

AUGMENTED EXPRESSION OF APELIN/APJ IN THE PARAVENTRICULAR NUCLEI OF
RATS AFTER MYOCARDIAL INFARCTION

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Title

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ABSTRACT

Heart failure (HF) is a disease condition in which insufficient blood is pumped through the body. The pathophysiology of HF is multisystematic and includes a collection of different responses to compensate for the inability of the heart to pump the blood with the most important outcome being increased sympathetic nervous system (SNS) activity. Increased SNS activity leads to reclaim the reserved cardiac function. However, this adaptive response is short term and deleterious. The central mechanisms that lead to increased SNS activity during conditions of HF remain enigmatic. APJ, a G-protein-coupled receptor and its endogenous ligand, is a novel neuroendocrine system. Previous studies from us and others indicated that central administration or over expression of apelin in brain cardiovascular regulatory areas resulted in an increase in blood pressure, sympathetic nerve activity and cardiac hypertrophy. The main objective of this study is to determine whether the Apelin/APJ system is involved in increased SNS activation during HF. We created HF rat models by left coronary artery ligation. Apelin and APJ receptor mRNA levels were measured in cardiovascular regions of the brain of sham and myocardial infarction (MI) rats. Results showed a significant increase in the levels of Apelin/APJ mRNA levels in paraventricular nuclei (PVN) and rostral ventrolateral medulla (RVLM) in MI rats as compared to sham rats. To determine the functional role of elevated APJ receptor in these cardiovascular regulatory regions of the brain during HF, we constructed a lentiviral vector carrying an APJ shRNA (Lenti-APJ-shRNA) to knockdown the APJ receptor. Efficiency of the lentiviral vector to knockdown the APJ receptor was confirmed *in vitro* by transducing a Cath.a cell line and a primary neuronal cell culture with Lenti-APJ-shRNA. In order to determine the effect of silencing of the APJ receptor *in vivo*, Lenti-APJ-shRNA virus was injected into the PVN of the MI and sham rats. Results showed knockdown of APJ receptor improved left

ventricular function and decreased myocardial fibrosis and hypertrophy in MI rats. Thus, this study shows that PVN plays an important role in sympatho excitation and pathophysiology of HF and these findings may help in developing effective therapies for HF.

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LIST OF ABBREVIATIONS

ACTH.....	adrenocortico trophin hormone
AHA.....	american heart association
AR.....	adrenergic receptor
ATCC.....	american type cell culture
ATP.....	adenosine tri phosphate
AVP.....	arginine vasopressin
BW.....	body weight
CAD.....	coronary artery disease
Ca ⁺⁺	calcium
CHF.....	congestive heart failure
CO ₂	carbon dioxide
cDNA.....	complementary deoxyribonucleic acid
cAMP.....	cyclic adenosine monophosphate
DNA.....	deoxyribonucleic acid
dNTP.....	deoxynucleotide triphosphate
EDTA.....	ethylenediaminetetraacetic acid
ELISA.....	enzyme linked immunosorbent assay
FBS.....	fetal bovine Serum
GAPDH.....	glyceraldehydes 3-phosphate dehydrogenase
GABA.....	γ-amino butyric acid
GPCR.....	G-protein couple receptor

HBSS.....hank's balanced salt solution
HIF-1.....hypoxia-inducible factor-1
HW.....heart weight
i.p.....intra peritoneal
i.c.v.....intracerebroventricular
LAD.....left anterior descending
LTCC.....L type calcium channels
LVSP.....left ventricular systolic pressure
LVEDP.....left ventricular end-diastolic pressure
LV dp/dt max.....left ventricular dp/dt max
LV.....lentiviral
LV-APJ-shRNA.....lenti APJ shRNA
LV-SCR-shRNA.....lenti Scrambles shRNA
HF.....heart failure
 μ L.....micro liter
 μ g.....micrograms
ml.....milliliter
mRNA.....messenger RNA
nl.....nano liter
MI.....myocardial infarction
NCHS.....national center for health statistics
NHLBI.....national heart lung and blood institute
NE.....norepinehprine

NO.....nitric Oxide
NTS.....nucleus tractus solaris
PCR.....polymerase chain reaction
PBST.....phosphate buffer saline plus tween 20
PVN.....paraventricular nuclei
RT.....reverse Transcriptase
RT PCR.....real Time PCR
RNA.....ribonucleic acid
RNAi.....ribonucleic acid interference
RVLM.....rostral ventrolateral medulla
SD.....sprague-dawley
SDS.....sodium dodecyl sulfate
shRNA.....small hairpin RNA
siRNA.....small interference RNA
SON.....supra optic nucleus
SNS.....sympathetic nervous system activity
TBST.....tris buffered saline plus tween20
TEMED.....N, N, N, N-tetraethylmethyldiamine

CHAPTER 1. INTRODUCTION

Heart primary function is to pump the blood to different parts of the body thus bringing nutrients and oxygen to the tissues and removing waste products. Heart failure (HF) occurs when the heart is not pumping effectively enough to meet the body's needs for oxygen rich blood. Congestive Heart Failure (CHF) is a syndrome often associated with a buildup of body fluid in the lungs and reduced cardiac output (Cohn et al., 1984)

1.1. Etiology of Congestive Heart Failure

CHF is a disease condition in which the heart can no longer pump enough blood to the body. There are estimated 5.7 million people in the United States and an incidence of 10 per 1000 population after age 65 with CHF. Over the past decade, the rate of hospitalizations increased by 159 percent (Foody et al., 2002; Roger et al., 2011). Studies also found that 75% of HF cases have hypertension and coronary artery disease (CAD) indicating that both are common risk factors (Ho et al., 1993; Kannel et al., 1994). In 2005, a study conducted by the National Center for Health Statistics (NCHS) and National Heart Lung and Blood Institute (NHLBI) demonstrated that total mortality of HF was 292,214 and that 80% of people with HF died within 8 years of diseases onset. Men when compared to women had a lower survival rate. There are estimated to be 550,000 new cases each year in the United States, based on the report from American Heart Association (AHA) (Lloyd et al., 2010). The rise in incidence and prevalence of HF globally is the result of improved care of myocardial infarction combined with the ageing of the population and the emerging pandemic of cardiovascular disease in developing countries (Zannad et al., 2009).

Heart failure has huge impacts on the health related economy and appears to be the biggest economic burden. According to the AHA, in the USA, 17% of medical expenditures equaling \$149 billion annually and nearly 30% of care cost is credited to stroke, hypertension, and HF. It is estimated that 1-2% of all health care expenditures is on CHF in developed countries. In the USA, expenditure on HF increased from \$30.2 billion dollars to \$37.2 billion dollars in 2009 (Lloyd et al., 2010).

1.2. Pathophysiology of Congestive Heart Failure

CHF is a disease with an assortment of different symptoms resulting from abnormal cardiac structure, function, contractility, or conduction (Jessup et al., 2003; Clyde et al., 1988). This disease is usually caused by ventricular dysfunction, myocardial infarction, chronic hypertension, and anthropogenic factors, such as food, alcohol, and tobacco (Jhonson et al., 2002). Additionally, CHF is often reported in old people who have complications including chronic lung disease, diabetes, hypertension, coronary heart disease, and renal dysfunction (Gottdiener et al., 2009). The pathophysiology of CHF includes the inability of the heart to deliver oxygen and an assortment of different responses in order to compensate for the lack of oxygen delivery (Dougherty et al., 1984). The characteristic of CHF is decreased cardiac output measured by the volume of blood pumped per minute. Cardiac output is determined by a number of factors including stroke volume, heart rate, preload, contractility, and after load as shown in figure 1.

A heart with normal systolic function will maintain an ejection fraction over 50-55%. Stroke volume is determined by preload, contractility, and after load. Change in any one of these factors will result in a change in stroke volume.

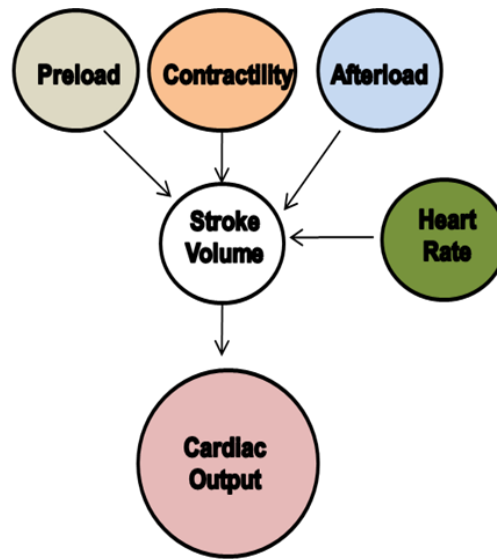


Figure 1. Determinants of Cardiac output. Cardiac output is the amount of blood ejected by the left ventricle of the heart in one minute. Stroke volume and heart rate together govern the cardiac output. Heart rate is intrinsic factor affected by autonomic, humoral and local factors. Stroke volume is determined by preload contractility and after load

Preload can better be defined as the stretching of cardiomyocytes prior to contraction and is an indirect measurement for sarcomere length. As sarcomere length cannot be determined in an intact heart, preload is usually measured using end diastolic pressure or volume. Changes in preload affect the stroke volume of the heart by the Frank-Starling mechanism that is defined as the ability of the heart to change its force of contraction and stroke volume in response to alterations in venous return (Rowell et al., 1993; Linappa et al., 2000). Preload is increased by a rise in central venous pressure, ventricular compliance, sympathetic stimulation of atria, increased ventricular filling time, or by pathological conditions such as ventricular systolic failure, aortic stenosis, and aortic regurgitation (pulmonary valve stenosis and regurgitation). The increase in preload activates the frank-starling mechanism to compensate partially for the reduction in stroke volume caused by the increase in after load. Preload is reduced by a decrease in central venous pressure and ventricular filling time, atrial arrhythmias, valve stenosis, and ventricular diastolic failure (Maurico et al., 2007).

After load is the burden that the heart has to work against to eject blood. After load is generally determined by mean arterial pressure and mostly related to ventricular wall stress. After load can be defined by the following equation $\sigma = P \cdot r/h$, where P is ventricular pressure, r is the ventricular radius, and h is ventricular wall thickness. Increases in aortic pressure or systemic vascular resistance strengthen after load. Increased after load results in an increase in end systolic volume/pressure and a decrease in an stroke volume, After load decreases stroke volume but increases end-systolic volume (LVEDP) by adding to the venous return into the ventricle (Klabunde et al., 2005)

Inotropy or contractility is the ability of the heart muscle fiber to contract at a given fiber length. Changes in inotropy alter the rate of force and pressure development by the ventricle and change the rate of ejection. Increase in inotropy result in elevation of ejection fraction, which is used as an index in conditions of HF (Klabunde et al., 2005; Fuster et. al., 2001).

The cardiac output in a normal individual is 3 - 7 liters per minute. If the cardiac output falls, the heart rate and stroke volume are increased in order to maintain organ blood supply, leading to structural abnormalities of the heart, activation of neurohormonal system, and stimulation of the renin angiotensin system. All of those alterations induce sodium and water retention in the kidney and vasoconstriction in peripheral microcirculation thereby, increasing cardiac contractility. Initially these compensatory responses are good for maintaining the blood supply but long-term activation of these systems lead to pathological alterations in the cardiovascular system. The stressed myocardium undergoes remodeling, which in turn leads to cardiac decompensation, causing cardiac complications including mitral regurgitation due to valvular annulus stretching, cardiac arrhythmias, and other detrimental effects on functioning of

heart lungs, kidneys, muscles, blood vessels and other organs leading to typical CHF clinical symptoms (Bonow et al., 1997).

An early symptom of CHF includes fatigue because of the decompensation of cardiac function. The patient's ability to exercise is diminished. As CHF progresses, patients have elevated end diastolic pressure that results in blood accumulation in pulmonary microcirculation leading to pulmonary hypertension and edema. Fluid accumulates in the lungs, thereby causing shortness of breath, particularly during exercise and when lying flat. In some instances, patients are awakened at night gasping for air. Long-term activation of the rennin angiotensin system leads to increased sodium retention, incidence of peripheral edema, and development of pleural effusions. With acute expansion of the heart, the pulmonary capillary membrane may succumb to increased pressure with the shearing of the capillary and subsequent release of fluids into alveoli. The lungs respond by cough to expel the fluid from the alveoli. Chronic conditions of elevated venous pressure in the lung lead to interstitial fibrosis with the thickening of the alveolar membrane leading to interstitial fibrosis (Zaret et al., 1992).

1.3. Sympathetic Nervous system in Congestive Heart Failure

HF is the inability to pump enough blood to meet the body's metabolic needs. The heart employs several compensatory mechanisms to overcome this inability and one such mechanism is enhanced sympathetic nervous activity. Increasing sympathetic nervous activity proliferates cardiac contractility and boosts output (Pepper et al., 1999). The increase in SNS activity is evidenced by an increase in circulating levels of norepinephrine that improve cardiac output but long-term dependence on this compensatory mechanism is harmful and deleterious to heart function. Enhanced plasma norepinephrine levels lead to catecholamine toxicity (Todd et al.,

1985) including cardiac interstitial fibrosis, cardiomyocyte apoptosis, and pump dysfunction (Brauri et al., 2004). Long-term exposure of cardiomyocytes to norepinephrine results in death of the cells from oxidative damage from reactive intermediates formed through auto-oxidation of catecholamine's (Neri et al., 2007).

1.4. Sympathetic Nervous System in Cardiovascular Regulation

The Sympathetic Nervous System (SNS) is part of the autonomic nervous system that mobilizes energy stores in times of need. The sympathetic division receives innervation from cell bodies located from the first thoracic (T1) and second lumbar (L2) regions of the spinal cord and thus are called the thoracolumbar division. The SNS has a wide range of cardiovascular actions, which lead to increases in heart rate and cardiac contractility, decreases in venous capacitance, and constriction of resistance vessels (Mann et al., 2005; Malpas et al., 2009). The sympathetic nerve fibers are located epicardially and run along the major coronary arteries representing predominant autonomic components of the ventricles. On the contrary, the parasympathetic fibers run along the vagus nerve subendocardially, mainly present in the atrial myocardium and less abundant in the ventricles (Zipes et al., 2008). The ventricular sympathetic innervations are characterized by a gradient from base to apex (Pierpont et al., 1985). The neuronal cardiac regulatory system is composed of specially distributed cell stations comprising afferent, efferent, and interconnecting neurons acting as control systems (Armour et al., 2004). The neurons connecting to the central nervous system and heart are in constant communication and each neuronal cell station is involved in cardio-cardiac reflexes that control spatially organized cardiac regions (McAllen et al., 1974).

The most important level of amalgamation of SNS efferent activities to the cardiovascular system resides in the dorsolateral reticular formation of the medulla. The hypothalamus modifies the activity of the medullary centers and is important for stimulating cardiovascular responses to emotion and stress. The motor flow of the SNS is formed by two serially connected sets of neurons. The first set of preganglionic neurons originates in the brain stem or spinal cord. The second set, composed of postganglionic neurons, lie outside the central nervous system in collections of the nerve cells that are called sympathetic ganglia. The sympathetic preganglionic neurons originate in the lateral horns of the 12 thoracic and first two or three lumbar segments of the spinal cord. The short and myelinated axons exit the spinal cord in the ventral roots then synapse on either sympathetic ganglion cells or chromaffin cells in the adrenal gland that release epinephrine. The sympathetic ganglia's include two major categories, paravertebral, and prevertebral. The paravertebral ganglia (three in the cervical region including the right and left stellate ganglia, 10 to 11 in the thoracic region, four in the lumbar region, four in the sacral region, and a single unpaired ganglion lying in front of the coccyx) sit on each side of the vertebrae and are connected to the sympathetic chain or trunk. The prevertebral ganglia provide axons distributed with the three major gastrointestinal arteries namely the celiac, superior mesenteric, arising from the aorta to supply abdominal and pelvic viscera. The predominant neurotransmitter of the sympathetic preganglionic neurons is acetylcholine (AC), whereas the predominant neurotransmitter of most sympathetic postganglionic neurons is norepinephrine (NE). Sympathetic activity is attenuated by the arterial baroreflex and the cardiopulmonary reflex and increased by the cardiac sympathetic afferent and arterial chemoreceptor reflexes (Triposkiadis et al., 2009).

