

PCR is provided in **Table 2.9**. The cDNA sequences of Atp2a2a and RyR1b were obtained from NCBI Gene Bank (accession numbers provided within brackets in **Table 2.9**) and the primers were designed using the OligoPerfect^{TM} Designer software (Life Technologies, Canada). Primers were validated and optimized for highest primer efficiency and annealing temperature. The respective annealing temperatures for Atp2a2a and RyR1b are also provided. The cDNA synthesized was used as the template to determine the relative expression of these genes. SsoAdvanced SYBR Green SuperMix (Bio-Rad, Canada) was used for PCR. The reaction volume per well was 20 µl. The components of the reaction set up are provided in **Table 2.10**. The samples were quantified using CFX Connect machine (Bio-Rad) and the protocol set up for quantitative PCR is given in **Table 2.11**.

Table 2.9. Primer pairs for Atp2a2a and RyR1b

Genes	Forward (5'-3')	Reverse (3'-5')	Annealing Temperature
Zf Atp2a2a (NM_200965.1)	Atttacttgtgcggattcttcta	cacgatgtctttggctttga	62°C
Zf RyR1b (AB247454.1)	Ccgctcctttggacctcaat	atcctcaacaccatgaccgc	60.7°C

Table 2.10. Components of PCR reaction set up per well.

Components	Volume per reaction
SsoAdvanced SYBR Green Supermix	10 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Sterile water	8 µl
cDNA Template	1 µl
Total Volume	20 µl

Table 2.11. Reaction protocol optimized for PCR in CFX Connect.

	Step 1	Step 2	Step 3	Step 4	Step 5
Temperature	95°C	95°C	Annealing Temp	73°C	4°C
Time	5 seconds	10 seconds	30 seconds	30 seconds	Hold at infinity

2.2.7. Statistical Analysis

The ultrasound analysis was carried out using One-way ANOVA followed by Fisher Exact Post Hoc test. Data were normalized to the body weight of fish. All other data were analyzed using one-way ANOVA with Tukey's Multiple Comparison Test. $P \leq 0.05$ was taken to indicate a significant difference. All data were expressed as mean ± standard error of mean (SEM).

2.3. Results

2.3.1. Zebrafish Heart Expresses NUCB2 mRNA

A band of approximately 200 bp representing the NUCB2 mRNA was detected (**Figure 2.1**). The no template negative control showed no bands representing the amplicon of interest.

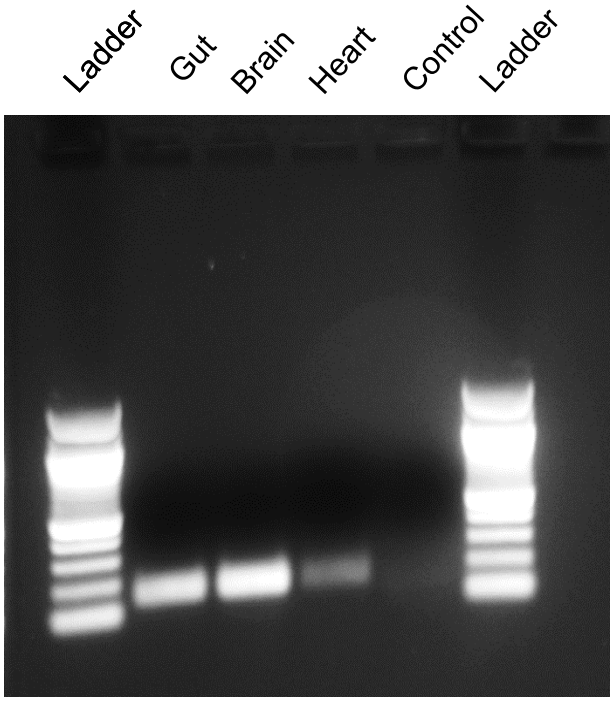

Figure 2.1. Gel electrophoresis image showing the presence of NUCB2 mRNA in zebrafish heart. The amplicon size of the band was approximately 200 base pairs. The presence of a band provides evidence that NUCB2 mRNA is expressed in zebrafish heart suggesting that zebrafish heart is a source of NUCB2/nesfatin-1.

2.3.2. NUCB2/nesfatin-1-like Immunoreactivity (ir) is Present in Zebrafish Cardiomyocytes.

Confocal microscopy imaging at a magnification of 63X, showed the existence of NUCB2/nesfatin-1 immunopositive cells in both atria and the ventricles. The figure below shows a magnified image of the ventricle. Nuclei were stained blue with DAPI and the immunopositive cells were stained red. NUCB2/nesfatin-1 like ir was observed in the cytoplasmic regions of the cardiomyocytes **(Figure 2.2 – (A-C)).** A negative control showed no presence of NUCB2/nesfatin-1 like immunoreactivity **(Figure 2.2 - D).**

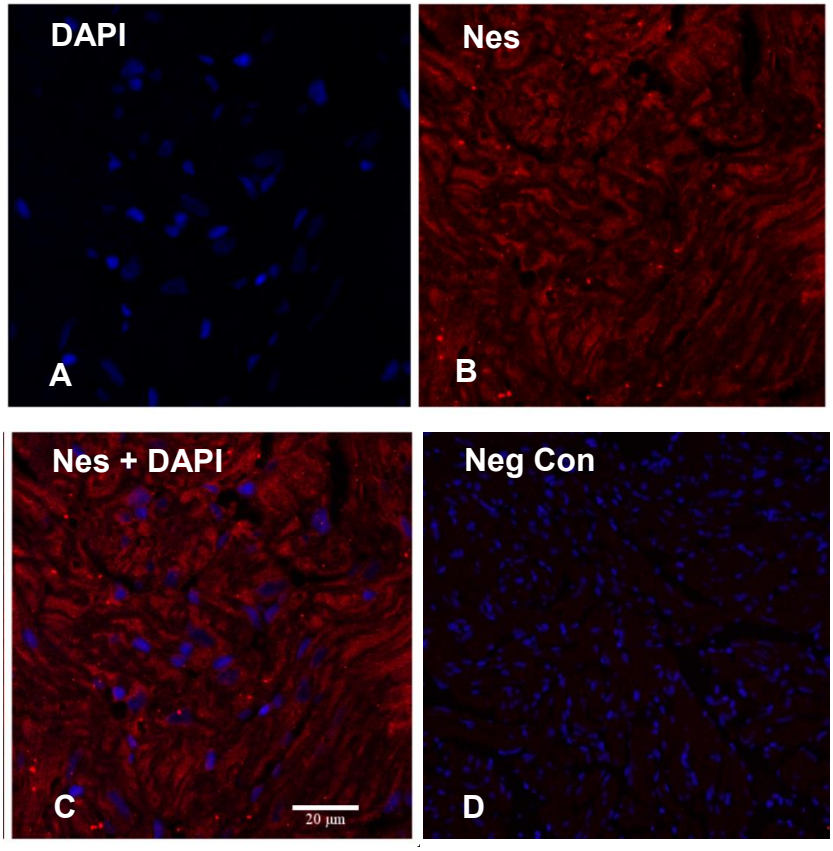

Figure 2.2. Characterization of NUCB2/nesfatin-1 like immunoreactivity in the zebrafish ventricles. Image **A** shows staining of the nuclei by DAPI. Immunohistohemical staining of zebrafish cardiomyoctye for nesfatin-1 ir is depicted in **B**. Red staining of the ventricle shows the presence of nesfatin-1 like immunoreactivity. Merged images of nesfatin-1 and DAPI staining are shown in **C**. No-primary antibody negative control, labeled only with the secondary antibody is shown in **D**.

2.3.3. Chronic Food Deprivation Does Not Modulate NUCB2 mRNA Expression in Zebrafish Heart

There were no significant differences in NUCB2 mRNA expression in zebrafish heart (**Figure 2.3)** in the fed control fish compared to the food-restricted fish.

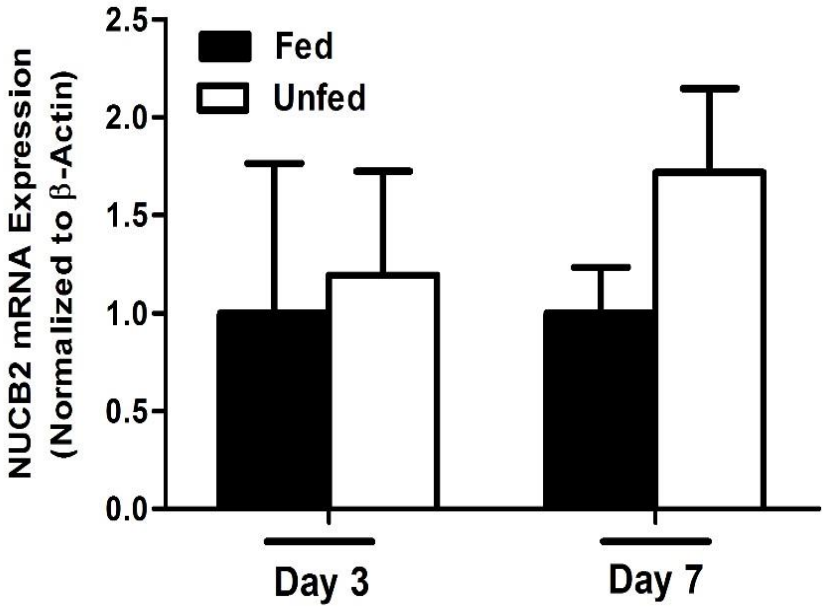

Figure 2.3. Food deprivation in a chronic manner does not affect nucleobindin-2 (NUCB2) mRNA expression in zebrafish heart. There was no significant difference in NUCB2 mRNA expression in the heart of fed and unfed zebrafish on day 3 and day 7. The samples were normalized using β-Actin as the reference gene. Statistical analysis: one-way ANOVA with Tukey's Multiple Comparison Test (n = 8 zebrafish/group).

2.3.4. Modulation of NUCB2 mRNA Expression in Zebrafish Heart Before and After Feeding

A significant difference was observed among groups at 0 hour feeding and (+1) unfed group (+1UF). The highest expression of NUCB2 was observed at zero time point (**Figure 2.4).**

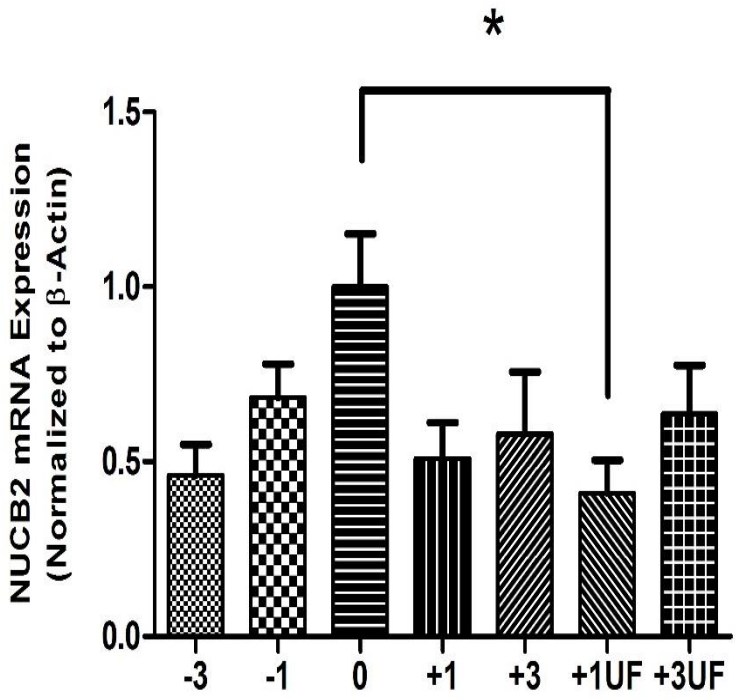

Figure 2.4. Periprandial expression of nucleobindin-2 in zebrafish heart. Highest expression of NUCB2 mRNA was observed at zero time point (at feeding) and a significantly low level of NUCB2 mRNA, when compared to 0 hour (at feeding), was observed in unfed group sampled at +1 hour after the scheduled feeding time. (+1)UF: post 1h unfed group; (+3) UF: post 3h unfed group. Statistical analysis: one-way ANOVA with Tukey's Multiple Comparison Test; Significance: *; $P < 0.05$ (n = 9 zebrafish/group).