1.5. Cardiovascular Adrenergic Receptors (ARs)

The sympathetic transmitter, NE, binds to seven trans membrane heteromeric G-protein coupled receptors called adrenergic receptors. By using a pharmacological classification scheme, NE receptors (adrenergic receptors) have been divided historically into two major subtypes that are termed alpha and beta-adrenergic receptors (Bylund et al., 1998). Beta-adrenergic receptors have been further subdivided into beta-1, beta-2 and beta-3 subtypes. Similarly, alpha-adrenergic receptors have been subdivided into alpha-1 and alpha-2 (Bylund et al., 1994). NE transporter 1 recycles about 80% of the NE released by sympathetic nerves and the remainder is released into the systemic circulation (Skeberdis et al., 2008). Thus, plasma NE levels are usually measured as a marker of chronic sympathetic activation.

The human heart has beta-1, beta-2, and beta-3 receptors (Bylund et al., 1998). Among beta-adrenergic receptors, the beta-1 and beta-2 receptors are predominantly at a 70:30 ratio. Activation of these receptors results in increases in cardiac contractility, heart rate, relaxation, and impulse conduction through the atrioventricular node. Beta-3 adrenergic receptors are usually inactive during normal physiologic conditions (Skeberdis et al., 2008) but stimulation these antagonizes the actions of beta-1 and beta-2 receptors through the nitric oxide synthase pathway (Gauthier et al., 1998) thus acting as a feedback mechanism during intense adrenergic stimulation (Rozec et al., 2000).

In the heart, activation of beta-1 and beta-2 adrenergic receptors induces the most powerful physiologic response. Beta-1 activates the G_s subunit of G-protein and stimulates adenyl cyclase thereby resulting in dissociation of adenosine triphosphate (ATP) into a second messenger, cyclic adenosine monophosphate (cAMP), which in turn activates a cAMP dependent

protein kinase A. Protein kinase A stimulates several downstream targets such as L type calcium channels (LTCC) and ryanodine receptors thereby increasing intracellular Ca^{+2} levels (Zhao et al., 1996) figure 2.

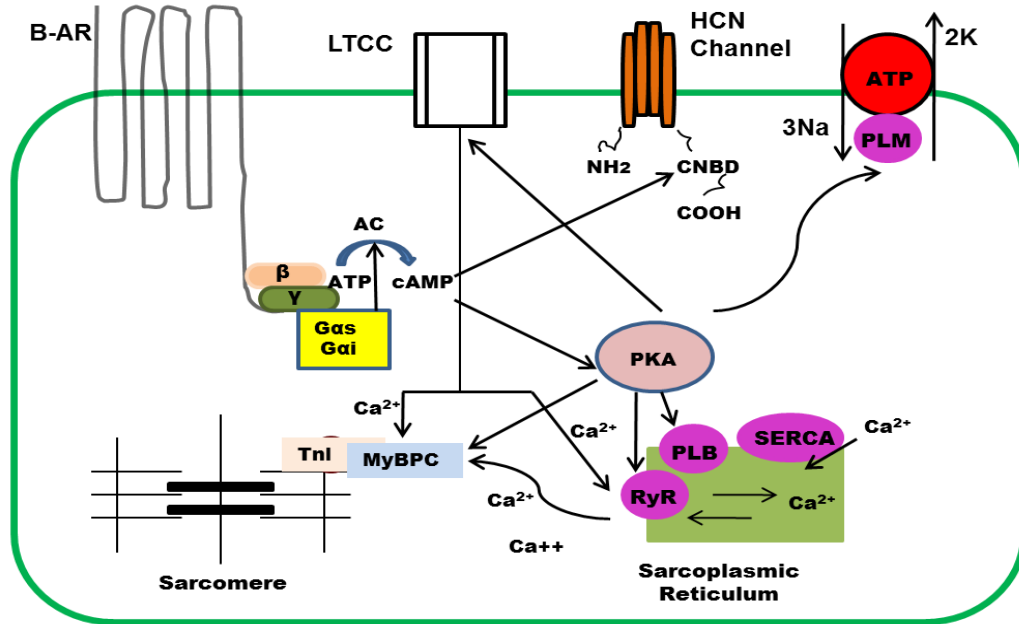


Figure 2. Beta-adrenergic receptor (β -AR) signaling pathways in cardiomyocytes. The major intracellular effect of the sympathetic transmitters norepinephrine and epinephrine is mediated by formation of 3',5'-cyclic monophosphate (cAMP), which increases the activity of the cAMP-dependent protein kinase A (PKA). PKA mediates a series of phosphorylations in diverse intracellular substrates, including the L-type Ca^{2+} channels (LTCC), hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, sarcoplasmic ryanodine receptors (RyR), phospholamban (PLB), myofibrillar proteins troponin I (TnI), cardiac myosin-binding protein C (MyBPC), and phospholemman (PLM).

Hyperpolarization-activated cyclic nucleotide-gated channels, which generate the hyperpolarization-activated cation inward current, affect the initiation and modulation of rhythmic activity in cardiac pacemaker cells. Phospholamban, a modulator of the sarcoplasmic reticulum associated ATP dependent calcium pump accelerates Ca^{2+} reuptake by the sarcoplasmic reticulum thereby accelerating cardiac relaxation (Sulakhe et al., 1995). Troponin I and myosin binding protein-C, which reduce myofilament sensitivity to Ca^{2+} , accelerate the

relaxation of myofilaments (Despa et al., 2005). Beta-2 adrenergic receptors activate both Gs and Gi proteins. Gs protein acts as a receptor accelerator. Gi protein acts as a receptor breaker, decreases cyclic AMP levels, activates mitogen activated protein kinases, and in turn regulates receptor signaling and nuclear transcription (Feldman et al., 2005).

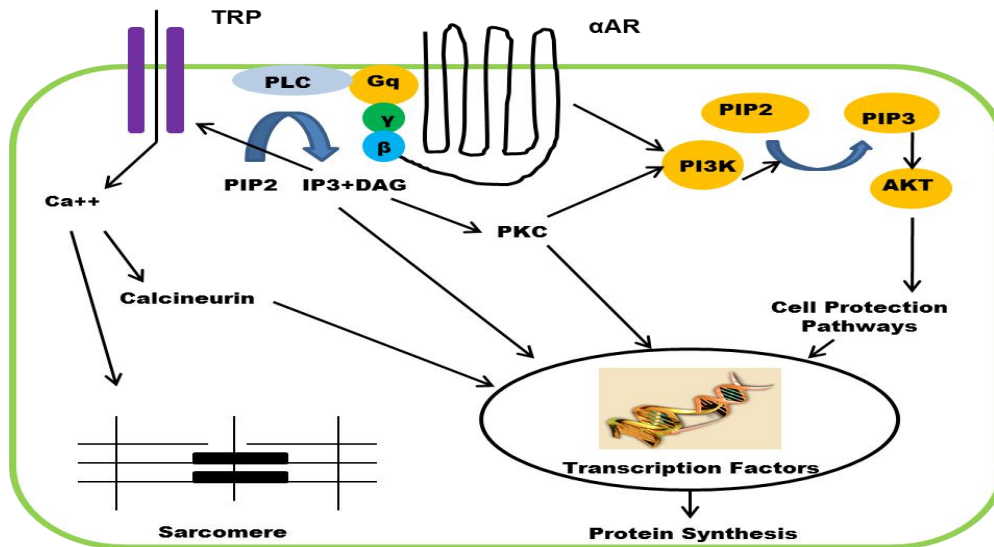


Figure 3. Agonist-induced stimulation of alpha-adrenergic receptor. (α AR) activates G_q and phospholipase C (PLC), resulting in hydrolysis of phosphatidylinositol bisphosphate (PIP_2), to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG). DAG, in turn, activates protein kinase C (PKC) to initiate a series of phosphorylations that alter channel activity and induce transcriptional changes. Moreover, IP_3 interacts with per nuclear inositol trisphosphate receptors (IP_3R) disinhibiting growth-related gene transcription. Both PIP_2 and DAG increase the permeability of the transient receptor potential (TRP) channel to Ca^{2+} , which enters the cell and activates calcineurin to initiate downstream growth signaling pathways. Ca^{2+} entry through TRP channels may also act on myofilaments enhancing contractile responses. The α AR also transactivates epithelial growth factor receptors, resulting in formation of phosphoinositide 3-kinase (PI_3K) and phosphatidylinositol trisphosphate (PIP_3), activation of the Akt pathway, and initiation of cell-survival signaling pathways.

The human heart expresses α_{1A} - and α_{1B} - adrenergic receptor at lower levels (~20%) than those of beta-adrenergic receptors (Woodcock et al., 2008). It is unclear whether cardiac α_1 - adrenergic receptors play a major role under physiologic conditions. The α_1 - adrenergic receptors heavily populate major arteries and activation of these receptors by NE is a major contributor to the regulation of blood flow by vasoconstriction (Shanno et al., 2006; Hein

et al., 2001). The pathways mediated by alpha-adrenergic pathways are summarized in figure 3. The release of NE from sympathetic nerves is controlled by pre-synaptic α_{2A} - and α_{2C} -ARs. Both pre-synaptic α_2 -ARs are essential, as deletion of α_{2A} - and α_{2C} -ARs leads to cardiac hypertrophy and failure due to chronically enhanced catecholamine release (Esler et al., 2001).

1.6. Symptoms and Treatment of Congestive Heart Failure

The manifestations of HF reflect the physiologic effects of the impaired ability of the heart to pump the blood and subsequent employment of compensatory mechanisms to increase cardiac output. The major symptoms of CHF are increased fluid build-up and salt retention due to the inability of heart to pump blood. Accumulation of fluid in the lungs causes pulmonary edema and dyspnea (breathlessness). Fatigue and limb weakness are early signs of HF due to falling cardiac output and sodium water retention. Reduced cardiac output also results in an insufficient supply of oxygen to the brain, leading to confusion, anxiety, and impairment of memory. In the final stages of HF, patients develop cardiac cachexia (malnutrition and tissue wasting) and cyanosis (bluish discoloration of skin) due to excess denatured hemoglobin (Bonow et al., 2011). Once HF is detected, different pharmacological agents are used for treatment of this disease based upon the symptoms displayed. The pharmacologic agents currently employed include diuretics, digoxin, angiotensin converting enzyme (ACE) inhibitors, and β -adrenergic blocking agents. Diuretics are a class of agents that promote excretion of edema fluid and improve cardiac output. Digitalis is one of oldest drugs used for the treatment of CHF via improvement of cardiac function by increasing the force and strength of ventricular contractions. The ACE inhibitors prevent conversion of angiotensin I to angiotensin II thereby attenuating the rennin angiotensin aldosterone system and decreasing workload of the heart. B-adrenergic

blocking agent is a relative new class of medicines that attenuate increased SNS activity, dilate peripheral microcirculation, decrease cardiac hypertrophy, and cardiac remodeling (Guyaat et al., 2004; Rich et al, 1999).

1.7. Hypothalamic Paraventricular Nuclei in Congestive Heart Failure

The paraventricular nucleus (PVN) of the hypothalamus is a key brain area that controls sympathetic outflow and is involved in sympatho-excitation in the CHF (Swanson et al., 1980). The PVN receives afferent neural projections from the nucleus tractus solitarius (NTS) (Swanson et al., 1983). The NTS receives vagal cardiac and baroreceptor afferent information and transfer these cardiac sensing signals to the PVN (Lovick et al., 1988; Lovick et al., 1989). Efferent projections from the PVN are sent to the pituitary gland, the rostral ventrolateral medullar (RVLM), and the spinal cord (Pyner et al., 1999) that contains pre-sympathetic neurons. The dorsomedial and ventromedial portions of the PVN (pPVN) contains parvocellular neurons, which project to the RVLM and spinal cord segments T1–T3 and T9–T11 (Swanson et al., 1980; Swanson et al., 1979), which give rise to sympathetic efferent fibers to the heart (Wurster et al., 1977) and kidneys (Taylor et al., 1992), respectively. Recent evidence, suggests that parvocellular neurons in the PVN are involved in the mediation of the neural component of cardiovascular reflexes by influencing sympathetic nerve discharge (Lovick et al., 1994; Haselton et al., 1994). However, dorsolateral portion of PVN contains magnocellular neurons, which generally have a single long varicose axon projecting into the posterior pituitary. There are two types of magnocellular neurosecretory cells, oxytocin producing, and vasopressin producing. It is well established that the magnocellular neurons of the PVN are responsible for the humoral component of the regulation of fluid-balance via control of vasopressin release (Poulain et al., 1982).

Most of the major central neurotransmitters and neuromodulators have been found in the PVN. Among them, glutamate (Hermes et al., 1996) and angiotensin II (Zucker et al., 2004) generally exert excitatory effects on cardiovascular reflexes. γ -aminobutyric acid (GABA) and nitric oxide (NO) (Stern et al., 2002) serve as PVN inhibitors to influence sympathetic activity. One might rationally speculate that the interactions of excitatory and inhibitory neurotransmitters within the PVN influence the regulation of sympathetic outflow. The balance of these interactions might play significant roles in the sympathetic dysfunction in CHF. However, the detailed mechanisms of activation of sympathetic nerve activity and increased vasopressnergic activity during HF remain unclear.

1.8. Apelin/APJ System Expression and Function

APJ is a G protein coupled receptor identified in 1993 through the human genome project and exhibits significant homology with the angiotensin II receptor type 1 (AT1) (O'Dowd et al., 1993). Despite showing considerable homology with the AT1 receptor, angiotensin does not bind to the APJ receptor (Tatemoto et al., 1999). The APJ receptor is found to be expressed both in the central nervous system and peripheral tissues, such as heart, lung, kidney, mammary glands (Klienz et al., 2005; Lee et al., 2006; Masri et al.; 2005).

Apelin, the endogenous ligand for the APJ receptor, was first identified in bovine stomach as a 36 amino acid peptide (Habata et al., 1999). Further studies showed that apelin mRNA is present in CNS and peripheral tissues such as heart, lung, kidney etc (Lee et al., 2006; Masri, 2005). The apelin gene is present on the human X chromosome and encodes for a 77 amino acid preproprotein, which is cleaved by unknown endopeptidases into apelin-36, apelin-17, apelin-13, and apelin-12 (Habata et al., 1999; Hosoya et al., 2000; Tatemoto et al., 2001).

The bioactive form of apelin was thought to be apelin-36; however, further studies showed that shorter isoforms of apelin are more potent than the longer ones.

Among the shorter isoforms, the most prominent one is apelin-13, which has a pyroglutamated N terminal end bind to APJ receptor with better efficiency than longer forms (Habata et al., 1999; Kawamata et al., 2001). The apelin gene is up regulated under the conditions of hypoxia, fasting, dehydration, and hypertension (Ronkainen et al., 2007; Reaux et al., 2001; Boucher et al., 2005).

The APJ/Apelin genes are expressed in different parts of the body and play diverse roles including glucose metabolism (Dray et al., 2008; Yue et al., 2010), thermoregulation (Jászberényi et al., 2004), and fluid balance (Roberts et al., 2002; Mitra et al., 2006). Central administration of apelin reduces food intake, suggesting importance in maintaining energy balance. More interestingly, apelin is also expressed in adipocytes and this expression is enhanced in mice suffering from obesity and diabetes suggesting that Apelin/APJ may play a role in the development of obesity and diabetes (Carpene et al., 2007; Clarke et al., 2009).

1.9. Apelin/APJ System in Cardiovascular Regulation

Injection of apelin results in a rapid transient fall of mean arterial blood pressure in rats (Lee et al., 2000; Cheng et al., 2003; Tatemoto et al., 1999; Lee et al., 2005). This response is abolished in mice lacking the APJ gene (Ishida et al., 2004). The transient fall in blood pressure is attenuated by treatment with L-NAME, a nitric oxide synthase inhibitor, suggesting that apelin-induced decrease in blood pressure may be mediated by a nitric oxide-dependent mechanism (Ishida et al., 2004; Tatemoto et al., 1999).

In humans, apelin has been shown to modulate cardiac contractility (Maguire et al., 2009). Apelin effects vascular tone *in vitro*, through endothelium-dependent vasodilator and endothelium-independent vasoconstrictor (Katugampola et al., 2001; Salcedo et al., 2007; Maguire et al., 2009). Apelin has been shown to cause vasodilation when infused into the human forearm (Japp et al., 2008). In isolated hearts, apelin increases contractility at sub-nanomolar ranges and induces sarcomere shortening in cardiomyocytes isolated from normal and failing hearts (Farkasfalvi et al., 2007). In rats, acute apelin intravenous infusion increases dp/dtmax and cardiac output as well as cardiac contractility (Szokodi et al., 2002).

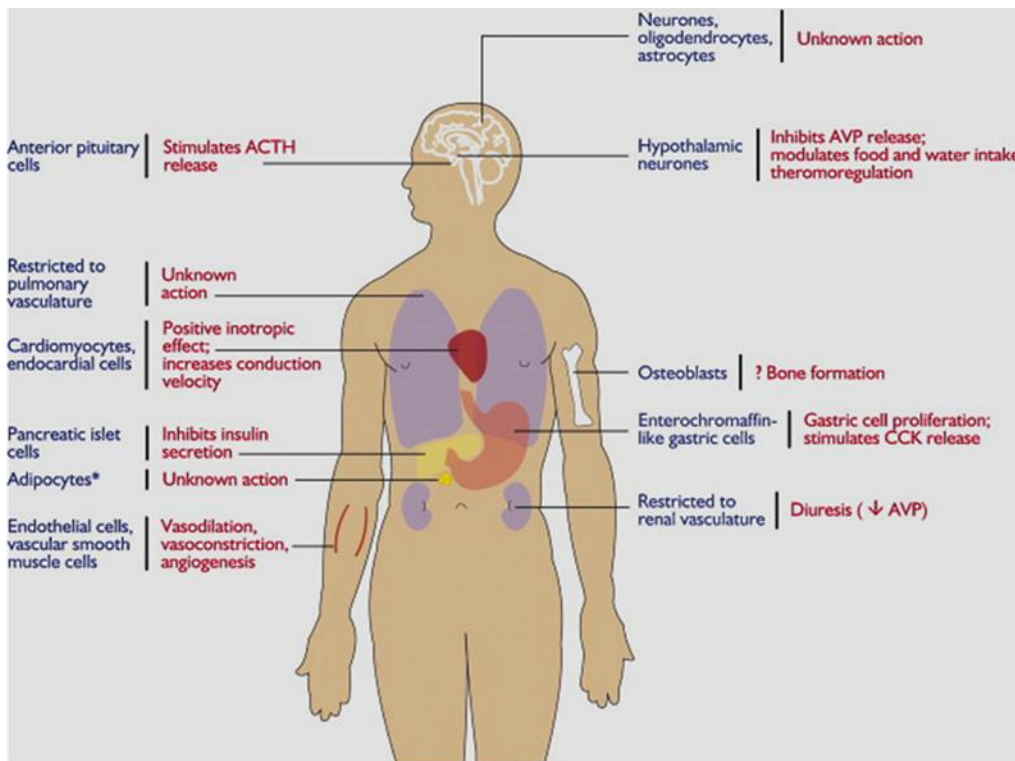


Figure 4. Expression and physiological functions of the Apelin/APJ system (most physiological effects not yet documented in humans). ACTH, adrenocorticotrophic hormone; AVP, arginine vasopressin; CCK, cholecystokinin.

Cardiac apelin is up-regulated by hypoxia (Ronkainen et al., 2007; Sheikh et al., 2008) and the expression of APJ is increased in ischemic HF in rats (Atluri et al., 2007). In a recent study, it was shown that mice lacking the gene encoding apelin develop impaired cardiac contractility in response to ageing or pressure overload (Kuba et al., 2007). In humans, apelin receptor APJ (APLNR) was found to be the most significantly up-regulated gene after mechanical offloading of failing myocardium (Chen et al., 2003) and the G212A variant of this gene is associated with slower heart failure progression (Sarzani et al., 2007). In summary, animal and human data suggest Apelin/APJ expression is up regulated in response to hypoxia and ischemia and increases in conditions of pressure overload and HF.

1.10. Brain Apelin/APJ System Expression and Cardiovascular Regulation

Immunohistochemistry and real-time polymerase chain reaction (PCR) studies showed that apelin and the APJ receptor are distributed predominantly in neurons of the hypothalamus and brainstem, including cardiovascular regulatory regions including the paraventricular nucleus (PVN), supraoptic nucleus (SON), circumventricular organs, nucleus tractus solitarius (NTS), and rostral ventrolateral medulla (RVLM) (Lee et al., 2000; Reaux et al., 2002; O'Carroll et al., 2000). Recent studies from this lab show that apelin levels are elevated in the RVLM of the spontaneously hypertensive rats as compared to normotensive rats and overexpression of apelin in the RVLM of the WKY rat brain resulted in an increase in elevated blood pressure, increased renal sympathetic nerve activity, and cardiac hypertrophy (Zhang et al., 2009).

In a recent study, APJ mRNA expression and apelin immunoreactivity was found in magnocellular neurons of the PVN and supraoptic nuclei (SON) and it is a well understood fact that these sites control the hypothalamic–neurohypophysial system (HNS), the pituitary gland

and the circumventricular organs (CVOs, structures involved in the control of drinking behavior; De Mota et al., 2000; Reaux et al., 2001; Brailoiu et al., 2002; O'Carrol et al., 2003).

Previous studies showed that expression of APJ to be differentially regulated in rats in response to fluid deprivation and increased dietary salt within brain areas such as the PVN and SON thus indicating a role for the apelinergic system in fluid homeostasis (O'Carrol et al., 2003). Moreover, in the neurohypophysial axis, the physiological effects of apelin look to be mediated by AVP but inconsistent effects have been reported. It is reported that repetitive i.c.v. injections of AVP resulted in increased apelin immunoreactivity in SON and PVN apelin and this effect was attenuated by i.c.v. pretreatment with the AVP V1 antagonist, indicating that like dehydration-induced effects, activity is mediated through AVP V1 receptors (De Mota et al., 2004). Apelin and APJ are linked to fluid regulation in particular for regulating the antidiuretic effect of AVP.

In a recent study conducted by Roberts et al. (2009), gene knockout mice lacking the APJ receptor showed abnormal fluid homeostasis and decreased urine output and vasopressinergic activity. C-fos activity measurement showed attenuation of expression within the subfornical organ and accentuated expression in the PVN indicating the importance of PVN in maintenance of the vasopressinergic activity. All these evidences demonstrate the importance of the Apelin/APJ system of the brain in maintaining cardiovascular homeostasis and vasopressinergic activity in the body.

1.11. Hypotheses of Current Study

Heart failure is a disease where the heart is unable to pump enough blood to meet the body's metabolic needs. HF constitutes major health problems in developed nations. Despite

considerable technical advancements, the effective treatment for HF has not been developed; hence, development of novel treatments for patients suffering from CHF remains a top priority.

The pathophysiology of HF is multifactorial in order to compensate for the inability of the heart to pump the blood and most importantly, this leads to increased sympathetic nervous system activity. This increased activity leads to the release of endogenous neurohormones, such as norepinephrine, resulting in increase in myocardial contractility, venous blood flow return, and retrieval of the reserved cardiac function. However, this adaptive response is short term and deleterious. Chronic elevation of SNS activity causes arrhythmias, activation of rennin angiotensin system, norepinephrine release, and increased peripheral resistance that enhances cardiac after load and preload. All of these factors cause alterations in myocardiocyte hypertrophy, apoptosis, fibrosis, and cardiac remodeling that then leads to further diminishing cardiac function and presenting symptoms of CHF. Thus, overexcited SNS plays an important role in HF pathogenesis. However, the central mechanisms that lead to increased sympathetic excitation during conditions of HF remain an enigma.

Recently, the Apelin/APJ system has been discovered in the brain cardiovascular regulatory regions, such as PVN, RVLM, and NTS. Studies from this laboratory demonstrate that over expression of apelin in the RVLM leads to elevated blood pressure, an increase in RSNA, and cardiac hypertrophy. Previous studies demonstrated that APJ is expressed in the magnocellular neurons in the PVN (De Mota et al., 2000; Reaux et al., 2001; Brailoiu et al., 2002; O'Carroll et al., 2003). Apelin stimulates this neuronal activity via stimulation of APJ receptor, regulates the antidiuretic activity of the AVP release (Lee, 2000; Reaux et al., 2002; O'Carroll et al., 2000).

All these evidences lead to the hypothesis that the Apelin/APJ system in the PVN contribute to the increased sympathetic nerve activity, abnormal vasopressinergic activity, and increase the pathophysiologies of CHF and the selective silencing of this system could attenuate the pathophysiological manifestations of CHF. Thus, current study was designed to test this hypothesis via the following series of specific aims:

Aim 1. We established congestive heart failure (CHF) in rats by ligation of the left coronary artery. Left ventricular dysfunction was confirmed via cardiac catheterization.

Aim 2. The expression of the Apelin/APJ system in the brain cardiovascular regulatory regions (including PVN, RVLM, NTS) were examined using real-time PCR, western blot analysis, and Immunohistochemistry.

Aim 3. We constructed a lentiviral vector containing APJ shRNA (Lv-APJ-shRNA) and examined the efficiency of this viral vector in *vitro* in cultured cells and *in vivo* rat brain expression.

Aim 4. The effect of selective silencing APJ in the PVN with Lv-APJ-shRNA was examined on the cardiac function and the pathophysiologies of CHF vs. control sham rats.

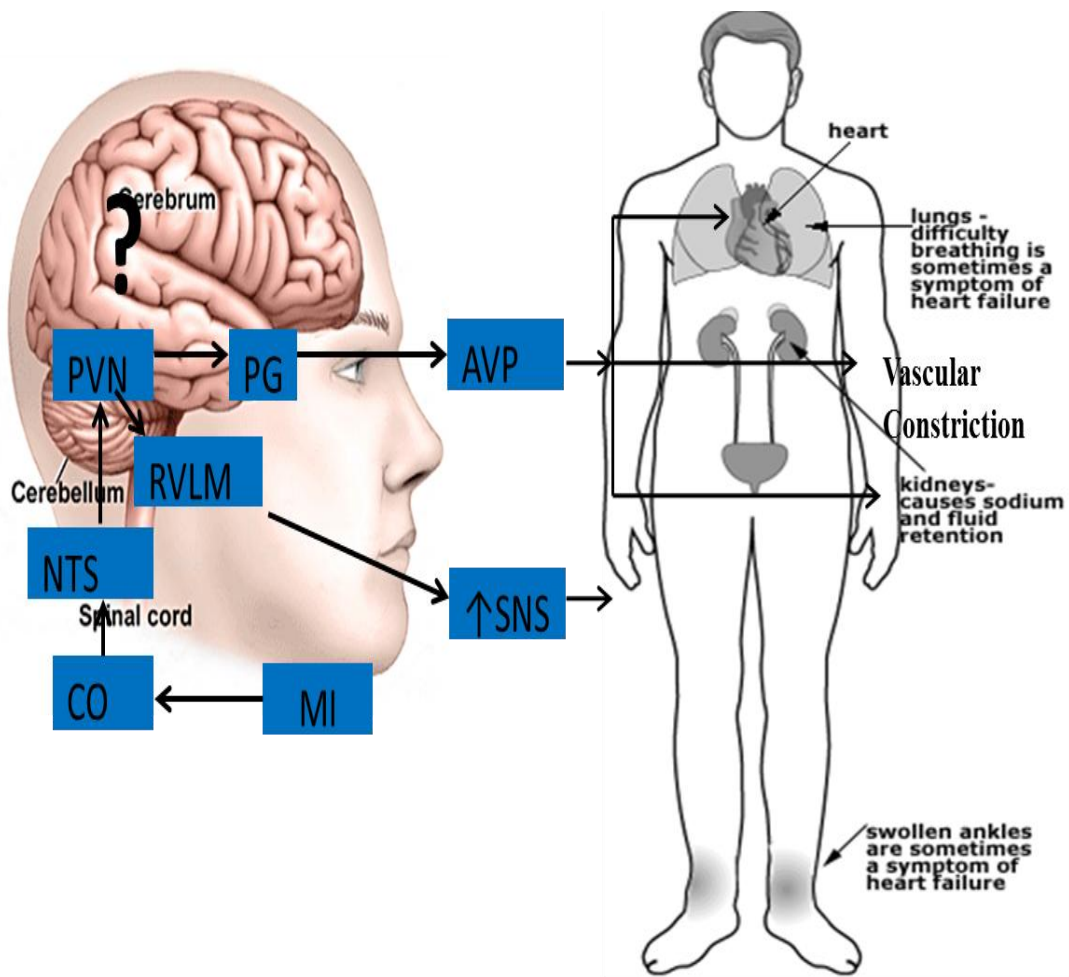


Figure 5. Apelin/APJ system possible mechanisms in pathophysiology of heart failure.

CHAPTER 2. ESTABLISHMENT OF CONGESTIVE HEART FAILURE

2.1. Introduction

Apelin/APJ is a novel neurohormonal system involved in cardiovascular homeostasis inducing both pressor and depressor responses (Lee et al., 2000; Cheng et al., 2003; Tatemoto et al., 1999; Lee et al., 2005). In a recent study, rats that were implanted with a left ventricular assist device showed both tissue and plasma up regulation of apelin and APJ levels in rats (Chen et al., 2003). Recent studies also show that rats subjected to HF by left anterior descending artery ligation presented elevated levels of APJ receptors (Atluri et al., 2007). Genetic studies show that the single nucleotide polymorphism G212A of the APJ receptor is associated with slower HF progression (Sarzani et al., 2007). Studies conducted in this laboratory show that over expression of the Apelin gene leads to increased sympathetic nerve activity and cardiac hypertrophy in normotensive rats (Zhang et al., 2009). In another study, it was shown that APJ knockout mice demonstrated abnormal fluid homeostasis and vasopressinergic activity. Thus, the emerging evidence indicates that the Apelin/APJ system may play significant roles in cardiovascular regulation and cardiovascular disease pathogenesis. However, the involvement of this neurohormonal system in neural control of cardiovascular function and HF remains poorly understood. In the present study, this research aims to create a MI rat model in order to understand the role of Apelin/APJ system in pathophysiology of HF.

Myocardial infarction rat models recently have become useful and popular for investigators because it closely represents many aspects of ischemic cardiomyopathy and HF in humans (Loreto et al., 2009). Myocardial infarction by coronary artery occlusion in small animals was invented in 1954 (Johns et al., 1954). Later methods to study infarct size and

ventricular function in rats after coronary artery ligation was developed in the 1970s and early 1980s (Pfeffer et al., 1979; Fishbein et al., 1978). The rat coronary artery ligation model generated much eagerness because the model has many pathophysiologic and clinical characteristics similar to the clinical syndrome of HF after MI in humans. Conceivably, the most clinically relevant observation is that the progression to HF in these rats is similar to what happens when a patient sustains a large MI, survives, but goes on to develop HF without another cardiac insult. In addition to being a good approximation of human disease and relatively low cost to establish an MI rat, this makes this attractive model (Goldman et al., 1995).

There are different methods available for establishing MI in rats but the most used is to ligate the left coronary artery of rats. The MI rat model created using left coronary artery ligation is very useful to study structural and functional changes in rats such as ventricular remodeling, hypertrophies, left ventricular dysfunction, and changes in peripheral circulation (Pfeffer et al., 1991; Fishbein et al., 1978).

The rat coronary ligation model has been used in studying neurohormonal changes and cardiovascular functions where studies showed coronary artery ligation leads to increased SNS activity and activation of brain renin angiotensin and aldosterone systems (Michel et al., 1988). Recently, the MI rat model has also been used to study the intercellular Ca^{2+} pumps activity (Litwin et al., 1992) and ACE 2 expression after MI (Burrell et al., 2005). All these evidences indicate that that MI rat model created by left coronary artery ligation is an ideal model for studying the role of Apelin/APJ system in HF.

2.2. Materials and Methods

2.2.1. Surgery

Adult male sprague-dawley (SD) rats (Charles River Laboratories, Wilmington, MA), weighing 220–280 g, were used in this study. All animals were housed under controlled conditions with a 12-h light/dark cycle. Food and water were available to the animals *ad libitum*. All protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee.

CHF rats were produced by ligation of cardiac coronary artery at the beginning part of the left front ascending branch via a survival surgery. Under isoflurane inhalation, rats were anesthetized with a mixture of oxygen (1 L/min) and isoflurane (3%). After an adequate depth of anesthesia was attained, the rat was fixed in a supine position with tape. A 5-0 ligature was placed behind the front upper incisors and pulled tightly so that the neck was slightly extended. The tongue was retracted and held with forceps and a catheter was inserted into the trachea. The catheter was then attached to a pressure-controlled ventilator (Topo, Kent Scientific Corporation, Torrington, CT) via the Y-shaped connector. Ventilation was performed with a tidal volume of 400 μ l and a respiratory rate of 133/min. 100% oxygen was provided to the inflow of the ventilator. Prior to the incision, the chest was disinfected with betadine solution and 70% ethyl alcohol. The chest cavity was opened by an incision of the left fourth intercostal space. Chest retractor was applied to facilitate the view. The heart was exposed and the pericardial sac was opened and pulled apart allowing the left anterior descending (LAD) artery to be visualized. Ligation was proceeded with a 6-0 polypropylene suture passed with a papered needle underneath the LAD artery about 2-3 mm from its origin. Acute MI was considered successful

based on blanching of the myocardial surface distal to the suture and by changes in cardiac contractility and rhythm. A drop of 1% lidocaine was placed on the apex of the heart to prevent arrhythmia. Lungs were overinflated, and the chest cavity, muscles, and skin were closed layer by layer with 6-0 nylon. The duration of the whole procedure lasted about 10-15 minutes. Control (sham) rats underwent the same surgical procedure without coronary artery ligation. MI and Sham rats were placed in separate cages and fed normal rat chow and water and allowed to recover post-surgery for 3 weeks.