2.3.5. Intraperitoneal Injections of Nesfatin-1 in Adult Zebrafish Modulated Various Cardiac Functions in a Dose Dependent Manner.

Acute i.p. injection with nesfatin-1 (in the 50 ng/g dose group) in adult zebrafish caused an initial non-significant increase in end-diastolic volume. This was followed by a dose-dependent decrease ($p<0.05$) in end diastolic volume compared to vehicle-injected control in 250 and 500 ng/g body weight dose (**Figure 2.5 A**). Accompanied by a significant increase in end-systolic volume at the lowest nesfatin-1 dose of 50 ng/g (**Figure 2.5 B**), but no significant effect on end-systolic volume at higher doses was observed. Despite the changes in end-diastolic and end-systolic volumes, stroke volume did not change significantly in any nesfatin-1-treated group (**Figure 2.5 C**). Similar to that observed for end-diastolic volume, heart rate was significantly decreased at the two higher doses (**Figure 2.5 D**). The net effect of these changes on cardiac output was decreased cardiac output in the 250 or 500 ng/g nesfatin-1 injected fish, compared to saline treated controls **(Figure 2.5 E)**.

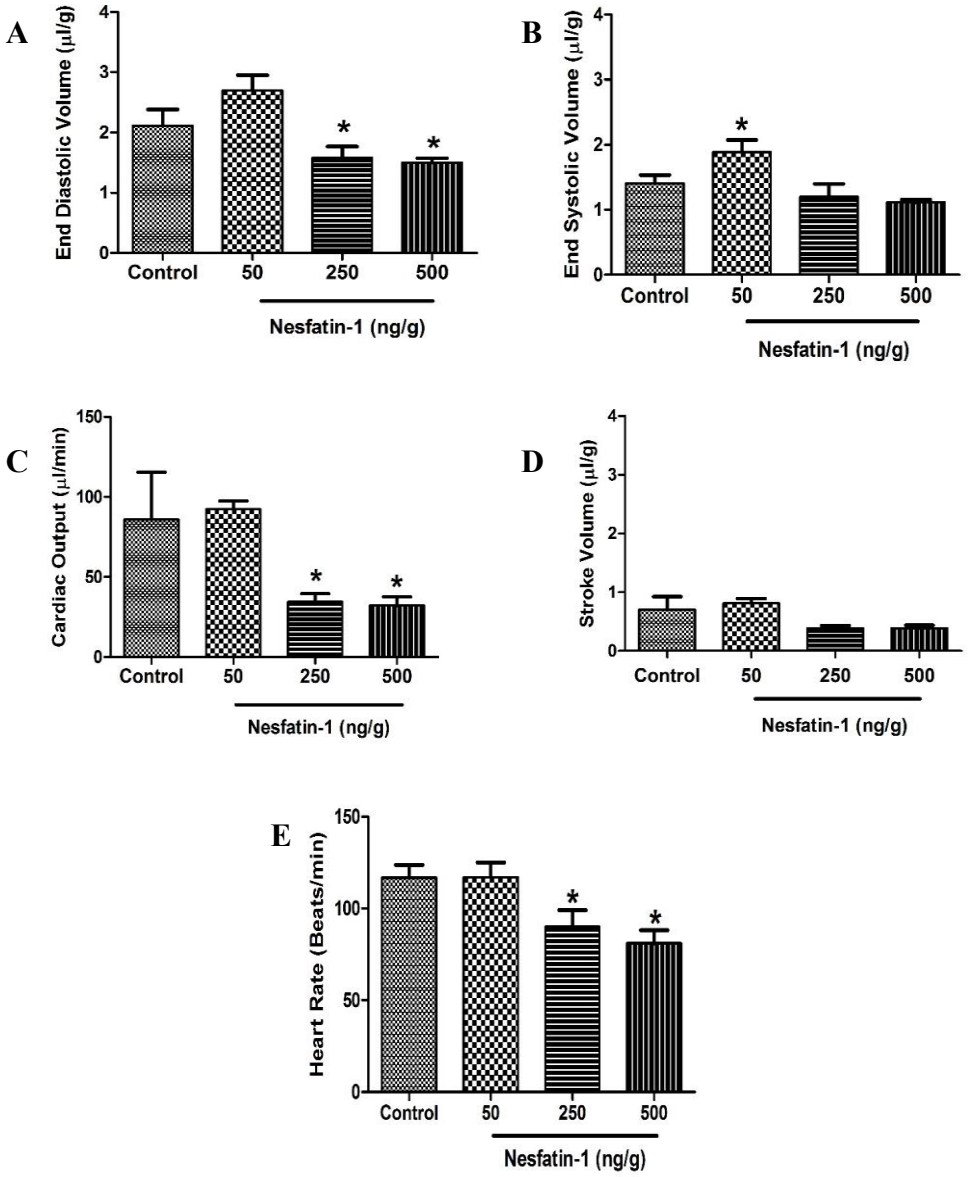

Figure 2.5. Effects of intraperitoneal injections of nesfatin-1 on various cardiac functions at doses 50 ng/g, 250ng/g and 500 ng/g body weight. All groups were compared to the control group. Statistical Analysis: one way ANCOVA with Fisher Exact post hoc test, Statistical significance: *, $P < 0.05$ (n = 8 fish/treatment)

2.3.6. Modulation of SERCA2a and RyR1b in Zebrafish Cardiac Tissue Post-Nesfatin-1 Injection

The Atp2a2a mRNA expression in zebrafish heart was significantly elevated in the group that received the highest dose of nesfatin-1 (500 ng/g) (**Figure 2.6 A)**. However, the expression of RyR1b did not change with nesfatin-1 treatment (**Figure 2.6 B)**.

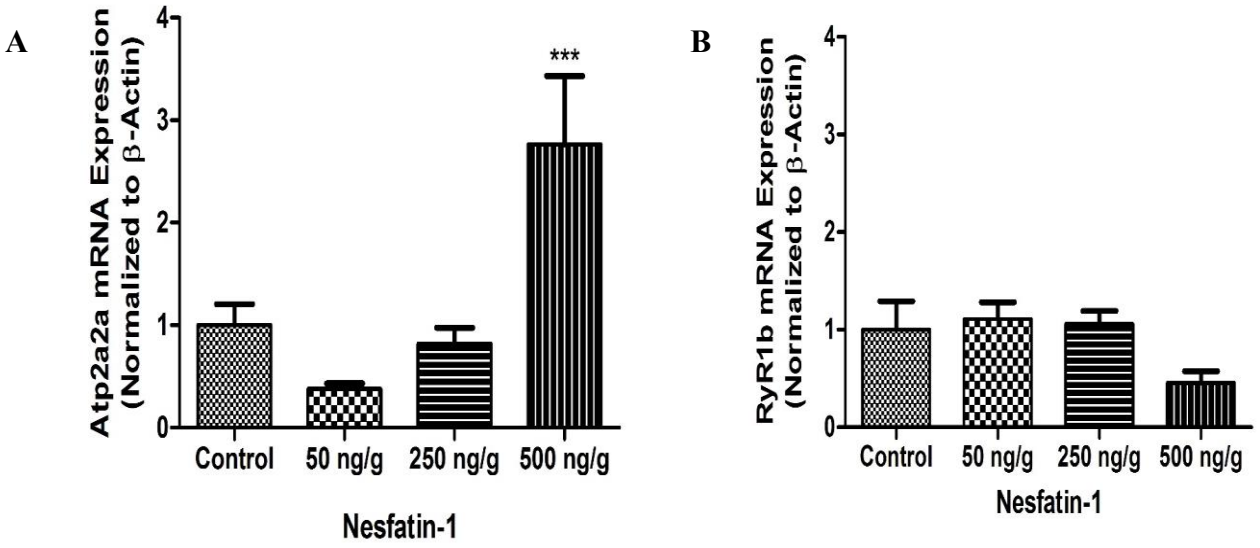

Figure 2.6. **Modulation of cardiac SERCA2a and RyR1b receptor following intra-peritoneal injection of nesfatin-1**. The mRNA expression of Atp2a2a was significantly increased in the group that received the highest dose of nesfatin-1 (500ng/g). However the ryanodine receptor (RyR1b) mRNA expression did not change when compared to the control group. The samples for quantitative PCR were assayed in duplicates and analyzed using one-way ANOVA followed by Fisher Exact test. Statistical Significance ***; $P < 0.05$ (n = 8 fish/group)**.**

2.4. Discussion

Nesfatin-1 is an anorexigenic peptide that is found in several peripheral tissues including stomach, pancreas, testis and gut (Oh-I et al., 2006; Stengel et al., 2009; Gonzalez et al., 2009). More recently, nesfatin-1 was found involved in regulating cardiovascular functions in rats and mice (Mimee et al., 2012; Yamawaki et al., 2012; Yosten et al., 2009; Feijóo-Bandín et al., 2014). NUCB2 mRNA and nesfatin-1 protein was detected in isolated rat hearts (Feijóo-Bandín et al., 2014). Presence of NUCB2 mRNA expression is also detected in goldfish (Gonzalez et al., 2010). We found the presence of NUCB2 mRNA in zebrafish heart. A 200 base pair amplicon representing NUCB2 mRNA was detected in zebrafish heart. We also observed that NUCB2/nesfatin-1 like immunoreactivity is present in zebrafish heart, suggesting that heart is also a source of endogenous nesfatin-1 in zebrafish. This result further strengthens our finding of NUCB2 mRNA in zebrafish. Whether heart derived nesfatin-1 is secreted in zebrafish remain unknown. Gonzalez et al., (2010) showed that chronic food deprivation in goldfish decreased and increased NUCB2 mRNA expression in the hypothalamus and liver respectively, suggesting a possible role of food intake in the expression of NUCB2 in hypothalamus and liver (Gonzalez et al., 2010). In this study, chronic food deprivation did not modulate the NUCB2 mRNA expression in the heart. This suggests that the cardiac NUCB2 mRNA expression is not meal responsive.