2.2.2. Measurement of Left Ventricular Hemodynamics

Left ventricle (LV) dysfunction and increased heart to body weight ratio are the hallmarks of a failing heart. We measured LV function using various parameters including LV end diastolic pressure, LV systolic pressure, and the contractility of the heart by measuring LV dp/dt_{max} . Briefly, three weeks after the Coronary artery ligations, rats were anesthetized with a mixture of 2% isoflurane and 98% oxygen, and the right carotid artery was cannulated with a catheter-tip pressure manometer (SPR-320, Millar instruments, Houston, TX) to record arterial pressure and heart rate. After that, the micromanometer was advanced into the LV for measuring ventricular function. The output signal from the Millar catheter was collected and analyzed with a Power Lab data-acquisition system (AD instrument, Colorado Spring, CO).

2.2.3. Assessment of Myocardial Infarction Size

Infarction and death of myocardial tissue in the heart due to occlusion of blood vessels and lack of oxygen supply is characteristic of MI. We measured MI size of the heart in treated and sham rats. Three weeks after left coronary artery ligation, the animals were anesthetized by injection of sodium pentobarbital and then euthanized. Hearts were removed. After the heart

weight and cardiac morphology were examined, hearts were fixed in 10% formalin/PBS and embedded in paraffin. Heart sections (10 μ m) were cut using a cryomicrostat and placed on a slide. Paraffin sections were deparaffinized and rehydrated by incubating three times for three minutes in xylene, three times for three minutes in 100% ethanol, three times for three minutes in 95% ethanol, and one time for three minutes in 80% ethanol, and the deionized water once for five minutes. Rehydrated tissues were stained by incubating with hematoxylin for three minutes, excess and unbound stain was removed by tap water. Slides were washed by dipping in 1% acid-ethanol solution, then washed with tap water and deionized water. Excess water was removed by blot paper and slides air-dried. Air dried slides were incubated in eosin stain for 45 seconds and washed for three times for five minutes in 95% ethanol, three times for five minutes and three times for 15 minutes in xylene. Slides were covered with a cover slip using a mounting medium and morphology and cellular dimensions were evaluated using a microscope equipped with a digital camera and a computer image recording and analysis system.

2.3. Results and Data Analysis

Since the HF rat model induced by left coronary artery ligation is a popular model for these types of studies, this team first characterized the MI rat model by ligation of the left coronary artery ligation as demonstrated in figure 6. Mean arterial pressure (MAP) and heart rate (HR) were measured in sham and MI rats using femoral arterial cannulation in figure 6. Heart rate was significantly increased in MI rats ($n=8$, 370.6 ± 18.5 bpm, $p<0.05$) as compared to sham rats ($n=5$, 341.7 ± 9.7 bpm). The mean arterial blood pressure (MAP) was slightly reduced in MI rats (84.8 ± 4.2 mmHg, $n=8$, $P>0.05$) as compared to sham rats (92.6 ± 3.9 mmHg, $n=5$). These results indicate that the HR was markedly increased in MI rats. This is a symptom mainly caused

by sympathetic nerve activation and other hormonal systems activation in the development of pathophysiological symptoms of the failing heart.

Increased heart/body weight ratio is one of the main symptoms of HF and is a sign of cardiac remodeling. Thus, we examined the heart/body weight ratio (HW/BW) in sham and MI rats. The results illustrated in figure 7 indicate that MI rats show a significant increase in HW/BW as compared to sham rats indicating chronic coronary arterial ligation significantly increases the heart weight, suggesting a significant cardiac hypertrophy and remodeling. This hypertrophy and remodeling could worsen the impaired cardiac contractility and oxygen consumption efficacy in the failing heart. HW/BW in sham and MI rats were 2.88 ± 0.12 mg/g (n=5) and 3.58 ± 0.07 mg/g (n=8) respectively ($P < 0.01$).

Left ventricle dysfunction is the major characteristic for a failing heart and can be measured using a cardiac catheter inserted through the left carotid artery. In the current study, left ventricular hemodynamic were measured using a cardiac catheter inserted through the right carotid artery. Left ventricular systolic pressure (LVSP) in myocardial infarction rats was significantly reduced in MI (94.9 ± 4.2 mmHg) as compared to sham rats (117.3 ± 5.7 mmHg) shown in figure 8, indicating decreased left ventricular functions in rats subjected to left coronary artery ligation.

Increased LVEDP is a progenitor for failing hearts and indicator for severe impairment of left ventricle function. Thus, this research examined LVEDP in sham and MI rats and the results are presented in figure 9. LVEDP was increased in MI (19.08 ± 1.96 mmHg, n=8, $P < 0.01$) as compared to sham rats (4.67 ± 1.22 mmHg, n=5). This result suggests that the cardiac output is

dramatically reduced, leading to the blood accumulation in the left ventricle at the end of contraction in the MI rats, a remarkable sign of HF.

The maximum and minimum rates of pressure rise (dp/dt max and dp/dt min) are pressure-independent measurements of cardiac contractility. Thus, this research also detected left ventricular (Lv) dp/dt max and dp/dt min in MI and sham rats by using a Millar catheter-tip pressure manometer through the right carotid artery. The results are presented in figure 10, indicating that Lv dp/dt max was significantly reduced in MI (4345.5 ± 170.0 mmHg/s, n=8, $p < 0.01$) as compared to sham rats (7635.6 ± 234.7 mmHg/s, n=5). In addition, the Lv dp/dp min was significantly increased in MI (-3752.5 ± 186.9 mmHg/s, n=8, $P < 0.01$) as compared to sham rats (7029.3 ± 243.3 mmHg/s, n=5). All the results demonstrate that the cardiac contractility is dramatically damaged in the MI as compared sham rats.

Additionally, death of tissue due to occlusion of blood supply and lack of oxygen is a major histopathological symptom associated with MI-induced HF. In current study, hearts were isolated from rats subjected to left coronary artery ligation or sham operation. The hearts were fixed and stained with eosin/haematoxylin. The infarct size was evaluated as described in the previous publications (Zhang et al., 2009). Results are shown in figure 11, indicating that left coronary artery ligation results in $36 \pm 2\%$ (n=8) of left ventricle tissue infarction, suggesting that left coronary artery ligation leads to significant MI in the hearts.

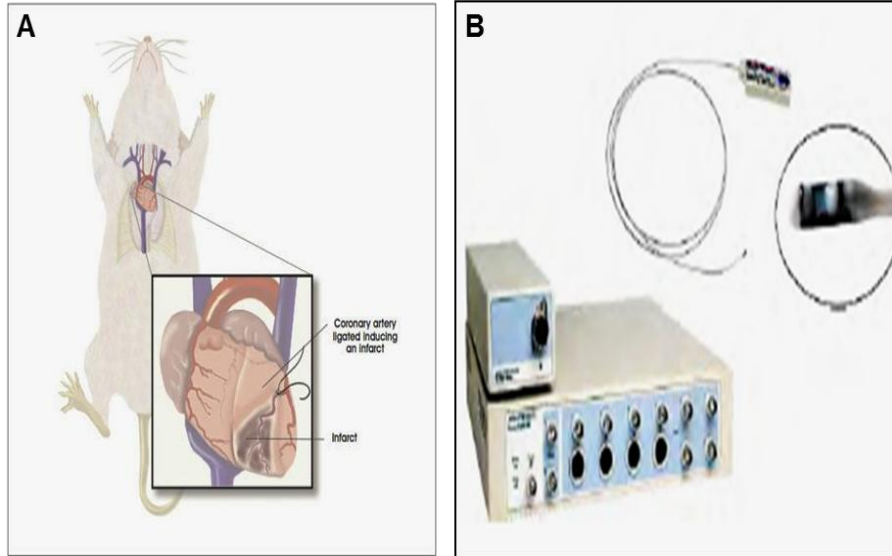


Figure 6. Myocardial infarction rat model and measurement of cardiac left ventricular function. Myocardial infarction rat model was created by ligation of the left coronary artery in anesthetic condition as shown in panel (A). Left ventricle hemodynamics were measured using a cardiac catheter inserted through the left carotid artery as shown in panel B.

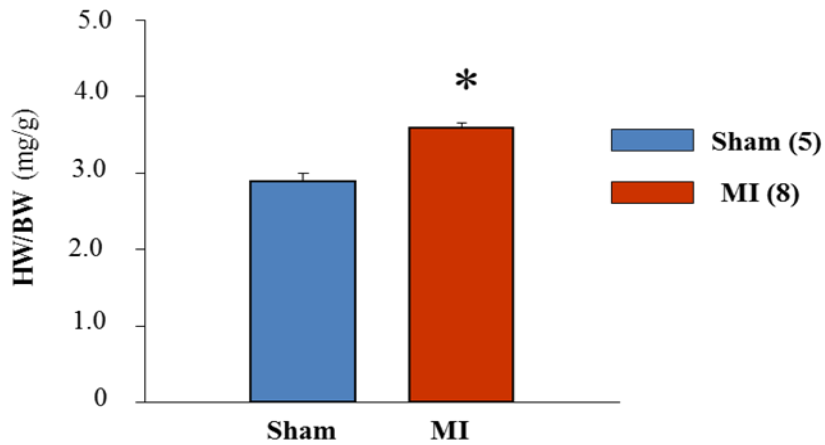


Figure 7. Heart weight to body weight ratio in sham and myocardial infarction rats. Heart weight to body weight ratio (HW/BW) were measured three weeks after ligation of left coronary artery or sham operation in rats. The number of rats in each group is indicated in parentheses. Data are mean \pm SE (n=5-8 rats). *P<0.05 compared with sham rats.

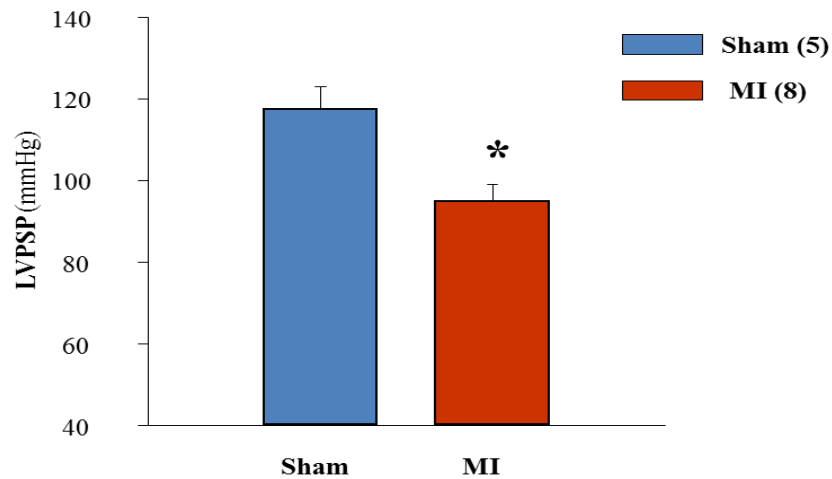


Figure 8. Left ventricular peak systolic pressure in sham and myocardial infarction rats. Left ventricular systolic pressure (LVPSP) in this experiment was measured three weeks after ligation of left coronary artery or sham operation in rats. The number of rats in each group is indicated in parentheses. Data are mean \pm SE (n = 5-8 rats). *P<0.05 compared with sham rats.

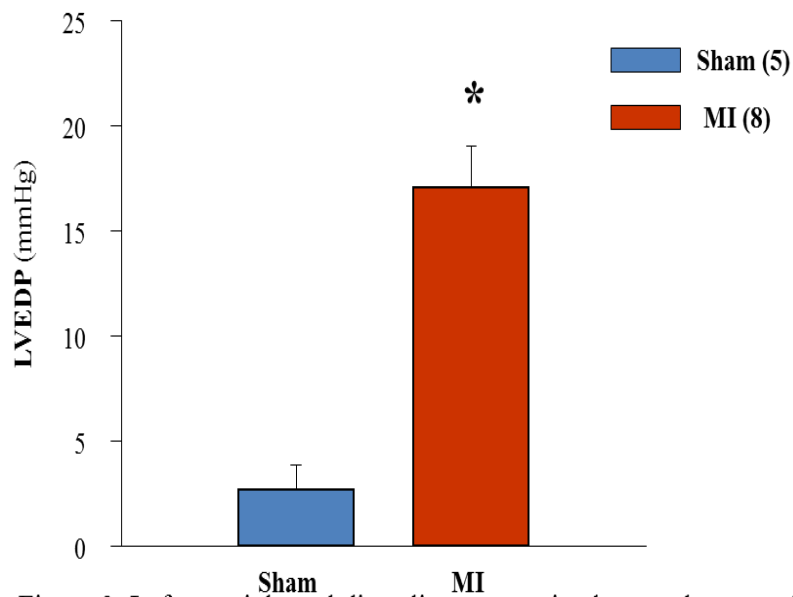


Figure 9. Left ventricle end diastolic pressure in sham and myocardial infarction rats. The left ventricular end diastolic pressure (LVEDP) was measured three weeks after ligation of left coronary artery or sham operation in rats using micromanometer blood pressure transducer. Number of rats in each group is indicated in the parentheses. Data are mean \pm SE (n=5-8 rats). *P<0.05 as compared with sham rats.

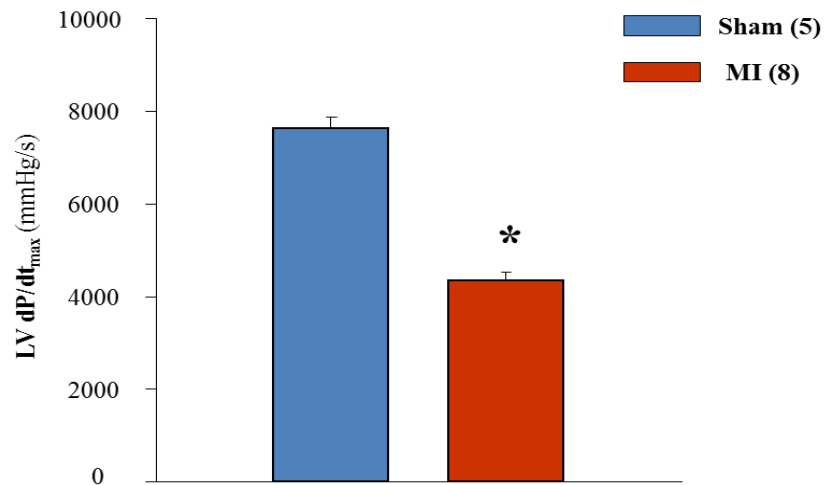


Figure 10. Left ventricle dp/dt_{max} in sham and myocardial infarction rats. Left ventricle de/dt_{max} (LV dp/dt_{max}) was measured three weeks after ligation of left coronary artery or sham operation in rats using micromanometer blood pressure transducer. Number of rats is indicated in parentheses. Data are mean \pm SE (n=5-8 rats). * $P < 0.05$ as compared with sham rats.

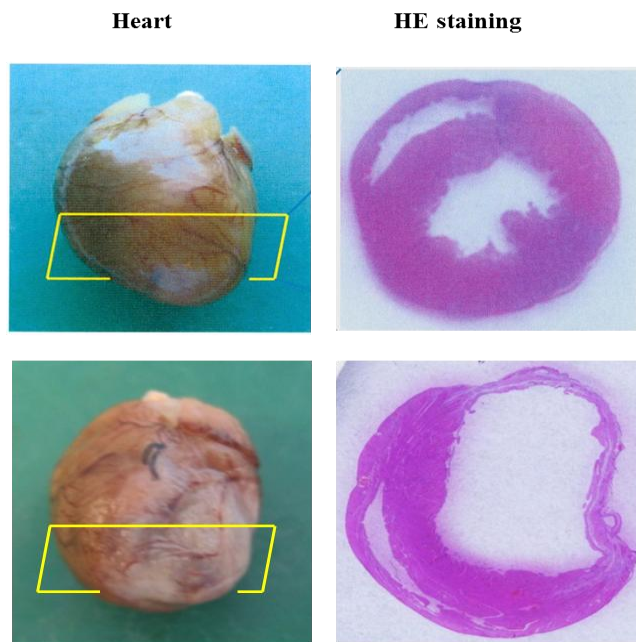


Figure 11. Eosin/ Heamtoxylin (HE) and Sirius Red staining of Heart sections derived from Sham and Myocardial Infarction rats. The heart sections were examined by HE or Sirius red staining three weeks after ligation of left coronary artery (MI) or sham operated rats. The histological staining indicate that ligation of left coronary artery induces significant left ventricle myocardial infarction and the normal myocardial tissue were replaced by fibers (fibrosis). Average MI size in eight rats are $36 \pm 2\%$ of LV.

2.4. Data Summary and Conclusion

Apelin/APJ system is a novel GPCR, its ligand was discovered recently, and the peripheral role of this system was well established. Recent *in vivo* animal studies demonstrate that apelin levels are elevated in rats with MI and ischemic injury (Atluri et al., 2007). However, the central role of the Apelin/APJ system remains a mystery. In a recent study from our laboratory, it was shown that over expression of apelin in the RVLM significantly elevated blood pressure and induced cardiac hypertrophy. Microinjection of apelin into the RVLM or PVN resulted in a significant increase in renal sympathetic nerve activity. These results led researchers to hypothesize hyperactive apelinergic system contribute to the sympathetic nerve activation and the pathogenesis of HF. Therefore, to elucidate the role of the Apelin/APJ system in the pathophysiology of HF, we established a MI rat model by ligation of left coronary artery. Left ventricular functions of MI rats were compared with sham rats, after three weeks of left coronary arterial ligation. MI rats demonstrated the following pathophysiologies.

- 1) The infarction size was directly confirmed by histological staining study, indicating that more than 30% of left ventricle wall was damaged and replaced by fibrous tissue.
- 2) HW/BW was significantly enhanced. This result suggested a cardiac hypertrophy and cardiac remodeling, which could impair oxygen consumption efficacy and as such, it contributed to the development of HF.
- 3) Heart rate was dramatically increased. This alteration could be induced by sympathetic nerve activation and other hormonal system stimulation. Sympathetic nerve system activation plays a very important role in the development of CHF including stimulation of renin-angiotensin-aldosterone system, cardiac remodeling, tachycardia, and peripheral arterial constriction.

- 4) Left cardiac contractility was significantly damaged. This was demonstrated by reduced left ventricular systolic pressure, elevated end-diastolic pressure, and reduced dp/dt max.

All these evidences suggest that the myocardial infarction rat model was successfully established by left coronary artery ligation.

CHAPTER 3. BRAIN APELIN/APJ EXPRESSION IN MYOCARDIAL INFARCTION RATS

3.1. Introduction

Heart failure is a disease condition where the heart is unable to pump enough blood to meet the body's metabolic needs (Jessup et al., 2003; Clyde et al., 1988). HF is often associated with employment of compensatory mechanisms to increase the ability of the heart to pump the blood (Bonow et al., 2011). Increased SNS activity is one such complementary mechanism that leads to increased heart rate in the body (Dzau et al., 1981), but long-term dependence this system is harmful to the body because it leads to catecholamine toxicity and phenotypic changes in heart (Todd et al., 1985). Apelin/APJ system is a recently discovered GPCR (along with its ligand) (O'Dowd et al., 1993) is expressed in the central and peripheral nervous systems (Klienz et al., 2005; Lee et al., 2006). Accumulated evidence demonstrates that the Apelin/APJ system plays a vital role in many functions of the body including Arginine vasopressin (AVP) release, body fluid homeostasis, blood pressure regulation, cardiac contractility, and may also contribute to the pathogenesis of several diseases such as obesity and diabetes (Yue et al., 2010; Reaux et al., 2001; Boucher et al., 2005).

Recent studies from our lab demonstrated that over expression of apelin in the RVLM of the brain increases plasma NE levels and induces cardiac hypertrophy and interstitial fibrosis. More interestingly, microinjections of apelin directly into the RVLM or PVN areas result in a significant increase in renal sympathetic nerve activity. Thus, we researchers hypothesize that elevated levels or activity of the Apelin/APJ system in the PVN contributes to the elevation in sympathetic outflow, AVP and NE release, leading to sodium and fluid retention, cardiac hypertrophy, cardiac fibrosis and remodeling, and cardiac dysfunction. In the previous chapter,

this work characterized the MI rat model induced ligating left coronary artery. In this MI rat model, left ventricular function is significantly reduced and associated with cardiac hypertrophy, fibrosis, remodeling, and increased HR. The aim of the current study was to investigate APJ receptor expression in the PVN and other cardiovascular regulatory areas of MI and sham rats.

3.2. Materials and Methods

3.2.1. MI rat model

Adult male sprague-dawley (SD) rats (Charles River Laboratories Wilmington, MA) were used in this study. All animals were housed under controlled conditions with a 12-h light/dark cycle. Food and water were available to the animals *ad libitum*. All protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee. The MI rat model was created as described in the previous chapter. Left ventricular dysfunction was confirmed by measuring left ventricular hemodynamic as described in the previous chapter.

3.2.2. RNA Isolation and Purification

Both sham (n=6) and MI rats (n=6) after four weeks of surgery were euthanized using i.p. injection of 3% pentobarbital (50 mg/kg). The rat head was separated from the body using a guillotine. The brain was dissected and carefully from the skull. Isolated rat brain was placed on a frozen ice pad and cut into 1 mm sections using brain matrices (Kent Scientific Corporation, Torrington, CT). The PVN, RVLM, and NTS were micro punched from the brain with Harris Micro-Punches. Total RNA was isolated using the Qiagen RNA lipid kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Briefly, isolated brain tissues were homogenized in 500 μ l of the QIAzol lysis buffer using an ultra turex homogenizer (IKA Works, Wilmington, NC) for one minute and incubated the tube containing homogenate for 5 min at room

temperature. Chloroform (200 μ l) was added to the homogenate and incubated for three minutes at room temperature. Homogenate was then centrifuged for 15 min at 12,000Xg at 4°C. Top aqueous layer was transferred using a micropipetter into a new tube. To the aqueous layer add one volume of 70% alcohol was added and mixed well and transferred into RNAeasy column provided by the manufacturer and centrifuged for 15s at 8,000 X g and flow through was discarded. 700 μ l of buffer RW1 were added to the column, which was centrifuged for 15s at 8,000 X g. The flow through was discarded. 500 μ l of buffer RPE was added to the column and centrifuged for 15 sec and another two minutes, respectively. Residual amount of buffer RPE was removed from the column by spinning the column at full speed for one minute. RNA was eluted by adding 30 μ l of RNase free water to the center of the column and spinning at 8,000 X g for one minute.

After extraction, RNA was purified using Ambion turbo DNA free kit (Life Technologies, Grand Island, NY). Briefly, 1 μ g of extracted RNA is incubated with DNase enzyme and buffer for 30 minutes. After incubation, DNase inactivation reagent was added; and sample was centrifuged at 1000 g for 90s. The upper aqueous layer was isolated using a micropipette. Total concentration of RNA was determined by measuring absorbance at 260 nm in a spectrophotometer (Molecular devices, Sunnyvale, CA).

3.2.3. Reverse Transcription

Reverse transcription is a process where complimentary DNA is synthesized using a single stranded RNA as a template. The reaction is carried out in presence of an enzyme known as reverse transcriptase, primers, and dNTPs. A total of 1 μ g of RNA was converted into cDNA

by using Promega reverse transcription system per manufacturer's guidelines (Promega, Madison, WI). For negative controls, RNA without the Reverse Transcriptase enzyme was used.

3.2.4. Real -Time PCR

To determine whether the expression of APJ receptor mRNA levels in PVN is altered in the MI (the MI animal model has been characterized in the last chapter) as compared with sham rats, real-time (RT-PCR) was employed in this current study. RT-PCR allows detection of amplification during early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Theoretically, there is a quantitative relationship between amount of starting target sample and amount of PCR product at any given cycle number. RT-PCR detects the accumulation of amplicons during the reaction. The data is then measured at the exponential phase of the PCR reaction. Traditional PCR methods use agarose gels or other post PCR detection methods, which are not as precise for the detection of PCR amplification product at the final phase or at the end-point of the PCR reaction. The PCR reaction comprises cDNA, master mix, and gene specific fluorescent probes, which emit fluorescence upon amplification during each cycle. This fluorescence is captured by the detection system of the machine and reported as Ct values (Medrano et al., 2005; Bustin et al., 2002). Gene expression is calculated by using $2^{-\Delta\Delta Ct}$ method as explained previously (Livak et al., 2001; Livak et al., 2008). Isolated cDNA was subjected to a two-step RT-PCR (including reverse transcription and PCR) on an ABI 7500 Sequence Detection system (Applied Biosystems Inc, Foster City, CA) for 40 cycles (50°C, 2 min; 95°C, 10 min; 94°C 15 sec; 60°C, 36 sec) using Taqman probe-based assay (Applied Biosystems Inc, Foster City, CA) according to the protocol provided by the manufacturer. Briefly, 5 μ l of cDNA was added into each well in a 96 well PCR plate in triplicate. Then 10 μ l of standard master mix was added and either 1 μ l of APJ Probe

(Rn00580252_s1) or 1 µl of GAPDH probe (Rn00576699_m1) as an internal control. The total reaction volume was made up to 20 µl by adding water. Ct values were obtained by the analysis software after the reaction in ABI 7500 real time PCR machine. The relative changes in gene expression were calculated using $2^{-\Delta\Delta ct}$ method.

3.2.5. Western Blot Analysis

To determine whether the expression of APJ receptor protein levels in PVN is altered in the MI as compared with sham rats, western blot analysis was performed. Both Sham (n=3) and MI rats (n=3) were euthanized by intraperitoneal (i.p) injection of 3% pentobarbital (50 mg/kg). The rat head was separated from the body using a guillotine and brain was dissected from skull and placed on a frozen ice pad. The PVN was isolated from brain sections using micro punching tools and cortex tissue was isolated for use as a negative control (less effective brain area in the control of blood pressure). Isolated brain tissue was homogenized in a 2 x lysis buffer. Total protein concentration was determined by using a modified lowry method (Lowry et al., 1951; assay kit was obtained from Bio-Rad (Bio-Rad Hercules, CA). Protein samples were electrophoresed using a SDS-PAGE and proteins were transferred electrophoretically for 2 hours at 350 mV at room temperature onto a nitrocellulose membrane. Membranes were blocked at room temperature for 1 hr in a 5% solution of non-fat dry milk with 0.1% tween-20. Subsequently, the blots were incubated with a rabbit anti-APJ primary antibody (Santa Cruz Biotech, Santa Cruz, CA) in 2% milk overnight at 4°C. Following the incubation, the blot were washed three times in 0.5% milk plus tris buffer saline and tween(TBST) at intervals of 15 minutes then blots were incubated with an goat anti-rabbit peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA) for 1 hour at room temperature. The primary antibody used in this study was raised against amino acids 1-300 at the N-terminus of the APJ protein.

Immunoreactivity was detected by enhanced chemiluminescence autoradiography Pierce ECL western blotting detection kit (Thermo Fisher, Rockford, IL). Protein band density was analyzed using Quantity One Software (Bio-Rad, Hercules, CA).

3.2.6. Immunohistochemistry

To determine whether the APJ receptor is located on the neurons within the PVN, immunohistochemistry was performed on the PVN containing brain sections. Rats were perfused transcardially with 50 ml of saline followed by 50 ml of 4% paraformaldehyde solution for 1 h. The brain was removed, fixed in 4% paraformaldehyde solution for 24 h, and transferred to PBS containing 20% sucrose. Frozen brain tissues were embedded and the tissue in OCT medium(Sakura Finetek, Torrence, CA) and cut into 10 micron coronal sections using Leica CM1950 cryostat (Leica Microsystems, Buffalo Grove, IL). The PVN sections, identified with a rat brain atlas, were incubated with phosphate buffered saline(PBS) plus 0.5% tween 20 containing 5% goat serum for 60 min at room temperature to quench the non-specific binding. Slices were incubated with primary antibodies (mouse anti-Neun monoclonal antibody (Millipore, Billerica, MA) 1:500; rabbit APJ polyclonal antibody (Santa Cruz Biotech) 1:500 overnight at 4°C. After being washed with Phosphate buffered saline with tween(PBST), the sections were incubated with secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Grand Island, NY), 1:1000; Alexa Fluor 594 goat anti-mouse IgG (Life Technologies), 1:1000] for 2 hours at room temperature. The sections were washed with PBST and staining was detected with an Olympus FluoView FV300 fluorescent microscope (Olympus, Melville, NY) connected to a computer to capture and analyze images with FlouView Software(Olympus, Melville, NY)

3.3. Results and Data Analysis

3.3.1. APJ mRNA Levels in the RVLM, PVN, and NTS of MI and Sham Rats

APJ mRNA levels were determined from micro punches of brain cardiovascular regulatory area including RVLM, PVN, and NTS in Sham and MI rats using RT-PCR. Data is presented in figure 12 that demonstrates the APJ receptor is expressed in PVN, RVLM, and NTS of MI and sham rats. APJ mRNA levels are enhanced in the PVN, RVLM, and NTS of MI rats by 130% ($P<0.01$), 60% ($P<0.05$), and 30% ($P>0.05$) respectively, as compared with sham rats ($n=4$ in each group). Taken together, these observations demonstrate that APJ receptor expression is enhanced in the PVN and RVLM brain areas of MI rats. MI-induced enhancement in APJ receptor expression is much stronger in the PVN as compared with the RVLM. Thus, the following experiment will focus on the brain PVN area.

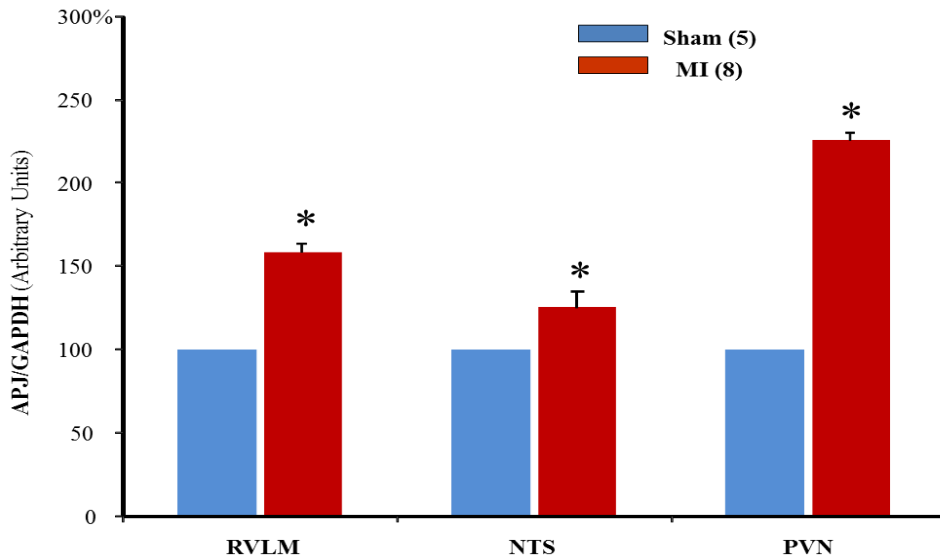


Figure 12. APJ mRNA expression in the brain cardiovascular regulatory areas of rats after myocardial infarction. Messenger RNA levels or APJ receptor in cardiovascular regulatory regions of brain using real-time RT-PCR in the rats three weeks after ligation of left coronary artery. The APJ receptor mRNA levels were normalized to internal control GAPDH. PVN, paraventricular nucleus; NTS, nucleus tractus solitari; RVLM, rostral ventrolateral medulla. The number of rats in each group is indicated in parentheses. Data are mean \pm SE ($n=5-8$ rats). * $P<0.05$ as compared with sham control.

3.3.2. APJ Protein Levels in the PVN of MI and Sham Rats

In previous experiment we measured APJ mRNA levels in various cardiovascular regulatory regions of the brain and found that APJ receptor mRNA levels were significantly increased in MI compared with sham rats. Next western blot analysis was performed to determine whether there was a change in APJ receptor protein levels in the PVN of MI compared sham rats .