Periprandial NUCB2 mRNA expression profiling in zebrafish heart showed that NUCB2 mRNA in the unfed group at 1 hour post-feeding time was significantly lowered compared to the fed group. NUCB2 mRNA expression was higher after 15 minutes post feeding (0h group). Stengel et al., (2009) observed a decrease in NUCB2 mRNA in the gastric cells of rats when they were fasted. An increase in the expression of NUCB2 was observed after feeding (Stengel et al., 2009). This is in accordance to our finding, where a decrease in NUCB2 expression was observed in the unfed group. In contrast, Gonzalez et al., (2010) observed that, NUCB2 mRNA expression was significantly increased in fed groups after +1 and +3 hours of feeding. The same was observed in liver as well, where NUCB2 mRNA expression increased significantly in the unfed groups at +1 and +3 hours. This increase in NUCB2 mRNA in the liver could indicate a possible role of nesfatin-1 in the physiology of liver during fasting (Gonzalez et al., 2010). In a recent study conducted in our lab, NUCB2 mRNA expression in the brain of zebrafish was significantly increased in the fed group at 3h post feeding as compared to the unfed group (+3UF) (Hatef et al., 2014). However, a

decrease in NUCB2 mRNA expression was observed in the gut and liver after regular feeding point. Food deprivation for 7 days decreased the mRNA expression of NUCB2 in the brain and gut of zebrafish. Contradicting to this was observed in zebrafish liver, where a significant up-regulation of NUCB2 mRNA was detected in the group deprived of food for 7 days (Hatef et al., 2014). We observed that, in zebrafish heart NUCB2 mRNA expression did not change despite being fed or unfed. This suggests that, expression of NUCB2 mRNA in zebrafish is highly tissue specific. Further analysis of serum nesfatin-1 in circulation would be an interesting addition to this study.

Nesfatin-1 is involved in regulating a number of cardiovascular functions (Mimee et al., 2012; Yamawaki et al., 2012; Yosten et al., 2009; Feijóo-Bandín et al., 2014). It modulated blood pressure and peripheral arterial contractility in rats (Yamawaki et al., 2012). Intravenous exposure of rats to 6.2 µg/kg of nesfatin-1 also induced an increase in systolic, mean and diastolic blood pressure (Yamawaki et al., 2012). Yosten et al., (2008) determined that in conscious rats, nesfatin-1 increased the mean arterial pressure. Intracereberoventricular injections of 60 pmol – 180 pmol nesfatin-1 caused a significant increase in the mean arterial pressure within 2 minutes of injection (Yosten et al., 2009). All these data provide evidence that nesfatin-1 is involved in the regulation of cardiovascular system. A major focus of this chapter is on the monitoring of cardiac functions in zebrafish using a high frequency ultrasound imaging. Ultrasound bio-microscopy of zebrafish heart post-nesfatin-1 injections at different doses showed that there is a dose dependent effect of nesfatin-1 on the cardiovascular functions in zebrafish. A lower end-diastolic volume after injection with nesfatin-1 in adult zebrafish is indicative of either decreased preload (controlled largely by venous pressure) or increased ventricular wall stiffness (Berne and Levy, 2010). An effect of nesfatin-1 on venous tone or body fluid balance to decrease preload cannot be ruled out at this point. However, if the effect of nesfatin-1 were directly on the heart, nesfatin-1-mediated increase in wall stiffness needs to be considered. Due to the acute nature of this experiment (48 hours after initial nesfatin-1 injection), there was insufficient time to develop structural changes in the ventricular wall such as fibrosis (Schwerte, 2009; Baicu et al., 2012). However, an increased sympathetic or other cardio stimulatory tone accompanied by slower time to relaxation could acutely increase wall stress, thereby decreasing efficiency of ventricular filling during diastole (Berne and Levy, 2010). However, heart rate would have also been affected by nesfatin-1 injection if wall stiffness resulted from the cardio stimulatory effects of the sympathetic nervous system,

endothelin or angiotensin II (Vierimaa et al., 2006; Schwerte et al., 2009). This was not observed with nesfatin-1, making this latter possibility unlikely. Vagal afferent neurons act in a para-sympathetic way to decrease heart rate. Iwasaki et al., (2009) observed that, nesfatin-1 activated the vagal afferent neurons by activating Ca^{+} channels in the brain and hence elicits its anorexigenic actions. Intraperitoneal injection of nesfatin-1 at higher doses (250 ng/g and 500 ng/g) in zebrafish resulted a decrease in heart rate. This could possibly suggest that nesfatin-1 acts on the vagal afferent neurons to in turn decrease the heart rate, suggesting that nesfatin-1 effects on heart are not direct.

Impaired ventricular relaxation remains a viable explanation for nesfatin-1 effects in the adult zebrafish heart if a change in expression of key regulatory proteins were detected. For example, expression of the sarco-endoplasmic reticular Ca-ATPase (SERCA2a), responsible for sequestering intracellular calcium during ventricular relaxation, is altered by other environmental perturbations such as temperature fluctuations and toxicants in zebrafish (Vornanen et al., 2002; Zhang et al., 2013). A disruption in ventricular SR calcium handling, either through ryanodine receptors or SERCA, has been shown to decrease first diastolic function, then systolic function with increasing perturbation in fish (Tiitu and Vornanen, 2002). The expression of SR calcium handling proteins was examined in heart collected post-ultrasound monitoring. The expression of SERCA2a was increased at the higher dose of nesfatin-1 (500 ng/g). Increased expression of Atp2a2a in the group that received the highest dose of nesfatin-1 (500ng/g) could be a direct effect of nesfatin-1 on Atp2a2a gene or it could be a compensatory mechanism as a result of nesfatin-1 induced changes in zebrafish cardiac functions. It has been shown that both increase and decrease in SERCA2a in the heart can result in heart failure and arrhythmia (Erkasap et al., 2007). Cardiac myocyte dysfunction was caused as a result of decreased SERCA2a (Erkasap et al., 2007). Chen et al., (2004) on the other hand, showed that transgenic overexpression of SERCA2a increases the risk of acute arrhythmias and sudden death in rats. Importance of SERCA pump is extensively studied in animal and human hearts with heart failure. Expression of SERCA is decreased in patients with heart failure. This suggests that calcium handling has an important role in the physiology of hearts (Chen et al., 2004). The expression of SERCA is regulated by hormones. For example, SERCA pump expression and muscle contractility is regulated by thyroid hormone. In studies using rodents suffering from hypothyroidism, SERCA2a expression was decreased. In rodents with hyperthyroidism, there was an increase in SERCA2a expression. These data suggests

that, an increase or decrease in thyroid hormone can regulate the SERCA2a expression (Periasamy et al., 2007). On the other hand, we did not find any changes in ryanodine receptor 1b in zebrafish heart that received nesfatin-1 treatment. It was observed that inhibition of this receptor, causes excitation-relaxation coupling impairment and skeletal muscle deformities. Ryanodine receptors are involved in excitation-contraction coupling in both skeletal and cardiac muscles (Brennan et al., 2005). In our study, we observed that dose-dependent nesfatin-1 treatment of zebrafish heart did not alter the expression of RyR1b among any groups as compared to the control.

Nesfatin-1 also decreased end-systolic volume, albeit at only the highest dose used (500 ng/g), indicating that ventricular contractility can also be acutely impaired by this peptide. This effect of nesfatin-1 is most likely through some direct cardiac actions rather than a vascular effect mentioned earlier while discussing diastolic volume changes. The changes in end diastolic and end systolic volumes further diminish stroke volume and then cardiac output in nesfatin-1 treated zebrafish. Considering Occam's razor, the simplest explanation for an effect on both end diastolic and end systolic volumes is for a single target of nesfatin-1. Therefore this part of my thesis provided results suggesting that zebrafish heart is a tissue source of nesfatin-1. It also, for the first time in a non-mammal, shows that various cardiac functions, including end diastolic and systolic volumes, heart rate and cardiac output are all modulated by exogenous nesfatin-1.

TRANSITION

At the time my thesis research was initiated, there was no information on the cardiac expression and regulation of nesfatin-1 in mouse. As a parallel project to the research outlined in this chapter, I studied nesfatin-1 in HL-1 cells, a mouse cell line, and the heart of mice. NUCB2 mRNA and nesfatin-1 like immunoreactivity were detected in mice cardiomyocytes. Further, we found that acute glucose load increases cardiac NUBC2 mRNA, but no effects for chronic high fat diet on NUCB2 mRNA expression was detected in mice heart. I also determined whether nesfatin-1 treatment modulates mRNAs encoding proteins involved in apoptosis. While nesfatin-1 appears to have both stimulatory and inhibitory effects on various proteins, no significance was reached due to the low sample number. Chapter 3, which follows this page, details my research on nesfatin-1 in mice and HL-1 cells.

Contributions: Neelima Nair conducted all studies, analyzed data and wrote the manuscript. Suraj Unniappan funded the project, provide the idea, infrastructure, helped with data analyses and manuscript writing. Haneesha Mohan, a graduate student in our lab provided samples from the acute and chronic diet studies using mice.

Publication: Nair, N., Unniappan, S. Murine Cardiomyocytes are a Source of Nesfatin-1 and Glucose Stimulates NUCB2 mRNA Expression in HL-1 Cardiomyocytes and Mouse Heart. Frontiers in Endocrinology (Nature Publishing Group).

CHAPTER 3: Nesfatin-1 in Mouse Heart and Murine Cardiomyocytes

3.1. Introduction

Nesfatin-1, its tissue localization and functions, especially those pertaining to cardiac functions were discussed in the chapters 1-2. In this chapter, I characterized NUCB2 mRNA in mouse heart and HL-1 cardiomyocytes. A pilot study was conducted to determine the role of macronutrients on the cardiac expression of NUCB2 mRNA in C57BL/6 mice.

3.2. Materials and Methods

3.2.1. Cell Culture

HL-1 is a cardiac muscle cell line, containing a centrally located nucleus with surrounding myofibrils that are contractile in nature. It is derived from AT-1 mouse atrial tumor lineage (Claycomb et al., 1998). AT-1 cells are a cell line that was derived from a transgenic mouse with growing atrial tumor. The expression of simian virus 40 (SV40) large T antigen acting via the ANF promoter, targeted the atrial cardiomyocytes. AT-1 cells had an advantage of being able to maintain their cardiac phenotype and yet be differentiated in culture. One limitation is that these cells could not be passaged. However, these difficulties were overcome by originating HL-1 cell lines. Triple staining of HL-1 cells with Hoechst 33258 dye showed that even beyond 30 passages, HL-1 cell were able to maintain their homogenous nature (Claycomb et al., 1998).

HL-1 cells have a number of advantages, including the ability to be passaged approximately 240 times. They are able to retain their differentiated phenotype while still being in culture. They have intercalated discs, voltage dependent currents and undergo cytoplasmic reorganization and myofibrillogenesis (Claycomb et al., 1998). Their ability to retain the phenotype makes them an excellent model for studies related to the cardiovascular system. A potassium current channel is also present in the arterial derived HL-1 cells. It was observed that they express L- and T-type calcium channels, which is a characteristic of a cardiac cell. Hence this cell line is used to study electrophysiological changes that occur in atrial fibrillation. They possess G-protein-gated inwardly rectifying potassium currents that are analogous in properties and receptor –mediated regulation to its counterpart in native atrial myocytes (Claycomb et al., 1998).