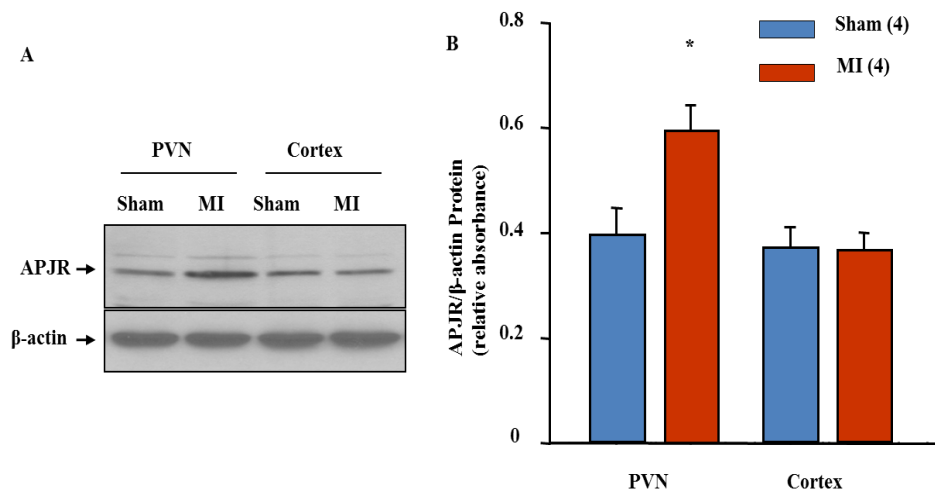


Figure 13. APJ Protein levels in the PVN of MI and sham rats. APJ receptor protein levels in the paraventricular nucleus (PVN) and cortex of rats subjects to either coronary artery ligation (MI) or sham operation (Sham) were examined using Western Bolt analysis. In this experiment, the less cardiovascular regulation-related brain area, cortex, was used as negative control. A, Representative autoradiogram of APJ protein levels in the PVN and Cortex. APJ protein levels were measured using Western analysis in 20 μg of total cell lysate isolated from PVN micropunches of Sham and MI rats. Data were normalized using β -actin. B, Bar graphs summarizing the quantitation of the APJ protein band density. Data are means \pm SE of APJ versus β -actin protein in the PVN, RVLM, or NTS in the rats three weeks after ligation of left coronary artery. Number of rats in each group is indicated in the parentheses. * $P < 0.05$ as compared with sham control rats.

The APJ receptor protein levels in the PVN were examined using regular western blot analysis in the rats four weeks after ligation of the left coronary artery. The brain area with less responsibility for blood pressure control, the cortex, was used as negative control. The results show that APJ protein levels were significantly increased in the PVN of MI as compared with

sham rats figure 13. However, APJ protein levels in the cortex were comparable in the PVN of MI and sham rats. These results confirmed the RT-PCR results, indicating APJ receptor expression (in both mRNA and protein levels) was enhanced in MI as compared with sham control rats.

3.3.3. Localization of APJ Receptor

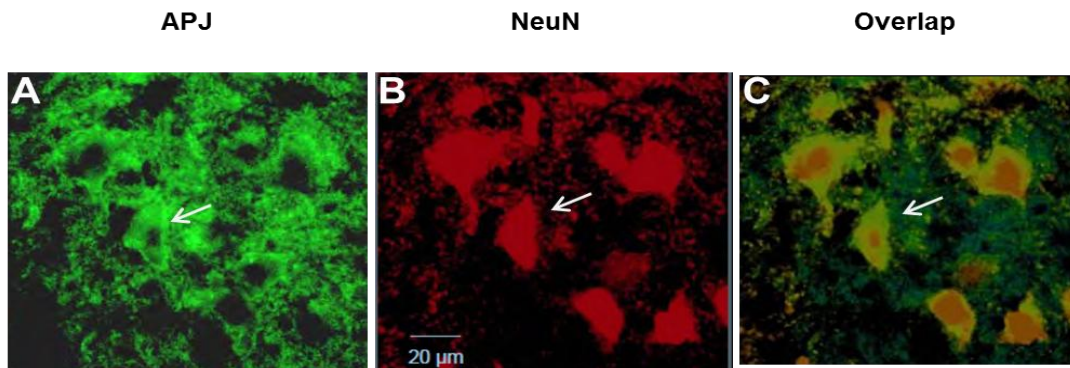


Figure 14. Immunohistochemistry of APJ receptor in the PVN. A Immunofluorescence images showing APJ protein in the PVN of rats. Fluorescence micrographs (x40 magnification) demonstrating neurons within the PVN stained with specific a specific anti-APJ receptor antibody (A, green), a anti-NeuN antibody (B, red), and overlap of A and B (C), indicating that APJ receptor is localized on the cytoplasmic membrane of neurons within the PVN (as indicated by the narrow in the images).

In the previous experiments, data demonstrated that mRNA and protein levels were elevated in the PVN of the brain of the rats that underwent left coronary artery ligation as compared to sham rats. Immunohistochemistry analysis was used to determine whether the APJ receptor was localized on the neurons within the PVN. The double immunostaining technique was used to detect APJ receptor localization in the neurons of the PVN with APJ receptor specific antibody and Anti-NeuN antibody as a neuronal marker. The results shown in figure 14 shows localization of APJ receptor in neurons of PVN (panel A), Panel B of figure 14 demonstrates immunoreactivity of the neurons to Anti-Nue N antibody and Panel C of figure

shows merged image of panel A and panel B which clearly demonstrate that neurons in the PVN show APJ receptor-like immunoreactivity and APJ receptors are localized on the cytoplasmic membrane of neurons within the PVN.

3.4. Data Summary and Conclusion

APJ receptor is a GPCR and its ligand, apelin was discovered recently. The physiological role of the Apelin/APJ system is well established in the peripheral system (Lee et al., 2000; Cheng et al., 2003; Tatemoto et al., 1999; Lee et al., 2005). Recent studies demonstrated that the Apelin/APJ system regulate peripheral vascular tone and has inotropic effects on cardiac contractility (Farkasfalvi et al., 2007; Szokodi et al., 2002). However, the central role of the brain Apelin/APJ system remains a mystery. Studied from our lab show that that central injection of Apelin-13 elevates blood pressure, heart rate, and sympathetic nerve activity and that overexpression of apelin in the brain cardiovascular regulatory region induces a long-term elevation of blood pressure, cardiac hypertrophy, and cardiac fibrosis (Zhang et al., 2009). On the other hand, enhanced sympathetic nerve activity has been observed in CHF patients and animal models (Chen et al., 2003; Atluri et al., 2007). Increased sympathetic nerve activation plays a critical role in the pathogenesis of CHF (Triposkiadis et al., 2009). Thus, in the present experiment, this team examined the expression of Apelin/APJ in different cardiovascular regions of the brain, after MI, which controls sympathetic nerve activity or vasopressinergic activity in the brain. The results are summarized in the following:

- 1) APJ receptor gene expression was examined using RT-PCR in the PVN, RVLM, and NTS brain areas in MI and sham rats. The results indicated that APJ receptor mRNA levels were enhanced in the RVLM and PVN in the MI compared with sham rats. The

enhancement in the APJ receptor and PVN was much greater than that in the RVLM of MI rats.

- 2) The protein levels of APJ receptor in the PVN were confirmed using western blot analysis. Results showed that the expression of APJ receptor in the PVN was significantly enhanced in MI compared to sham rats.
- 3) The localization of APJ receptor in the PVN using double staining immunolocalization with a specific anti-APJ antibody and a specific neuronal marker anti-NeuN was examined next. The results clearly demonstrated that APJ receptors dominantly localized in the neuronal cytoplasmic membrane.

All the above results demonstrated that in MI rats, the Apelin/APJ system expression was enhanced in the PVN, a brain area that controls sympathetic nerve activity. Combined with previous results showing that overexpression or direct microinjection of apelin into this brain area increases sympathetic nerve activity, heart rate, and cardiac remodeling. Therefore we hypothesize that the enhanced Apelin/APJ system contributed to the enhanced sympathetic nervous activation, cardiac remodeling, and cardiac damage after MI and that selective silence of Apelin/APJ system attenuates these cardiac pathologies of MI.

CHAPTER 4. CONSTRUCTION OF LENTI-APJ-shRNA AND LENTI-SCR-shRNA VIRAL VECTOR

4.1. Introduction

In previous chapters, this work presented data on the expression of the Apelin/APJ system in the PVN of MI rats using RT-PCR and western blot. Results from these experiments demonstrated that the expression of the APJ receptor was increased in the PVN of MI as compared to sham rats. Moving forward, this study intends to determine whether down-regulation of this receptor in the PVN attenuates the development of HF.

A number of technologies have been used for down-regulation of gene expression. For example, site directed mutagenesis, anti-sense oligonucleotides, and ribozymes have been used for more than a decade to target specific gene sequences. These technologies worked satisfactorily in some simple experimental models. However, they have many drawbacks due to various reasons including lack of specificity or tropism to most types of cells and tissues. Recently, scientists developed a novel method of gene silencing using RNA interference (RNAi). The basic principle of this technique is to introduce into cells a double-stranded RNA (dsRNA) homologous to a specific gene that results in the post-transcriptional silencing of that gene. Two main steps mediate the gene silencing by RNAi. An enzyme that belongs to the RNase III family of nucleases, named Dicer, which initially recognizes the introduced dsRNA and cleaves into short double stranded fragments (20-25 bp) called siRNA. The siRNAs then separates into single strands which then binds to the RNA-induced silencing complex (RISC), a multi-protein complex with RNase activity, which guides the targeted mRNA to be degraded (Bantounas et al., 2004; Fire et al., 2004).

The dsRNA usually is introduced into cells by plasmid-based method (siRNA) or by viral-based expression of small hairpin RNAs (shRNA). Viral based expression has become a tool of choice recently because this method can transduce both cells and tissues. Viral vectors have become a popular approach for transduction of shRNA because they allow for longer-lived gene silencing effects. Several viral vectors have been used for this purpose including adenoviral vector, adeno-associated viral vectors (AAV), retroviral vector, and lentiviral vectors. These vectors have very good efficiency for the transduction of cells, but differ largely in integration into host chromosomal DNA. Retroviral and lentiviral vectors can mediate integration of the transgene into the host chromosome permitting long-term expression of the transgene. Adeno and adeno-associated viral vectors are also capable of integrating into host chromosomal DNA at a low frequency. However, these vectors also act through functional episome (extra chromosomal) expression. Adeno-associated viral vectors have several drawbacks that include being immunogenic (Sun et al., 2002), limited payload capacity to carry transgene, and difficulty in large-scale production of these viral vectors. All of these properties make them the least favorite choice for clinical studies (Merten et al., 2005). In contrast, lentiviral vectors are one of the oldest for transgenic expression of the genes. Lentiviral vectors transduce both dividing and non-dividing cells and integrate into host chromosome conferring long-term transgene expression. Replication incompetent lentiviral vectors have been developed recently. They have a low toxicity and low immunogenicity profile and are easily produced. These properties make them an ideal vector for transduction (Park et al., 2006; Logan et al., 2002). Hence, in the present study, the goal is to prepare a lentiviral vector-mediated shRNA for selectively silencing APJ receptor gene expression. Lentivirus containing scrambled shRNA was also prepared and used as a negative control. In the previous studies, this research team characterized a MI rat model and

studied the expression of the APJ receptor in the PVN of the brain using RT-PCR and western blot analysis. Results demonstrated that the expression of APJ receptor was enhanced in the PVN of MI rats.

The goal of the present study is to construct lentiviral vectors encoding APJ shRNA to be used to silence the APJ receptor *in vivo* in order to determine whether down-regulation of APJ receptor in the PVN attenuates pathophysiology of MI-induced cardiac damage. Thus, objectives of the current study are (1) To construct a lentiviral vector containing shRNA to effectively silence APJ receptor. (2) Determine viral titer using puromycin method. (3) Establish the ability of lenti shRNA viral vectors to effectively knockdown gene expression of APJ receptor *in vitro* by transducing primary cell cultured from hypothalamus of neonatal rats and neuron cell-line, Cath.a cell[CRL11179](ATCC CRL11179, Manassas, VA).

4.2. Materials and Methods

4.2.1. Construction of pLKO.1 Plasmid Containing APJ shRNA

To selectively silence APJ receptor, *E. coli* (DH5 α) bacteria carrying pLKO.1 plasmid encoding for APJ shRNA figure 15 or pLKO.1 plasmid containing scrambled shRNA with GFP marker (figure 16) from Thermo Open Biosystems (Huntsville, AL). *E. coli* (DH5 α) bacteria containing pLKO.1 plasmids were supplied as frozen glycerol stocks. *E. coli* from frozen stocks were streaked onto a lennox broth (LB) agar plate containing 100 μ g/mL of carbencillin and incubated at 37°C overnight. The following day, colonies were picked from each plate and seeded into two different culture tubes containing 5 ml of lennox broth (LB) media with 100 μ g/mL of carbencillin. Tubes were incubated for 16 hours at 37°C with vigorous shaking (250 RPM). After the 16-hour incubation, DNA was isolated from bacteria containing pLKO.1

plasmids with APJ shRNA or scrambled shRNA by using Qiagen plasmid mini kit (Qiagen, Valencia, CA). Briefly, bacteria were pelleted by centrifuging at 6,000 X g and resuspended in 0.3 ml of buffer P1 (Containing RNase A), 0.3 ml of buffer P2 was added and thoroughly mixed by inverting the tube several times and incubated for 5 minutes. Post incubation buffer P3 was added, contents in the tube are vigorously mixed by inverting the tube several time and incubated on ice for 5 minutes. After 5 min incubation, tubes were centrifuged at maximum speed and supernatants were collected and applied to a Qiagen tip 20 column equilibrated with QBT buffer. The supernatant was allowed to enter the resin of the column by gravity flow. Qiagen tip column was washed with 2 X 2 ml of buffer QC by gravity flow. DNA was eluted by adding 0.8 ml of buffer QF Eluate was collected in 1.5 ml tubes. Isolated DNA concentration was measured by measuring optical density (O.D.) at 260 nm in a UV spectrophotometer (Molecular Devices, Sunnyvale, CA). Briefly, 1 ml of a 1:100 dilution of isolated DNA was prepared by adding 10 μ l isolated DNA to 990 μ l DNAase/RNase free water. The samples were mixed well and pipetted into a quartz cuvette. O.D. was measured at 260 nm. DNAase/RNase free water was used as blank. Total DNA concentration of the sample was calculated using the following formula.

$$\text{Unknown mg/ml} = 50 \text{ mg/ml} \times \text{Measured A}_{260} \times \text{dilution factor}$$

The Integrity of the pLKO.1 plasmid DNA with APJ shRNA isolated from *E. coli* (DH5 α) was characterized with enzyme restriction analysis and agarose gel electrophoresis. Restriction enzymes BamHI and NdeI enzymes (New England Bio labs, Ipswich, MA) were chosen based on the plasmid map provided from the manufacturer and are set up for restriction reaction as indicated in table 1.

Table 1: Reaction Mixture Set for Restriction Digestion

Sterile, nuclease-free water	14.8 μ l
Restriction enzyme BamHI	1.0 μ l
Restriction enzyme BamHI10X	2.0 μ l
BSA (10X, 10 mg/ml)	0.2 μ l
DNA sample	1 μ g
Restriction enzyme NdeI 20U	1.0 μ l
Final volume	20 μ l

Plasmid DNA without restriction enzyme was used for negative control. All samples were centrifuged (8,000 X g) for 30 sec and incubated in a thermal cycler (Bio-Rad, Bio Hercules, CA) at 37°C for 2.5 hours for digestion. The digested plasmid DNA was loaded into the wells of a 0.8% agarose gel and digested DNA fragments were separated based on size.

4.2.2. Preparation of HEK293T Cell Cultures

Production of lentiviral vectors carrying shRNA for APJ receptors requires a helper cell-line HEK293T (Human embryonic kidney cell-line 293T), obtained from Thermo Open Biosystems (Huntsville, AL). HEK293T cell-line facilitates optimal lentivirus production.

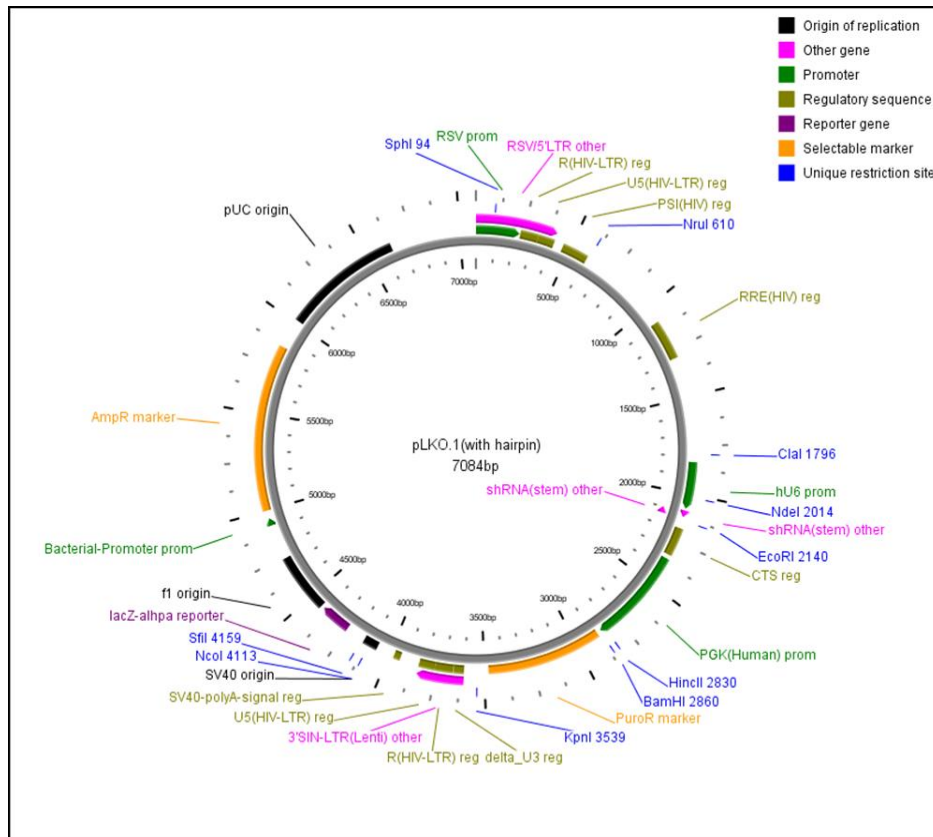


Figure 15. Vector back bone of pLKO.1 plasmid. Vector back bone of pLKO.1 plasmid carrying a APJ ShRNA driven by U6 promoter, loop and puromycin selection marker driven by hPGK promoter. Vector is flanked by RSV LTR at the 5' end and sin LTR at the 3' end.

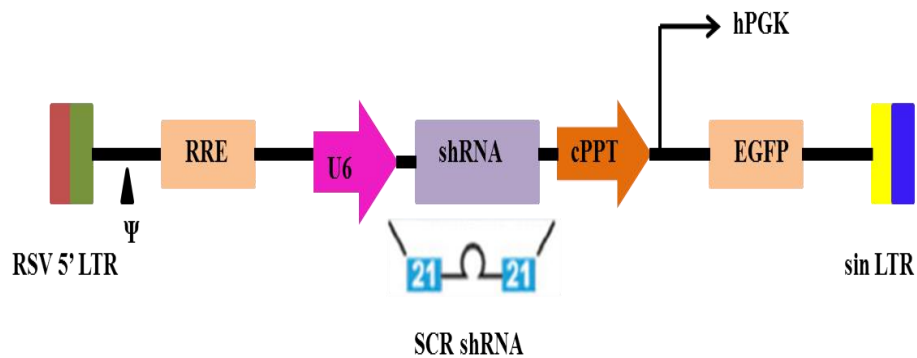


Figure 16. Vector back bone of pLKO.1 plasmid carrying SCR shRNA. pLKO.1 plasmid containing U6 promoter, 21 nucleotide SCR shRNA hair pin loop and GFP marker driven by hPGK promoter. Vector is flanked by RSV LTR at the 5' end and sin LTR at the 3' end.

The HEK293T cell-line stably and constitutively expresses the SV40 large T antigen, which helps in higher transformation efficiency and expression of genes. HEK293T cells were stored as a frozen stock and stored in liquid nitrogen. The cells were revived as per the protocol provided with the kit. Briefly, HEK293T cells were removed from liquid nitrogen and a ‘quick thaw’ was carried out by incubating the cryovial containing the cells in a 37°C water bath for 2 minutes until ~80% of cells thawed. Cells were pipetted from the cryovial to a 15 ml tube containing 10 ml Dulbecco’s minimal essential medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Bio, Lawrenceville, GA). The samples were centrifuged at 500 X g, and the media was discarded. The cell pellet was re-suspended in the medium and added to a T-25 flask containing DMEM supplemented with 5% FBS. Cells were cultured at 37°C with 5% CO₂. Culture medium was replaced with fresh medium every 3 days. When HEK293T cells were 90% confluent they were passaged to a ratio of 1:15 to 1:20 for general maintenance.

4.2.3. Lentiviral Packaging

We used a Trans-Lentiviral GIPZ Packaging System (Thermo Open Biosystems, Huntsville, AL) to make lentivirus containing APJ shRNA. This system allows creation of a replication incompetent HIV-1 based lentivirus. To produce a stably transduced cell-line expressing the shRNA, Lentivirus was produced by co-transfecting HEK293T with the optimized packaging plasmids containing pTLA1-Pak, pTLA1-Enz, pTLA1-Env, pTLA1-Rev and pTLA1-TOFF plasmids (the plasmid structures are shown in figure 17) and the transfer vector plasmid containing APJ shRNA transcript (the plasmid structure is shown in figure 15). Co-transfection of the trans-lentiviral packaging mix and the transfer vector containing the APJ shRNA or scramble control shRNA (SCR shRNA) encoding gene into HEK293T cells produces

a replication-incompetent lentivirus that can be used to silence APJ receptor expression *in vitro* and *in vivo* (Kappes et al., 2001; 2003).

After preparation of the lentiviral expression vector and packaging plasmids, HEK293T cells were subcultured at 1:2 so that the cells were in a "rapid" replication state, which allows for the transcriptional and translational machinery of the cell to function at the highest level when generating virus. The transfection reactions were set up as described in Table 2. HEK293T cells were plated at a density of 5.5×10^6 cells in 100 mm plates with full medium including 5% fetal bovine serum (Atlanta Bio, Lawrenceville, GA) and penicillin–streptomycin antibiotics (Fisher Scientific, Pittsburgh, PA). On the day of transfection, each plate was transfected with plasmid DNA (transfer plasmid and packaging plasmids) diluted in 1 ml (total volume) of serum-free medium. 187.5 μ l of Arrest-In (Thermo Open Biosystems, Huntsville, AL) was diluted into 1 ml (total volume) of serum-free DMEM in a separate micro centrifuge tube. The diluted plasmid DNA and Arrest-In were mixed rapidly and incubated for 15 minutes. An additional 1 ml of serum-free medium was added into the sample (total volume 3 ml). After the growth medium was aspirated from the cell culture plates, the 3 ml serum medium containing plasmid DNA and Arrest-in reagent were added. The cells were incubated for 4 hours in a CO₂ incubator with 5% CO₂. The transfection mixture was aspirated and fresh 12 ml's of DMEM cell culture medium with 10% FBS was added. After 48 and 72 hours post transfection, supernatants were collected for viral particle isolation. The viral particles were pelleted by ultra-centrifuging the supernatant at 23,000 rpm in a Beckman Coulter Ultracentrifuge (Beckman Coulter, Brea, CA) with a SW 28 swing bucket rotor for 1.5 hours at 4°C.

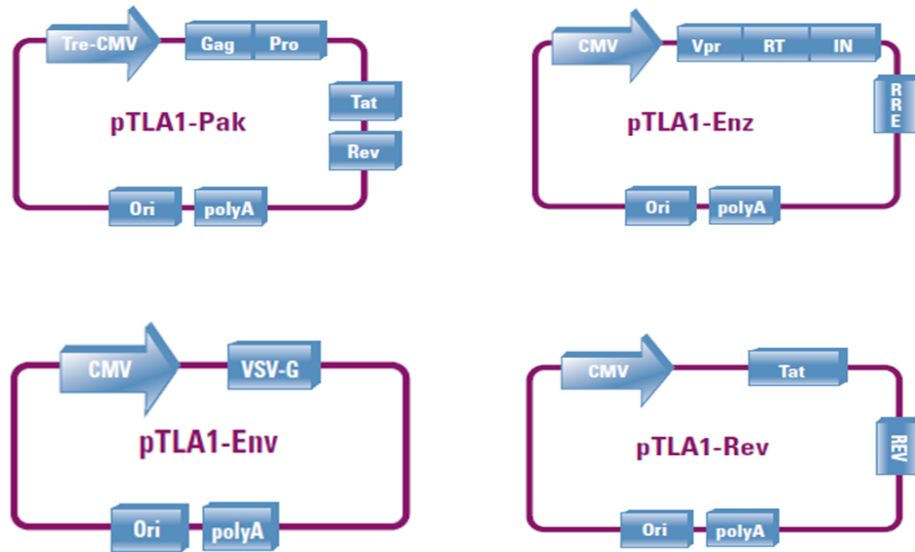


Figure 17. Viral packing plasmids. Packing plasmids. pTLA1-Pak plasmid, pTLA1_ENZ, pTLA1-ENV, pTLA1-REV of viral packing mix. Each plasmid encodes for lentiviral Gag, REV, ENV and ENZ proteins.

Table 2: Components Used for Lentiviral Packaging

Components	Amount
Tissue culture plate size 100 mm	100 mm (one per lentiviral construct)
Number of HEK 293T cells to transfect	5.5×10^6
Amount of Trans Lenti Viral Packaging mix	28.5 μg (30 μl * of packaging mix stock)
Transfer Vector	9 μg
Amount of Arrestin Transfection Reagent	187.5 μg (187.5 μl of 1 mg/ml stock)

4.2.4. Titration of Lentivirus

Viral titrating, isolation, and concentration of the viral particles from above experiments were accomplished using puromycin selection. In brief, HEK 293T cells were plated in 12 well cell culture plates and grown in a CO₂ incubator until 30-50% confluent. On the day of transduction (Day 2), lentiviral stock was thawed and serial diluted ranging 10² – 10¹⁰ were prepared. For each dilution, the lentiviral stock was diluted with complete culture medium to a final volume of 1 ml. Cell culture medium was aspirated from each well of the culture plate and the medium containing different dilutions of the virus were added into different wells in the 6-well plate. The following day (Day 3), the media containing virus was replaced with 2 ml of complete culture medium and continue to incubate at 37°C overnight in a humidified 5% CO₂ incubator. The following day, media was aspirated and fresh medium containing puromycin 2 µg/ml (Invivogen, San Diego, CA) was added. The puromycin-selection was carried out for two weeks by changing medium every 2 days. After selection, monolayer of cells was washed with PBS and stained with a crystal violet solution (1 ml per well) and washed with PBS. The total numbers of colonies were calculated by observation under a light microscope. Titer value was calculated by using the following equation.

$$\text{Viral Titer} = \text{Average of Number of colonies} \times \text{dilution factor of two consecutive wells}$$

4.2.5. Preparation of Cath.a Cell Cultures

In earlier studies, it was shown that APJ receptors are expressed in neurons of PVN. In order to determine the efficacy of the lentiviral vectors carrying APJ shRNA to knockdown APJ receptor, a Cath.a cell-line was used because of its neuronal origin. Cath.a cells express a variety of pan-neuronal markers, neurofilaments and GPCR like Angiotensin receptor AT1 and have

been used in many studies (Suri et al., 1993; Lazaroff et al., 1996; Yannping et al., 1997). Cath.a cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) as a frozen stock. Cells were revived by carrying out a 'quick thaw' by incubating the vial containing the cells in the 37°C water bath for two minutes until about 80% thawed. The cells were removed from the vial and added to 15 ml tube containing 10 ml RPMI 1640 media supplemented with 8% horse serum and 4% fetal bovine serum. The cells were then centrifuged at 1000 X g. Supernatant was discarded. The cell pellet was re-suspended in the fresh medium and transferred to T-25 flask containing RPMI 1640 medium with 8% horse serum and 4% fetal bovine serum. Cells were grown at 37°C with 5% CO₂ and passaged regularly when the confluence reached 90%.

4.2.6 Cath.a Cell Transduction and Detection of APJ mRNA Levels

Cath.a cells were plated onto a 100 mm dish the day before transduction. The medium was replaced with fresh medium and 3 µl of 5×10^6 transducing units (TU) per ml Lenti-APJ-shRNA(Lv-APJ-shRNA) virus was added onto the cells (n=3) and incubated for 18 hours at 37°C in a CO₂ incubator. At the same time, control plates (n=3) of untransduced Cath.a cells were prepared. After 18 hr incubation, medium was aspirated from transduced and untransduced plates and fresh media was added and incubated for 72 hours. Cell monolayer was washed with a hanks balanced salt solution (HBSS); trypsinized and resuspended in fresh medium. Cells were then pelleted by centrifuge. Total RNA was extracted using Qiagen RNAeasy Kit (Qiagen, Valencia, CA) by following the instruction provided by manufacturer. Briefly, isolated cells were homogenized in 350-µl lysis buffer for 40 sec then one volume of 70% alcohol was added and mixed well by pipetting. Homogenate was applied to the column provided by manufacturer and centrifuged for 15 sec at 8,000 X g. The flow through was discarded. 700 µl of buffer RW1

were added to the column and centrifuged for 15 sec at 8,000 X g. The flow through was discarded and 500 µl of buffer RPE was added and centrifuged for 15 sec and 2 min respectively. Residual amount of buffer RPE is removed from the column by spinning the column at full speed for 1 min. RNA is eluted out of the column by adding 30 µl of RNase free water to the center of the column and spinning at 8000 X g for 1 min. After extraction, RNA is purified by using Ambion Turbo DNA free kit (Life technologies, Grand Island, NY). Total concentration of RNA was determined by measuring absorbance at 260nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA).

A total of 1 µg of RNA was converted into cDNA by using the Promega Reverse Transcription system kit (Promega, Madison WI). RNA without Reverse Transcriptase was used as negative control. The reverse transcription reaction was performed as per the guidelines provided by the manufacturer as described previously. cDNA was subjected to two step RT-PCR using an ABI 7500 Sequence Detection System (Applied Biosystems Inc, Foster City, CA) for 40 cycles (50 °C, 2 min; 95°C, 10 min; 94°C 15 seconds; 60°C, 36 sec) with Taqman probe based assay (Applied Biosystems Inc, Foster City, CA) according to the protocol provide by the manufacturer. Briefly, 5 µl of cDNA was be added into each well in a 96 well PCR plate in triplicates. 10 µl of standard master mix and 1µl of APJ Probe (Rn00580252_s1) obtained from Applied Biosystems Inc (Foster City, CA) were added. GAPDH probe (Rn00576699_m1) obtained from Applied Biosystems Inc (Foster City, CA) was used as an internal control. The total reaction volume was made up to 20 µl by adding water. Ct values was obtained by carrying out the reaction in ABI 7500 real time PCR machine (Applied Biosystems, Foster City, CA) and relative change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ method.

4.2.7. Preparation of Primary Neuronal Cultures

In the last experiment, the efficiency of knockdown of APJ receptor using Lv-APJ - shRNA virus in dividing cells was examined. In this study, the efficiency of Lv-APJ-shRNA was determined in silencing APJ receptor expression in non-dividing primary cultured neurons. Primary cultured neuronal cells were prepared from the hypothalamus containing PVN area taken from 1-day-old rats as described previously (Sumners et al., 1991). Briefly, trypsin (375 U/ml) and DNase I (496 U/ml)-dissociated cells were resuspended in DMEM containing 10% PDHS (Plasma derived horse serum) and plated on 35-mm Nunc plastic tissue culture dishes pre-coated with poly-L-lysine. After the cells were grown for three days at 37°C in a humidified incubator with 95% O₂ and 5% CO₂, they were exposed to 1 μM ARC(β-cytosine arabinoside) for two days in fresh DMEM containing 10% PDHS. ARC was then removed; and the cells were incubated with DMEM (plus 10% PDHS) for an additional 11–14 days before use.

4.2.8. Primary Cultured Neuron Transduction and Detection of APJ mRNA Levels

Primary cultured neurons in 35 mm culture dishes were transduced by addition of 2μl of 5 X 10⁶ TU/mL of viral particles. Plates transduced with viral particles were incubated for 72 hours thereby allowing sufficient time for down regulation of mRNA and at same time a control plate of rat primary cell culture neurons was prepared without adding Lv APJ shRNA virus media was aspirated and cell layers were washed with fresh cold HBSS, after washing fresh medium was added to the plates and neuronal cells were scrapped from the cell culture dish using a fresh autoclaved Walter stern autoclaved policeman (VWR Scientific, Batavia, IL) and collected the cell supernatant using a micropipette. Cells were pelleted by centrifugation in a centrifuge and total RNA was extracted using Qiagen RNAeasy kit (Qiagen, Valencia, CA) as

described earlier. After extraction, RNA was purified by using Ambion Turbo DNA free kit (Life Technologies, Grand Island, NY). Total concentration of RNA was determined by measuring absorbance at 260nm in a spectrophotometer as described previously.