HL-1 cells have been used to study the expression of a wide range of genes. It was found to express MHC, α-cardiac actin, ANF and connexin43 (Claycomb et al., 1998). HL-1 cells were

a generous gift from Dr. William C Claycomb (Loiusiana State University Medical Center, New Orleans). The cells were cultured in Claycomb Medium (Sigma-Aldrich, Catalog # 51800C). The medium is supplemented with final concentrations of 10% Fetal bovine serum (Sigma – Aldrich, Catalogue # 72442, Batch 12J001); 100 U/ml penicillin/streptomycin (Sigma – Aldrich, Catalogue # P4333); 0.1mM Norepinephrine (10mM stock, Sigma – Aldrich, Catalogue #A0937); 2mM L Glutamine (200mM stock; Sigma – Aldrich, Catalogue # G7513). The cells were maintained in a T-25 or T-75 flasks, which is pre-coated with gelatin/fibronectin, at 37°C in an atmosphere of 95% O_2 and 5% CO_2 in a humidified incubator. Cell culture media was changed every 24 hours. The cells were sub-cultured once they reached 90-100% confluency using 0.05% trypsin in 0.02% EDTA-Na (Sigma – Aldrich, Catalogue # T3924).

3.2.2. Detection of NUCB2/nesfatin-1 like immunoreactivity in HL-1 cardiomyocytes.

NUCB2/nesfatin-1 like immunoreactivity was detected in HL-1 cardiomyocytes using immunohistochemistry and fluorescence microscopy techniques.

3.2.2.1. Immunohistochemistry

HL-1 cells were cultured on gelatin/fibronectin coated chamber slides to determine indirect immunofluorescence. Cells were fixed with 2.5% paraformaldehyde (PFA) for 15 minutes at 4°C and washed with Dulbecco's Phosphate Buffered Saline (DPBS, Sigma – Aldrich, Catalogue # D8537). Once the cells are fixed, primary antibody was added with dilution ratio 1:200 using DAKO Antibody Diluent (DAKO, North America). The primary antibody used was Phoenix Nesfatin-1 (Phoenix, Catalogue # H-003-22, Lot # 01142-6). Six hours later, the slides were washed with DPBS. Following this secondary antibody was added with a dilution ratio of 1:200. Texas Red-Anti-Rabbit IgG, was diluted with DAKO antibody diluent and used as a secondary antibody. The slides were maintained at 37°C in a humidified incubator for one hour. Slides were then washed with DPBS and allowed to dry at room temperature for 30 minutes. The chamber was then peeled off, and a drop of DAPI (Vectashield, Vector laboratories) was added to the slide and covered with a coverslip.

3.2.2.2. Fluorescence Microscopy

Once the cells were fixed and stained for NUCB2/nesfatin-1, the slides were viewed under a fluorescence microscope to detect indirect immunofluorescence. The slides were viewed at 40X

magnification with blue and green filters specific for the stains used. DAPI stains the nuclei of the cells, and this was seen in the presence of the blue filter. A green filter was used for viewing cells stained with Texas Red, showing cells immunopositive for nesfatin-1/NUCB2.

3.2.3. Characterization of NUCB2 and the proposed GPCR receptor mRNAs in HL-1 cardiomyocytes

The cells were cultured in two T25 flasks as described earlier. The procedures used for total RNA extraction, cDNA and qualitative PCR are described below.

3.2.3.1. Total RNA extraction

Once reached 90-100% confluency, cells were washed with DPBS to remove any dead cells and 2 mL of TRIzol® Reagent (Life Technologies, Canada) was added and cells were lifted from the matrix and collected in a 1.5 mL Eppendorf tube. Then 200 µL of chloroform was added in order to separate RNA from protein and DNA. The clear supernatant was separated and precipitated with isopropanol, and 70% ethanol was added to remove any phenol impurities from the pellet. Purified RNA was isolated using RNase-free water (20 µL). The quality and quantity of the extracted mRNA was determined using a NanoDrop 2000C (Thermo Vanta, Finland). Total RNA was quantified by measuring the optical density (OD) absorption ratio (A260nm/A280nm) of diluted RNA. Pure RNA has a ratio between 1.9 and 2.0. All RNA samples used for cDNA synthesis and qualitative RT-PCR had high quality. Isolated RNA was stored at -80° C until further analysis.

3.2.3.2. cDNA Synthesis

Synthesis of cDNA was conducted using iScript DNA synthesis kit as described by the manufacturer (Bio-Rad, Canada). The components of the tube and synthesis is described in chapter 1 **(Table 2.1 and 2.2).** The samples are stored in -20° C until required.

3.2.3.3. Real Time Qualitative PCR

The cDNAs synthesized were used as templates for determining the qualitative expression of NUCB2 and GPCR receptor mRNAs. cDNA sequences for NUCB2, GPCR3, GPCR6 and GPCR12 were obtained from National Center for Biotechnology Information Gene bank (NCBI) and specific primers were synthesized using OligoPerfect^{TM} Designer software (Life

Technologies, Canada). The forward and reverse primer sequences of the genes with their respective GenBank accession numbers (within brackets) are provided in **Table 3.1**.

Table 3.1. Primer pairs for real time RT-PCR with respective annealing temperatures.

Genes	Forward (5′-3′)	Reverse (3′-5′)	Annealing Temperature
Mouse βActin (EF095208.1)	ccactgccgcatcctcttcc	ctcgttgccaatagtgatgacctg	60°C
Mouse NUCB2 (NM_001130479.2)	ccagtggaaaatgcaaggat	gctcatccagtctcgtcctc	60°C
Mouse GPCR3 (NM_008154.3)	gggagcaggaagctctatgg	gtctgctacggccaagctac	64.5°C
Mouse GPCR6 (NM_199058.1)	gggtcactgggctgtcttc	tcagcagtggccagactacc	64.5°C
Mouse GPCR12 (NM_001010941.2)	gagtcctggtccgtgtttcc	cccctgtcctcttcaacg	51.8°C

Once the primers were obtained, the expression of these genes was determined using Reverse transcription polymerase chain reaction. The samples were run on a CFX Thermocycler (Bio-Rad, Canada). The components of the mastermix and the PCR reaction protocol are described in the previous chapter **(Table 2.4, 2.5 and 2.6).** The samples were stored at -20 °C until further required. Following PCR, the samples were run on 1% agarose gel. The procedure for gel electrophoresis is similar to that discussed in the previous chapter (section 2.2.3.4) and thus the expression of genes of interest was analyzed.

3.2.4. Effects of High Glucose Concentration on NUCB2 and GPCR3 mRNA Expression in HL-1 Cardiomyocytes

HL-1 cardiomyocytes were cultured and maintained as described in section 3.2. The cells were then trypsinized and re-suspended in media and plated in 6-well plates. Cells were treated with 100 mM of D-glucose prepared in media. The cells used as control groups received normal growth media. Cells were kept in a 37 °C humidified incubator during a one hour incubation period. Cells were taken out, the media was aspirated and 2ml TRIzol® Reagent (Life

Technologies, Canada) was added and the cells were collected for further analysis. Total RNA was extracted from the cells using TRIzol Reagent as previously described in the previous section. RNA purity was measured using NanoDrop 2000C (Thermo Vanta, Finland). Isolated RNA was stored at -80° C until further analysis. Synthesis of complimentary DNA sequence was conducted using iScript DNA synthesis kit (Bio-Rad, Canada). The reaction was set up is described in the previous chapter (section 2.2.3.2). The cDNA was stored at -20°C. The relative expression of NUCB2 and GPCR3 in response to high glucose treatment was determined using real time quantitative PCR. SsoAdvanced™ SYBR Green Supermix (Bio-Rad, Canada) was used for polymerase chain reaction. All reactions were performed with a final volume of 20µl as per **Table 2.7**. The reaction protocol was set up in the CFX connect as per **Table 2.8**. Forward and reverse primers for NUCB2 and GPCR3 were used (see **Table 3.1** for gene sequences and respective annealing temperatures).

3.2.5. Dose Dependent Effect of Nesfatin-1 on GLUT4 and GPCR3 mRNA Expression in HL-1 Cardiomyocytes

GLUT4 is the main glucose transporter of the heart (James et al., 1988). In this study, we studied the effect of different doses of nesfatin-1 on GLUT4 and GPCR3 mRNA expression in HL-1 cardiomyocytes. HL-1 cells were cultured and maintained in supplemented Claycomb medium in a T-75 flask, until ready to be sub cultured. The cells were then trypsinized and subcultured and plated in 6-well plates. On the day of the study, the growth media was supplemented with different doses of nesfatin-1. Doses of 0.01 nM, 0.1 nM and 1 nM of synthetic rat nesfatin-1 (200µg, purity > 95%), was mixed with the growth media and added to cells (n = 6 wells/dose). The cells treated as control group received the normal growth media. Cells were incubated for one hour in a 37 °C humidified incubator with 5% CO_2. After one hour, the cells were taken out, media was aspirated and 2ml of TRIzol was added to extract RNA and stored at -80°C until further analysis.

Table 3.2. Primer Pairs for Mouse GLUT4 and GPCR3 genes.

Gene	Forward (5′-3′)	Reverse (3′-5′)	Annealing Temperature
GLUT4 (AB008453.1)	tcattcttggacggttcctc	gacagaagggcagcagaatc	62.5°C
GPCR3 ((NM_008154.3)	gggagcaggaagctctatgg	gtctgctacggccaagctac	64.5°C

The mRNA was reverse transcribed to its complimentary DNA with iScript DNA synthesis kit (Bio-Rad, Canada). The reaction protocol for the synthesis of cDNA is described in chapter 1 (Section). The cDNA produced is used as a template for studying the quantitative expression of GLUT4 genes in HL-1 cardiomyocytes. The primers used for GLUT4 gene and GPCR3 gene is provided in **Table 3.2.** The primers were validated and optimized for highest primer efficiency and annealing temperature. The relative expression of GLUT4 gene was conducted using SsoAdvanced^{TM} SYBR Green Supermix (Bio-Rad, Canada) on a CFX Connect system (Bio-Rad, Canada). Expression of GLUT4 and GPCR3 was normalized with mouse β Actin gene (internal control/housekeeping gene). The reaction protocol for quantitative analysis is described in section 3.2.4. PCR products were analyzed using $-\Delta\Delta C_T$ method.

3.2.6. RT2 Profiler PCR Array for Mouse Apoptosis

A PCR array is a combination of performance of a polymerase chain reaction and a microarray. A microarray helps in the analysis of various genes simultaneously and is a one-time procedure. A combination of both PCR and microarray, helps in analyzing and quantifying the expression of several number of genes. A mouse apoptosis array profiler consists of 84 different genes that are involved in apoptosis (www.SABiosciences.com/support_publication.php#pcrarray). Apoptosis, otherwise known as programmed cell death, is a process where the cell undergoes different morphological changes including cell shrinkage, nuclear disintegration and blebbing. Apoptosis is a result of aging and development of cells, and also occurs in order to maintain the homeostasis of cell proliferation in tissues (Elmore et al., 2007). A defect in apoptosis mechanism can lead to an increase in number of cell death or cancer. Therefore apoptosis can have either an advantage, where it is a natural process of cell death, without damaging the other cells, or a disadvantage, where no apoptosis

causes increase in number of cancer cells. Several genes and pathways are involved in the mechanism of apoptosis. Some of these genes include annexin A5, genes of the caspase family and TNF family ((www.SABiosciences.com/support_publication.php#pcrarray). 96-well PCR array plate consists of 84 genes related to mouse apoptosis, five different housekeeping genes, one genomic DNA control (GDC), three reverse transcriptase control (RTC) and three positive PCR controls (PPC). Housekeeping genes (Actb, B2m, Gapdh, Gusb and Hsp90ab1) are provided for normalizing the data. A genomic DNA control assay helps in the detection of contamination by genomic DNA. RTC assay is used to determine the reverse transcription efficiency. PPC is helpful in detecting the efficiency of the polymerase reaction as a whole. HL-1 cells were cultured in a T75 flask and grown until they reached 100% confluency. Once the cells were confluent, they were subcultured in 12 well plates. Cells were treated with 10 nM of nesfatin-1. The group of cells that received the normal media (supplemented Claycomb media) was treated as control. Following treatment, the cells were incubated for one hour. The cells were then extracted using TRIzol Reagent for RNA extraction and stored at -80°C and further analysis. Total RNA extraction was carried out as described in the previous chapter. Synthesis of cDNA was conducted using iScript DNA synthesis kit as directed by the manufacturer. The reaction set up for cDNA synthesis and the protocol set up in the Thermo cycler (Bio-Rad, Canada) are described in the previous section.

3.2.6.1. RT2 Profiler PCR Array (for 96-well)

The cDNA synthesized in the previous step is used as the template for the real time PCR array. The components required for PCR array set up is as follows:

Table 3.3. Components required for setting up the PCR array plate (96-well). Adapted from Qiagen

Array components	Volume
2 x RT2 SYBR Green Mastermix	1350 µL
cDNA template	102 µL
RNase-free water	1248 µL
Total	2700 µL

The tube containing RT2 SYBR Green Mastermix should be centrifuged prior to use. Two 96-well PCR plates were used: one for the nesfatin-1 treatment group, and a second plate for control (untreated) group. The components of the PCR were made in a tube, which was later transferred to a loading reservoir. Using an 8 channel pipettor, 25 µL of the PCR components were added to each well. Following this, the array is sealed tight using the optical thin-wall 8-cap strips. The plate was centrifuged at 1000 g for 1 min at room temperature to remove any possible bubbles. Protocol setup used for CFX Connect was as follows:

Table 3.4. Reaction Setup in CFX Connect. Adapted from Qiagen.

Number of cycles	Time	Temperature
1	10 min	95°C
40	15 seconds	95°C
	30-40 seconds	55°C
	30 seconds	72°C

The threshold values were calculated and the C_T values are exported to an excel sheet. A melting curve analysis is also carried out at 65 °C to 95 °C at 2°C/min. Data is analyzed using a

web portal software which is available at www.SABiosciences.com/pcrarraydataanalysis.php. This software performs the analysis using $\Delta\Delta C_T$ method. The fold change for genes in each well from the treatment group (group 1) and the control group is calculated. A fold change greater than 1 implies up-regulation, and a fold change less than 1 implies down-regulation.

3.2.7. Effects of Macronutrients on NUCB2 mRNA Expression in Male C57BL/6 Mice Heart.

A pilot study was conducted to determine whether the expression of NUCB2 mRNA in C57BL/6 mice (Charles River Laboratories, Quebec, Canada) heart is modulated by macronutrients. A chronic and an acute study were conducted. In the chronic study, the mice were housed in a temperature and humidity controlled vivarium in a 12 h light: 12 h dark cycle for 17 weeks. Prior to the start of the study, the mice were fed with control diet for one week. Mice were divided into two groups (n = 7 mice/group): control (Product # D12451) and high fat (Product # D12492; 60% energy derived from fat) diet (Research Diets, New Brunswick, NJ). They were fed *ad libitum* with their respective diets. Hearts were collected from only two groups (control and high fat) and stored at -80 °C until further required. In the acute diet study, mice were acclimatized for a week prior to the study. The mice were fed with *ad libitum* mouse chow for 11 days. Two days prior to the study, the mice were acclimatized with oral gavaging procedure. Mice were divided into two groups (n=7/group): control (no gavage), high carbohydrate (D-Glucose; BioShop; Catalogue # GLU501.500). Hearts were collected from control and high carbohydrate groups after 4 hours and stored at -80 until further required. RNA was extracted with TRIzol Reagent as discussed in the previous chapter (section 2.2.3.1). cDNA was synthesized using iScript DNA synthesis kit as described in the section 2.2.3.2. The samples were stored in -20 °C until used as a template for quantitative PCR. The expression of NUCB2 mRNA was conducted using SsoAdvanced SYBR Green SuperMix kit (Bio-Rad, Canada). All reactions were performed with a final volume of 20 µl as per **Table (2.7 and 2.8)** and the reaction protocol was set up in the CFX connect is discussed in the previous section 2.2.4.2. Forward and reverse primers for NUCB2 and Beta Actin were used (see **Table 3.1** for gene sequences and respective annealing temperatures).

3.3. Statistical Analysis

All data were analyzed using Student's – t test and one-way ANOVA with Tukey's Multiple Comparison Test. $P \leq 0.05$ was taken to indicate a significant difference. All data were expressed as mean ± standard error of mean (SEM).

3.4. Results

3.4.1. NUCB2/nesfatin-1 like Immunoreactivity (ir) in the Cytoplasm of HL-1 Cardiomyocytes.

Imaging of HL-1 cells at a magnification of 40X, showed the presence of NUCB2/nesfatin-1 like immunoreactivity in them. The cells were stained positive at the cytoplasmic regions **(Figure 3.1B**) and nucleus is stained blue with DAPI **(Figure 3.1A).**

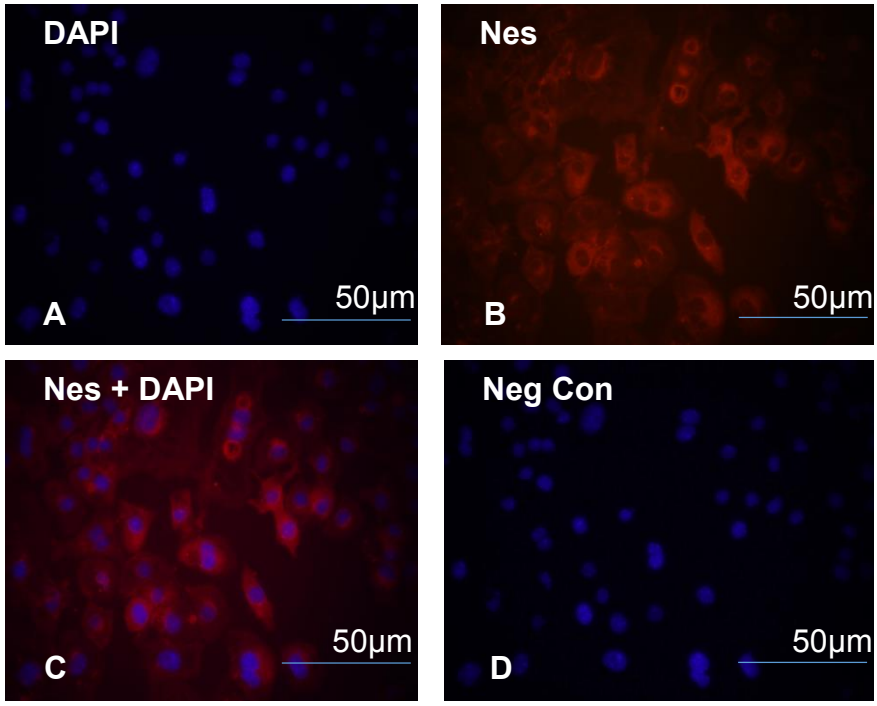

Figure 3.1: Characterization of NUCB2/nesfatin-1 like immunoreactivity within the cytoplasm of HL-1 cardiomyocytes. Immunohistohemical staining of HL-1 cardiomyoctyes for nesfatin-1 ir is depicted in (**B**). Red staining of the ventricle shows the presence of nesfatin-1 like ir. **(A)** shows staining of the nucleus by DAPI. Merged image of nesfatin-1 and DAPI is shown in (**C**). No-primary antibody negative control, labeled only with the secondary antibody is shown in **(D).**

3.4.2. Characterization of NUCB2 mRNA and GPCR mRNAs in HL-1 cardiomyocytes

The gel electrophoresis image shown below determines the presence of NUCB2 mRNA in these HL-1 cell lines. A band corresponding to approximately 200 base pairs shows the presence of NUCB2 mRNA. It was observed that HL-1 cardiac muscle cell line expressed GPCR3 receptor. The expression of GPCR6 and GPCR12 was not observed **(Figure 3.2)**

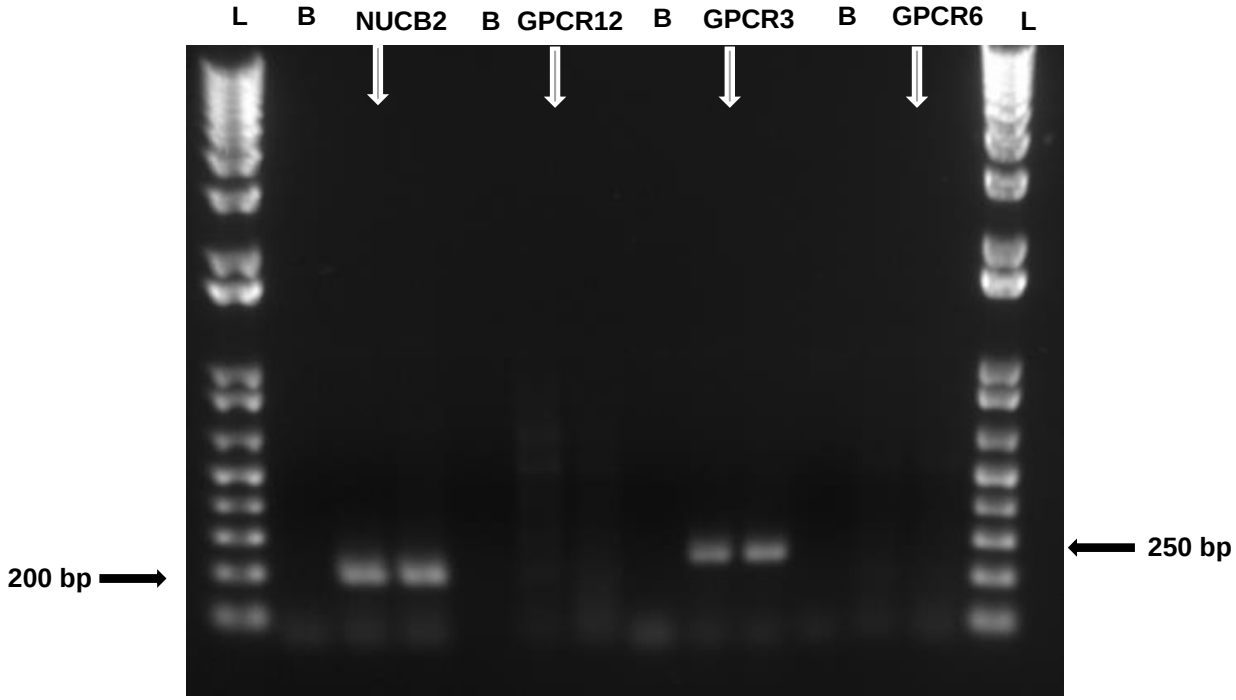

Figure 3.2: Gel electrophoresis image showing the expression of NUCB2 and GPCR receptors in HL-1 cardiomyocytes. A single band (approximately 200bp) showing the expression of NUCB2 is seen. HL-1 cardiomyocytes also expressed GPCR3 mRNA (approximately 250 bp). However GPCR 6 and GPCR 12 were not expressed by HL-1 cardiomyocytes**. L: Ladder; B: Blanks.**

3.4.3. High Glucose Upregulates NUCB2 and GPCR3 mRNA Expression in HL-1 Cells

HL-1 cells were treated with 100 mM of D-glucose and the control group was treated with normal Claycomb media that contains 25 mM of glucose. NUCB2 mRNA expression increased significantly in the group that received 100 mM of glucose (**Figure 3.3A)**. GPCR3 mRNA was also up-regulated in the presence of high glucose **(Figure 3.3B)**

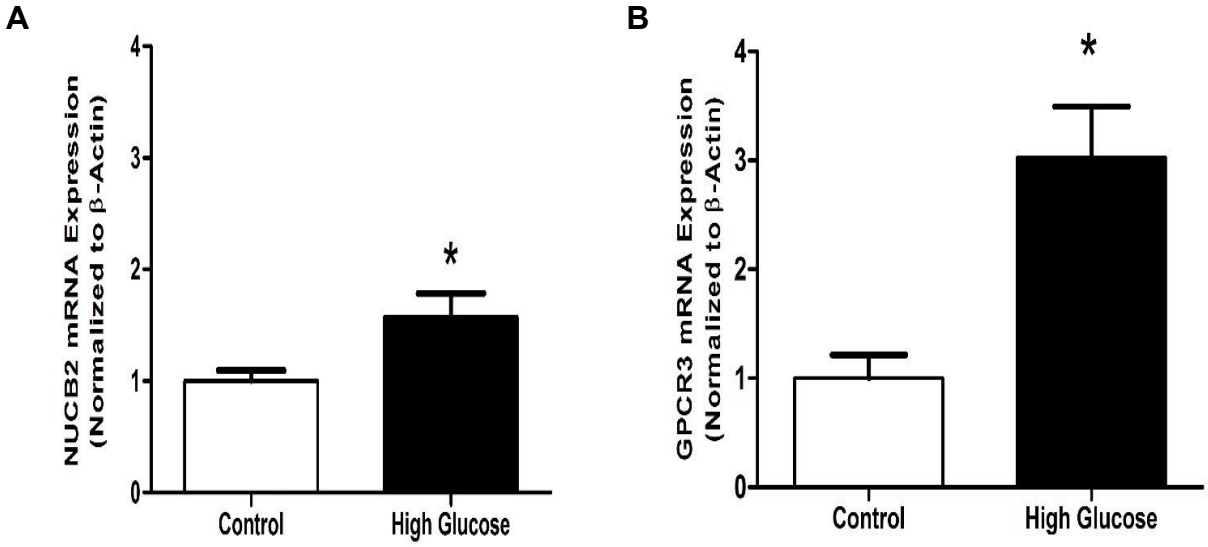

Figure 3.3: Mean ± SEM of the effects of high glucose (100mM) on NUCB2 mRNA expression (a) and GPCR3 mRNA expression in HL-1 cells. Mean was compared to the control group (25mM) using Student t-test analysis. The graph depicts that both NUCB2 and GPCR3 mRNA levels were significantly increased in the presence of high glucose concentration. Statistically significant *; $P < 0.05$.

3.4.4. GLUT4 mRNA Expression in HL-1 Cells is Not Affected by Nesfatin-1

It was observed that GLUT4 mRNA expression **(Figure 3.4)** was not altered in the presence of different (0.01, 0.1 and 1nM) nesfatin-1 doses.

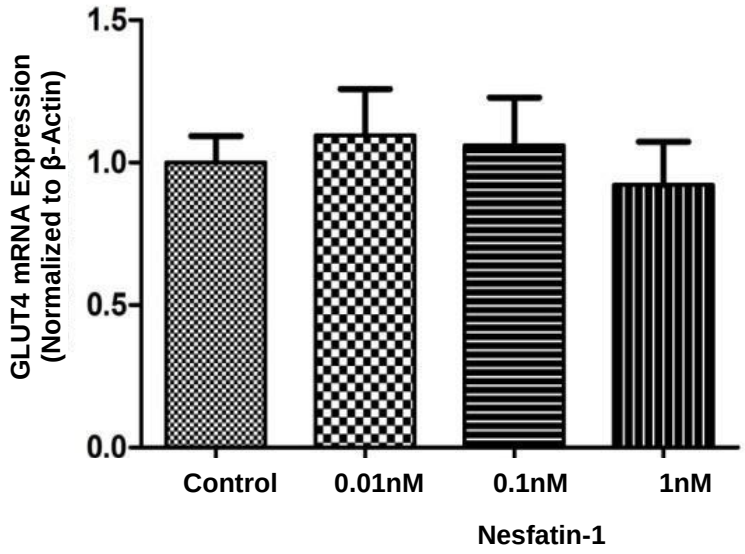

Figure 3.4: Mean ± SEM of the effects of dose dependent treatment of nesfatin-1 on GLUT4 mRNA expression in HL-1 cells. The GLUT4 mRNA was unaltered in all nesfatin-1 treated groups compared to the control. Mean was compared to control group and analyzed using one-way ANOVA and Tukey's Multiple Comparison test. Statistical Significance: Non-Significant; $P > 0.05$.

3.4.5. Dose-dependent Effects of Nesfatin-1 on GPCR3 mRNA Expression in HL-1 Cardiac Muscle Cell Line.

The receptor present in HL-1 cardiomyocytes was characterized to be GPCR3, among the other receptors of the GPCR family. GPCR3 mRNA expression was not significantly different among any of the groups that received nesfatin-1 dosage. This was compared to the controls, which received only the normal media **(Figure 3.5)**

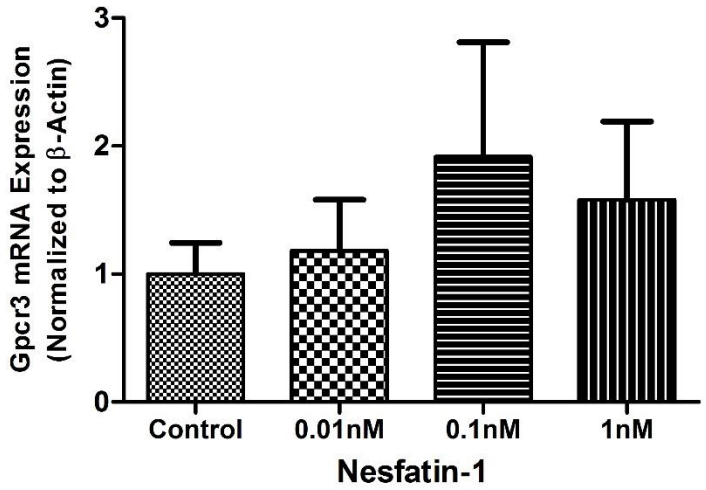

Figure 3.5: Mean ± SEM of the effects of dose dependent treatment of nesfatin-1 on GPCR3 mRNA expression in HL-1 cells. GPCR3 mRNA levels did not significantly differ in nesfatin-1 treated groups as compared to control. Mean was compared to control group and analyzed using one-way ANOVA and Tukey's Multiple Comparison test. Statistical Significance: Non-Significant; $P > 0.05$

3.4.6. Nesfatin-1 (10nM) Modulates Several Apoptosis Genes.

The genes that are depicted as red circles are genes that are upregulated in the presence of 10 nM of nesfatin-1 when compared to the control. They had a fold change value greater than 1. The p-value is determined using Student's t-test. However none of the p-values were less than 0.05. The significance of this study can be determined only in the presence of replicates. The data requires further assays and gene expression analysis (of genes that are up-regulated) in order to determine the effect of nesfatin-1 on apoptosis of HL-1 cardiomyocytes. The list of genes that were up-regulated and down-regulated are provided in **Figure 3.6** and **Figure 3.7** depicts a scatter plot.

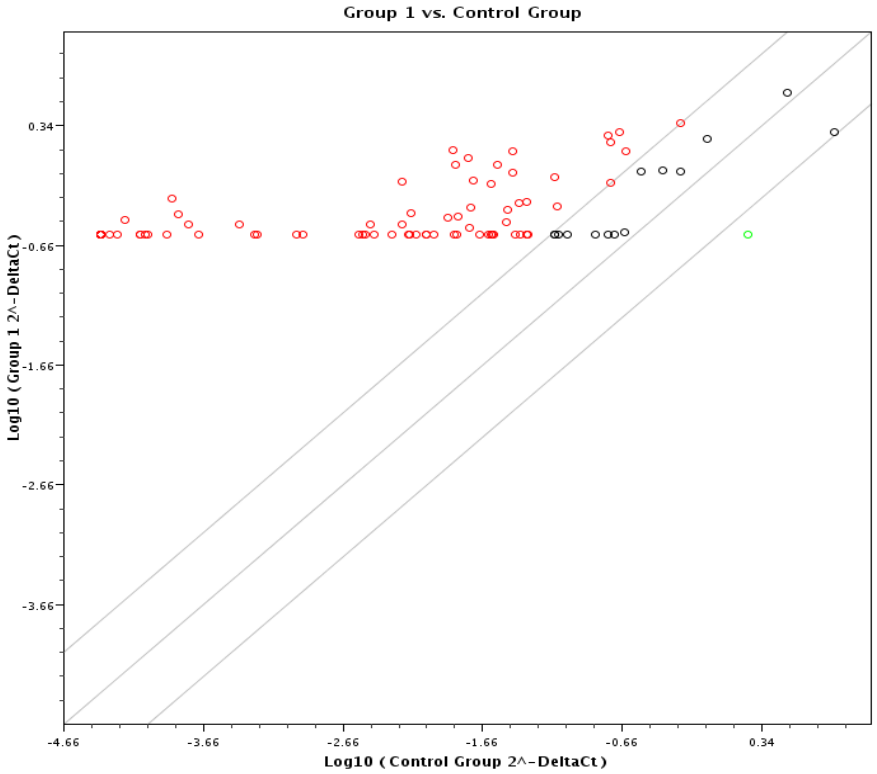

Figure 3.6. Scatter plot data showing the expression of 84 different genes. Red circles depict genes that are over expressed and green circle depicts the gene that was down-regulated. Data was analyzed using $\Delta\Delta C_T$ method (www.SABiosciences.com/pcrarraydataanalysis.php). However, due to the absence of replicates in this study, the data was not significant; P>0.05.

		Fold Change (comparing to control group)	
		Group 1	
Position	**Symbol**	**Fold Change**	**Comments**
A01	Abl1	74.7975	A
A02	Aifm1	1.5539	A
A03	Akt1	1.9253	A
A04	Anxa5	3.0551	A
A05	Apaf1	25.2347	A
A06	Api5	3.5778	A
A07	Atf5	10.5195	A
A08	Bad	5.9728	A
A09	Bag1	10.4391	A
A10	Bag3	5.873	A
A11	Bak1	3.7626	A
A12	Bax	17.3435	A
B01	Bcl10	6.6186	A
B02	Bcl2	36.9075	A
B03	Bcl2a1a	6754.7204	C
B04	Bcl2l1	60.3924	A
B05	Bcl2l10	6754.7204	C
B06	Bcl2l11	31.3703	A
B07	Bcl2l2	89.0771	A
B08	Bid	55.3634	A
B09	Birc2	10.5334	A
B10	Birc3	6754.7204	C
B11	Birc5	2.1842	A
B12	Bnip2	3.0459	A
C01	Bnip3	1.5889	A
C02	Bnip3l	1.2349	A
C03	Bok	11.3129	A
C04	Card10	240.9962	B
C05	Casp1	812.6504	B
C06	Casp12	11.2353	A
C07	Casp14	3481.1501	B
C08	Casp2	12.267	A
C09	Casp3	30.3273	A
C10	Casp4	506.9993	B
C11	Casp6	19.7992	A
C12	Casp7	11.1495	A
D01	Casp8	7.2189	A
D02	Casp9	102.5502	A
D03	Cd40	2255.4732	B
D04	Cd40lg	6754.7204	C
D05	Cd70	3545.3314	B

		Fold Change (comparing to control group)	
		Group 1	
Position	**Symbol**	**Fold Change**	**Comments**
D08	Cideb	40.5507	A
D09	Cradd	127.5663	A
D10	Dad1	9.2459	A
D11	Dapk1	27.6183	A
D12	Dffa	55.9274	A
E01	Dffb	96.1488	A
E02	Diablo	6.2482	A
E03	Fadd	24.5547	A
E04	Fas	28.59	A
E05	Fasl	6754.7204	C
E06	Gadd45a	73.3254	A
E07	Igf1r	3.7664	A
E08	Il10	1896.5912	B
E09	Lhx4	533.3964	B
E10	Ltbr	36.948	A
E11	Mapk1	1.4121	A
E12	Mcl1	37.8233	A
F01	Naip1	6754.7204	C
F02	Naip2	5093.1275	B
F03	Nfkb1	67.3293	A
F04	Nme5	6754.7204	C
F05	Nod1	31.6633	A
F06	Nol3	8.7354	A
F07	Polb	4.0937	A
F08	Prdx2	4.0598	A
F09	Pycard	5.8158	A
F10	Ripk1	94.934	A
F11	Tnf	2786.6194	B
F12	Tnfrsf10b	268.4895	B
G01	Tnfrsf11b	5887.9311	B
G02	Tnfrsf1a	13.0752	A
G03	Tnfsf10	1344.6272	B
G04	Tnfsf12	85.1024	A
G05	Traf1	5765.4718	B
G06	Traf2	13.1806	A
G07	Traf3	19.1199	A
G08	Trp53	42.4331	A
G09	Trp53bp2	10.7705	A
G10	Trp63	3091.3412	B
G11	Trp73	4096.5333	B
G12	Xiap	26.2886	A
H01	Actb	1.8821	A
H02	B2m	0.1556	A

H03	Gapdh	0.2661	A
H04	Gusb	10.2823	A
H05	Hsp90ab1	1.2478	A
H06	MGDC	6754.7204	C
H07	RTC	6754.7204	C
H08	RTC	6754.7204	C
H09	RTC	6754.7204	C
H10	PPC	7916.0697	OKAY
H11	PPC	16231.7753	OKAY
H12	PPC	8975.1289	OKAY

Figure 3.7. List of genes up-regulated and down-regulated in the presence of 10nM of nesfatin-1. A: This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30). These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result. This fold-change result may also have greater variations if p value > 0.05; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene. **B**: This gene's average threshold cycle is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples, and the p-value for the fold-change is either unavailable or relatively high (p > 0.05). **C**: This gene's average threshold cycle is either not determined or greater than the defined cut-off value (default 35), in both samples meaning that its expression was undetected, making this fold-change result erroneous and un-interpretable.

3.4.7. Effects of Macronutrients on NUCB2 mRNA Expression in Male C57BL/6 Mice Hearts.

In the chronic diet study, the NUCB2 expression in the heart of male C57BL/6 mice which received high fat diet did not significantly differ as compared to the control group which received normal diet. This suggests that NUCB2 mRNA expression in C57BL/6 mice heart was not altered in high fat induced mice **(Figure 3.8A)**. However, in the acute oral gavage study, the group that received high carbohydrate had a significant increase in the expression of NUCB2 mRNA when compared to control group (no gavage) **(Figure 3.8B).**

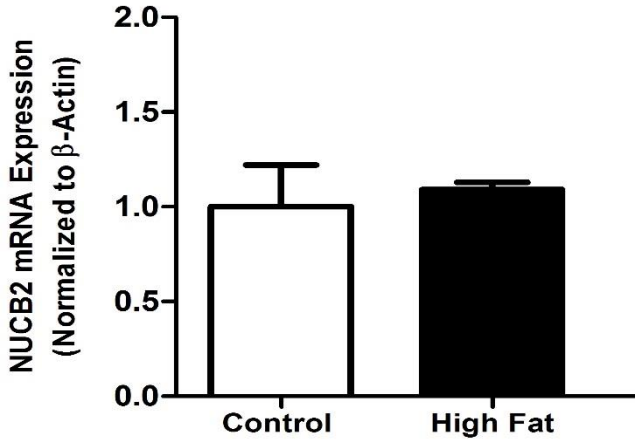

Figure 3.7A: Mean ± SEM of the effects of high fat diet on the NUCB2 mRNA in the heart of C57BL/6 mice (n =8). There was no significant difference in NUCB2 mRNA levels in high fat group as compared to the control group. Mean was compared to the control group which received the normal diet and analyzed using Student t-test; $P > 0.05$.

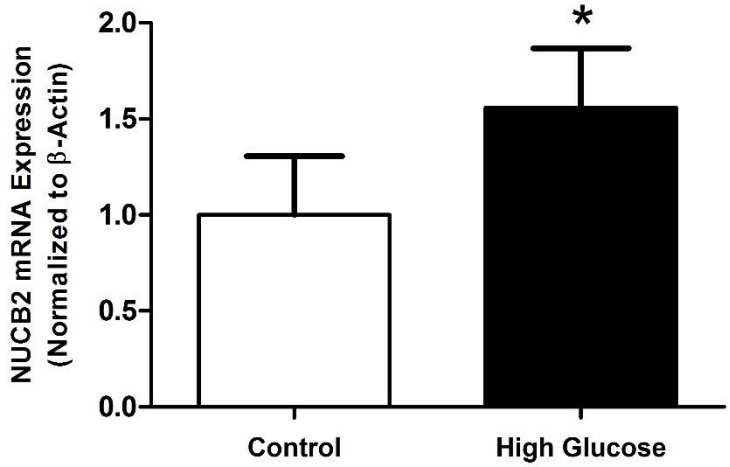

Figure 3.6b: Mean ± SEM of the effects of high glucose diet on NUCB2 mRNA in the heart of C57BL/6 mice (n=8). The group that received high carbohydrate diet had a significant increase in NUCB2 mRNA levels compared to the control group (No gavage). Mean was compared to the control group and analyzed using Student's t-test. Statistical significance: Statistically Significant; *; $P < 0.05$

3.5. Discussion

HL-1 cardiomyocytes expressed both NUCB2 and GPCR3 receptor mRNAs. GPCR6 and GPCR12 were not expressed in these cell lines. Feijóo-Bandín et.al. (2014) found that, nesfatin-1 is secreted and synthesized by the heart in rodents and humans. They also showed that HL-1 cardiomyocytes express nesfatin-1. This was supported by the presence of a 9.5 kDa band using Western blot. Both rodent and human heart tissues express nesfatin-1 peptide and NUCB2 mRNA. In agreement with this, my study found that HL-1 cells express NUCB2 mRNA. I found NUCB2/nesfatin1-like immunoreactivity in HL-1 cardiomyocyte cytoplasm. This suggests that heart is a source of circulating nesfatin-1. I also observed that NUCB2 mRNA expression was significantly increased in HL-1 cells treated with high glucose concentration compared to the control. Gonzalez et.al. (2011) observed that MIN6 cell line incubated with high glucose (16.7 mM) increased nesfatin-1 levels when compared to the control group (2.0 mM). Basal nesfatin-1 in circulation was also increased in human subjects treated with high glucose (Feijóo-Bandín et al., 2014). Similar to the endocrine pancreas and circulation, cardiomyocyte nesfatin-1 and its potential receptor GPCR3 are also glucose responsive. Together, my results indicate the presence of a glucose responsive nesfatinergic system in murine cardiomyocytes.

GLUT4 is the main glucose transporter of the heart (James et al., 1988). Nesfatin-1 is involved in the translocation of GLUT4 to the periphery of the cell from the cytoplasmic region (Feijóo-Bandín et al., 2014). A time and dose-dependent effect of nesfatin-1 on GLUT4 translocation was observed by Feijóo-Bandín et.al (2014). The nesfatin-1 doses used by this team were higher than compared to the doses used in our study. We observed that different doses of nesfatin-1 (0.01, 0.1 and 1 nM) did not significantly alter the expression of GLUT4 in HL-1 cardiomyocytes when compared to the control that received the normal media. This could be a result of the low dosage used in our study, suggesting that GLUT4 mRNA expression could be altered only in the presence of high concentrations of nesfatin-1. To determine the effect of nesfatin-1 on various apoptosis genes, we carried out a PCR array on HL-1 cells treated with 10nM of nesfatin-1. Our preliminary observations indicate that most of the apoptosis genes appeared up-regulated. Some of the genes that appeared to be up-regulated include TNF, Casp1-4, Casp6-10 and Akt1. Hsp90ab1 appeared down-regulated in the presence of 10nM of nesfatin-1 (Figure 3.6). However, none of the p-values were less than 0.05. The data is preliminary and requires further

assays and gene expression analysis (of genes that are upregulated) in order to determine the effect of nesfatin-1 on apoptosis of HL-1 cardiomyocytes.

A pilot in vivo study was conducted using C57BL/6 mice. We observed that NUCB2 mRNA expression in the heart of high fat fed, diet induced obese mice (DIO) was not significantly different from the control. However, in DIO mice, NUCB2 mRNA expression was significantly increased in the pancreas (Gonzalez et al., 2011). This suggests that NUCB2 mRNA expression in heart is not affected by diet-induced obesity in C57BL/6 mice. In the acute diet study, we observed that the group that received high carbohydrate diet had a significant increase in NUCB2 mRNA expression in the heart as compared to the no gavage group. This result is consistent with our data observed in HL-1 cells. Therefore, nesfatin-1/NUCB2 mRNA expression is glucose responsive in the heart. *In vivo* and *in vitro* data from this research show that glucose is an important stimulator of nesfatin-1 in the heart.

INTEGRATION OF FINDINGS

The aim of my thesis was to characterize and study the effects of NUCB2/nesfatin-1, a novel metabolic peptide, in the cardiovascular system of zebrafish and mice. The most significant findings of this research are reported in chapter 2. We detected the presence of NUCB2 mRNA and NUCB2/nesfatin-1 like immunoreactivity in zebrafish cardiomyocytes. This provides the first evidence that the heart is a possible source of endogenous nesfatin-1 in zebrafish. This research also considered the expression of NUCB2 in zebrafish heart during the regular meal -time and under calorie restriction. Chronic food deprivation did not affect NUCB2 mRNA, suggesting that cardiac NUCB2, at least at the mRNA level is not modulated by nutrient availability. Meanwhile, periprandial changes in NUCB2 mRNA were observed in the heart. The highest expression of NUCB2 mRNA was detected during feeding time point (0 hour time point-15 mins post feeding). We collected heart samples 15 minutes post feeding in the 0 hour group. Interestingly, this 0 h group had the highest expression of NUCB2 mRNA among all time points tested. Compared to this group (0 h), the lowest expression of NUCB2 mRNA was in the unfed group (+1 UF), and the expression at this point showed no difference between +1 UF and +1 F (and +3 UF and +3 F). This suggests that NUCB2 mRNA expression in the heart is not dependent on meal at 1 and 3 hours post-feeding time. It is possible that a circadian pattern also exists in NUCB2 mRNA expression in zebrafish heart. Exogenous nesfatin-1 showed a dose-dependent effect on the cardiac functions in zebrafish. It modulated functions including EDV, CO and HR. At the highest doses of ip injection of nesfatin-1 (250 and 500 ng/g BW), there was a significant decrease in EDV, CO and HR. However, the mechanism of action of nesfatin-1 in heart is yet to be elucidated, although we were able to determine the expression of two important cardioregulatory calcium handling proteins of the heart: SERCA2a and RyR1b. The gene encoding the SERCA pump (Atp2a2a) was significantly over-expressed in the group that received the highest dose of nesfatin-1 (500 ng/g BW). This increase in expression could possibly be a direct action of nesfatin-1 on Atp2a2a mRNA expression, or it is acting as a compensatory mechanism to maintain the changes in the homeostasis caused due to dosage of nesfatin-1. While this work requires further insight on the mechanistic actions of nesfatin-1 and the receptor through which it elicits its action, chapter 2 details zebrafish heart as a possible source of nesfatin-1, and a role for exogenous nesfatin-1 in regulating cardiac physiology in zebrafish. To the best of our knowledge, this is the first report on nesfatin-1 and its cardiovascular functions in a non-mammal.

At the time when we initiated the research in chapter 3, there were not many publications on nesfatin-1, especially cardiac nesfatin-1 in mice. We detected both NUCB2 mRNA and NUCB2/nesfatin-1 like immunoreactivity in mouse heart, providing further evidence that heart is a source of endogenous nesfatin-1. High glucose increased NUCB2 mRNA expression in HL-1 cells. In order to further confirm this, we conducted an acute high glucose study *in vivo* using C57BL/6 mice. Similar to the finding using HL-1 cells, we found that high glucose increases the expression of NUCB2 mRNA in the heart of mice. These data suggest that NUCB2 mRNA in mouse heart is glucose responsive. We then determined the effect of exogenous nesfatin-1 on the glucose transporter gene of heart (GLUT4). The doses used here were insufficient to cause any change to GLUT4 mRNA expression. Nesfatin-1 is said to act via a family of G-protein coupled receptors, of which, only GPCR3 was expressed in this cell line. This could possibly suggest that, nesfatin-1 acts via GPCR3 receptors in cardiomyocytes. However, this requires further investigation. In the PCR array analysis conducted to determine the role of nesfatin-1 in apoptosis, treatment of HL-1 cells using 10 nM of nesfatin-1 appeared to up-regulate and/or down-regulate certain genes. This requires further analysis, as all the p values were not less than 0.05. Use of replicates would eliminate this and provide a more appropriate result. Further, follow up using qPCR to determine the mRNAs of interest is required. In conclusion, heart is a source of endogenous nesfatin-1, both in mammals and fish, and it regulates cardiac functions in fish. **(Figure A)**.

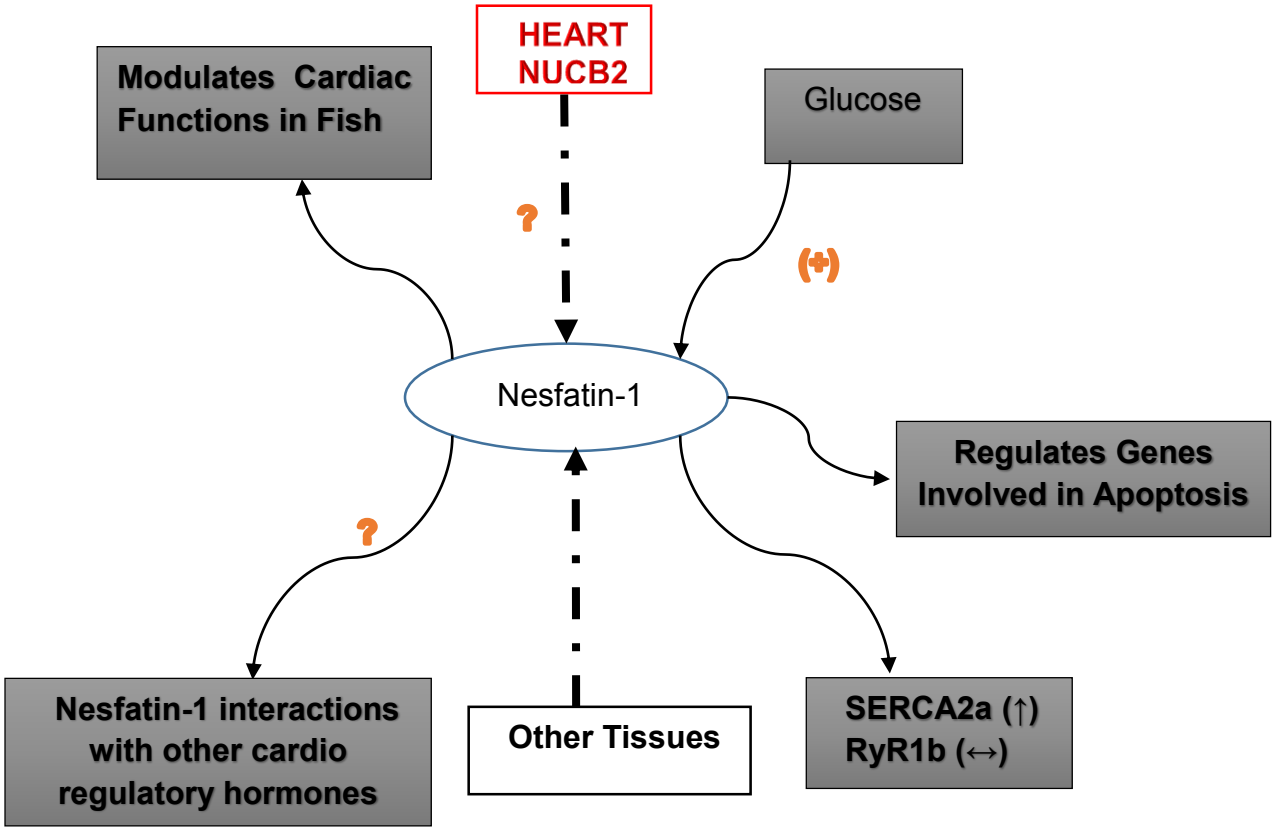

Figure A: Schematic representation depicting the role of nesfatin-1 in the cardiovascular system. Glucose stimulated nesfatin-1 levels in mouse Cardiomyocytes. Exogenous nesfatin-1 regulated genes involved in apoptosis (Eg: Akt1, Casp1, TNF), cardioregulatory proteins of the heart (SERCA2a) and modulated several cardiac functions in Zebrafish heart. This diagram concludes that heart is source of endogenous nesfatin-1 and is also involved in the regulation of cardiovascular system of mouse and fish.

FUTURE DIRECTIONS

This research results provide many leads for future research on nesfatin-1 and the cardiovascular physiology in fish and mammals. While several lines of research are possible at this point, some of the important follow-up studies, which are logical extensions of current findings, are listed below.

1. **A time-dependent study of nesfatin-1 doses on the cardiac functions of zebrafish:** One limitation of this research is that it considered the effects of nesfatin-1 on the CV system only at a single time point, 15 minutes post-injection. In a future study, a dose effective in regulating zebrafish cardiac functions (using US scan) should be tested at 15, 30, 45 and 60 minutes to determine whether the effects of exogenous nesfatin-1 reported in Chapter 2 continues to exist for longer durations.
2. **Determine serum nesfatin-1 levels in zebrafish post intraperitoneal injections:** While CV effects for some of the doses of nesfatin-1 tested was found, how much elevation in circulating nesfatin-1 was achieved with these injections was not tested. For this, it is essential to determine nesfatin-1 in circulation in using tissue extracts and Western blot or ELISA/RIAs.
3. **To elucidate the mechanism of action of nesfatin-1 in eliciting cardiac functions:** This research attempted to begin the search for mechanisms of nesfatin-1 action by studying the potential nesfatin-1 receptors, and by measuring the abundance of two proteins involved in calcium handling in cardiomyocytes. However, the on cellular mechanism mediating the effects of nesfatin-1 on cardiomyocyte should be studied using pharmacological and cell signaling methods.
4. **Interactions of nesfatin-1 with other CV regulatory hormones:** Whether nesfatin-1 modulates other CV hormones, including GLP-1, ghrelin and PYY is unclear. This aspect should be studied following expression and actions of CV regulatory peptides after nesfatin-1 administration.
5. **Cell growth and apoptosis:** Some changes in apoptosis genes after nesfatin-1 treatment were found. It suggests a possible role for nesfatin-1 on the life of these cells. This study should be repeated with an increased number of samples *in vitro*. Further studies are also important determine whether there are any effects for nesfatin-1 on cell growth and

apoptosis. Both cell counting and expression of proteins encoded by these mRNAs must be used.

6. **Absence of endogenous nesfatin-1:** My studies explored the effects of exogenous nesfatin-1 on zebrafish CV function *in vivo*, and a role for nesfatin-1 treatment on HL-1 cells *in vitro*. However, these studies do not help understanding whether endogenous nesfatin-1 is critical in CV physiology. NUCB2 gene knock-out and knock-down strategies should be used to delete or attenuate endogenous nesfatin-1, and determine how the CV system functions in the absence of this CV regulatory peptide.

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