A total of 1µg of RNA isolated from cultured neurons treated with or without Lv-APJ-shRNA, or untransduced control was converted into cDNA. The cDNA was used for RT-PCR to detect APJ receptor expression in cultured hypothalamic neurons treated under each condition. In brief, 5 µl of cDNA, 1 µl of APJ Probe, and 10 µl of master mix were mixed together. The total reaction volume is made up to 20 µl by adding water. GAPDH was used as an internal control. Ct values were obtained by running the reaction in ABI 7500 real time PCR machine (Applied Biosystems, Foster City, CA). The relative change in gene expression was calculated by using $2^{-\Delta\Delta Ct}$ method.

4.2.9. Determination of APJ protein Levels in Primary Cultured Neurons after Transduction.

To confirm the efficiency of Lv-APJ-shRNA vector on silencing APJ receptor expression in primary neuronal cultures from the hypothalamus of rats, cultured cells were transduced with 2 µl of Lv-APJ-shRNA and incubated for four days. Total proteins from neurons were isolated by homogenizing in a 2 x lysis buffer. Extracted proteins were separated on a SDS PAGE gel and transferred onto a membrane. Then, the membrane was incubated with a rabbit anti-APJ primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 2% milk overnight at 4°C. Following the incubation, the blot was washed three times in 0.5% milk plus tris buffer saline and tween (TBST) at intervals of 15 minutes at room temperature. The membrane then was incubated with an anti-rabbit peroxidase-conjugated secondary antibody (Bio-Rad, Hercules,

CA) for 1 hour at room temperature. The primary antibody used in this study is raised against amino acids 1-300 at the N-terminus of the APJ. Immunoreactivity was detected by enhanced chemiluminescence autoradiography using Pierce ECL western blotting detection kit (Thermo Fisher, Rockford, IL). The film was developed and analyzed with Quantity One Software (Bio-Rad, Hercules, CA).

4.3. Results and Data Analysis

4.3.1. Identification of PLKO.1 Plasmid

E. coli bacteria carrying pLKO.1 plasmid (7084 bp) that encodes for APJ shRNA was cultured overnight and plasmid DNA was isolated using the Qiagen plasmid isolation kit. Isolated plasmid DNA was restriction digested with BamH1 and Nde1 enzyme. Nde 1 cuts the plasmid at 2014 bp position where as BamH1 cut the plasmid at the 2860 bp position. Therefore, double digestion with these two enzymes will yield two fragments with 6.3 kb and 794 bp. Results are presented in Figure 18 showing digested plasmid DNA run on a 0.8 % agarose gel. Lane 1 shows 1 KB DNA ladder; lane 2 shows plasmid DNA digested with BamH1 and Nde1 enzymes, showing two fragments of size 6.3 kb and 794 bp; lane 3 shows uncut plasmid DNA; lane 4 shows plasmid DNA digested with BamH1 only showing 7kb fragment, lane 5 shows the plasmid DNA digested with Nde1 enzyme showing 7Kb fragment .

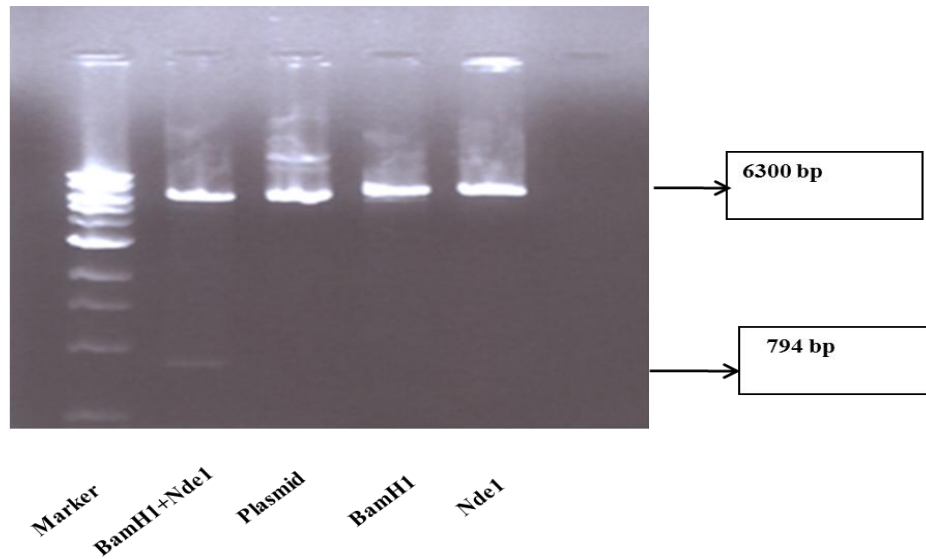


Figure 18. PLKO.1 vector characterization using agarose gel electrophoresis. Agarose gel electrophoresis was conducted to characterize PLKO.1 plasmid construct. Lane 1, 1 Kb marker; Lane 2, the plasmid enzyme restriction with BamH1 and Nde1 (BamH1+Nde1); Lane 3, unrestricted plasmid DNA; Lane 4, the plasmid enzyme restriction with Bam 1 only; and line 5, the plasmid enzyme restriction with Nde 1 only.

4.3.2. Viral Titration

Lentiviral vectors carrying transcripts encoding for APJ shRNA and SCR shRNA were prepared using a lentiviral packaging kit. Viral titration was performed to determine the number of viral particles in the concentrated viral stock. HEK 293 T cells plated in a 12 well plate were transduced with different dilutions of viruses described previously. Transduced cells were selected for 12 days using Puromycin 2 $\mu\text{g}/\text{mL}$. After 12 days of selection, the cell monolayer was stained with crystal violet and the number of colonies was counted. Results show that well receiving 10^5 dilution of Lv-APJ-shRNA virus there were 42 colonies counted and at well receiving 10^6 dilution of Lv-APJ-shRNA of virus six colonies were counted. Applying the formula mentioned previously total titer of our viral stock of Lv-APJ-shRNA was found to be 5×10^6 TU/mL figure 19. Results from viral titration for Lv-SCR-shRNA shows that 4.8×10^6

TU/mL (Data not shown). These results indicate that we have produced viral particles with high titer values.

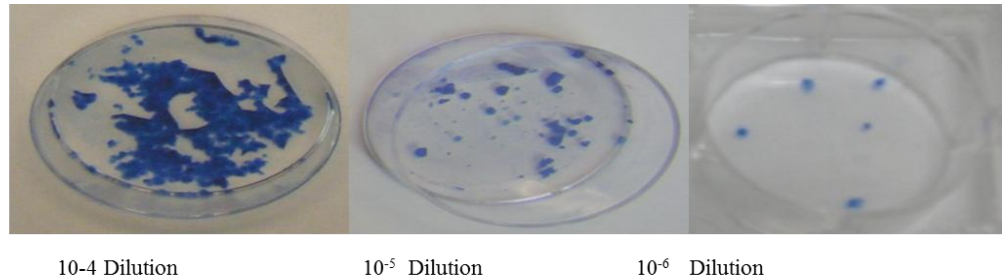


Figure 19. Titration of viral vector using HEK 293 T cells. HEK 293T cells transduced with virus dilutions of 10^4 , 10^5 , 10^6 of Lv-APJ-shRNA viral vector followed by section with puromycin and cell monolayer's are stained with crystal violet.

4.3.3. Effect of Lenti-APJ-shRNA on APJ mRNA Levels in Cath.a cells

Lv-APJ-shRNA virus was constructed to silence specific APJ receptor expression. In order to determine the efficiency of the lentivirus carrying APJ shRNA to knockdown APJ receptor expression. Cath.a cells were cultured and transduced with Lv-APJ-shRNA virus. Cath.a cells that were not transduced with lentivirus were used as controls. mRNA levels of APJ receptor were determined using RT-PCR and un-transduced Cath.a cells were used as control. RT-PCR data is shown in Figure 20 demonstrating that APJ mRNA levels were down regulated in the Cath.a cells transduced with lenti APJ shRNA virus by 1.4 folds ($P < 0.05$) as compared with control cells. Taken together, these observations demonstrate that APJ receptor expression is significantly decreased in Cath.a cells transduced with Lv APJ shRNA virus, showing that Lv-APJ-shRNA virus are effective in knocking down of APJ receptor; hence, lenti APJ shRNA virus can be used for silencing APJ receptor expression *in vivo*.

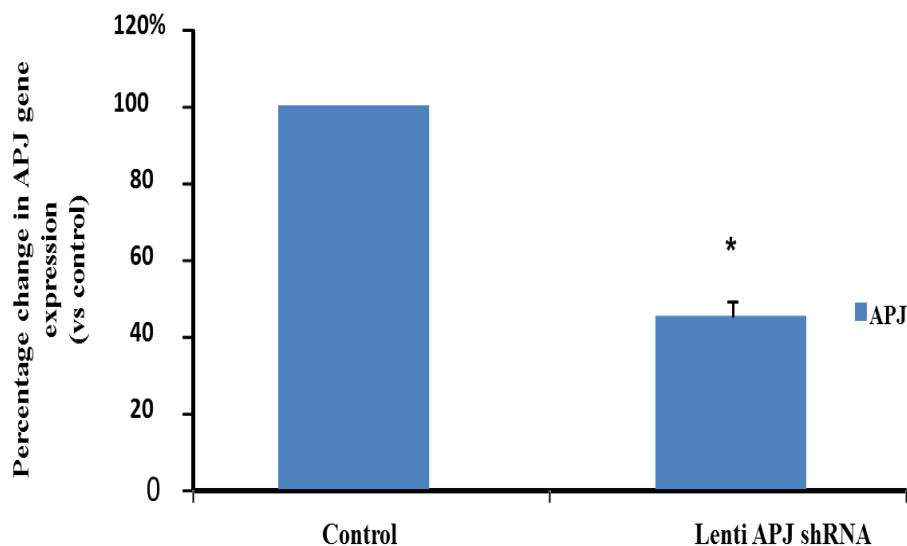


Figure 20: Knockdown of APJ mRNA in Cath.a cell line. APJ mRNA levels in Cath.a cell line with and without transduction with Lv- APJ-shRNA viral vector. The mRNA levels in Cath.a cells were measured with real time RT-PCR and were normalized to GAPDH internal control. Data are mean \pm SE. *P<0.05 as compared with control (n=3 incubations).

4.3.4. Effect of Lenti-APJ-shRNA on APJ mRNA Levels of Neuronal Cell Culture

In the previous experiment, the efficiency of the lenti-APJ-shRNA-shRNA virus to specifically knockdown the APJ receptor in Cath.a cell-line was tested and results showed a knockdown of APJ receptor expression. Immunohistochemistry studies from earlier studies showed that APJ receptor is expressed in neurons of the PVN. Thus, the effect of Lenti-shRNA-shRNA was examined on the APJ receptor expression in neurons cultured from the hypothalamus including PVN brain area to confirm the efficiency of lenti APJ shRNA vector to silence APJ receptor expression in non-dividing cells. Rat primary neuronal cultures were prepared and transduced with lenti-APJ-shRNA. The mRNA levels were determined using RT-PCR. Results showed that APJ mRNA levels were significantly reduced in the neurons transduced with lenti-APJ-shRNA virus by 1.5 folds, as compared with control neuronal cells (P<0.05). Taken together, these observations demonstrate that APJ receptor expression is down-

regulated in neuronal cells transduced with lenti-APJ-shRNA virus showing that lenti APJ virus are effective in knocking down of APJ receptor in primary cultured neurons.

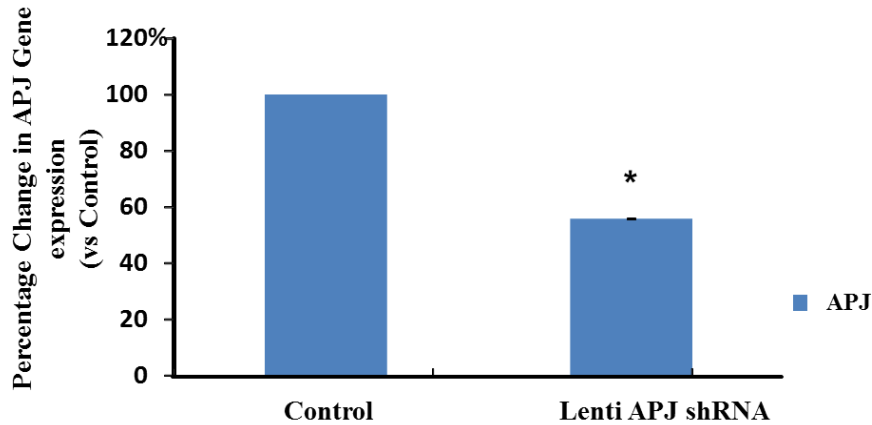


Figure 21. Knockdown of APJ mRNA expression in neuronal cells. Messenger RNA expression levels in Primary neuronal cells culture transduced with lenti APJ shRNA virus and normal neuronal cells. This figure represents RT PCR of in Primary neuronal cell culture transduced with lenti APJ shRNA virus and normal Primary neuronal cell culture normalized to GAPDH internal Control. APJ Receptor levels are down regulated in Primary neuronal cell culture that is transduced with Lenti APJ shRNA. Data are mean \pm S.E (n=3 p<0.05)

4.3.5 Effect of Lenti-APJ-shRNA on APJ Protein Levels of Neuronal Cell Culture

Previous experiment measured APJ mRNA levels in primary neuronal cultured cells. APJ receptor mRNA levels were significantly decreased in neurons transduced with Lenti-APJ-shRNA as compared to untransduced neurons. Western blot analysis was performed next to determine whether protein levels of APJ receptor were down-regulated in the neurons transduced with Lv-APJ shRNA as compared to untransduced control. The APJ receptor protein levels in the cultured neurons were examined using the regular western blot technique. The results showed that APJ protein levels were significantly decreased in the neurons transduced with Lv-APJ-shRNA as compared with control (Figure 22) ($P < 0.05$, n=3, each incubation). These results

confirmed the RT-PCR results, indicating APJ receptor expression in both mRNA and protein levels were decreased in neurons transduced with Lv-APJ-shRNA.

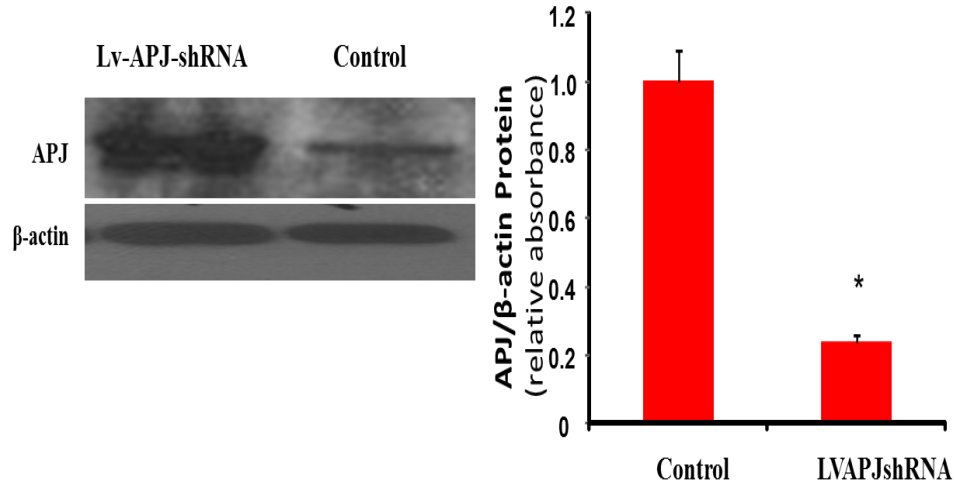


Figure 22. Effect of Lv-APJ-shRNA on the expression of APJ proteins in primary cell culture neurons. A, representative autoradiogram of APJ protein levels in primary cultured neurons transduced with Lv-APJ-shRNA or Lv-GFP. Data are mean \pm SE, normalized to β -actin (n=3). *P<0.05 as compared with untransduced control

4.4. Data Summary and Conclusion

Apelin/APJ system is a novel neurohormonal system expressed in both peripheral and central systems and involved in cardiovascular homeostasis (Klienz et al., 2005; Lee et al., 2006; Masri et al., 2005; O'Carroll et al., 2000). Animal and human studies showed that apelin levels are elevated in during heart failure and myocardial infarction (Atluri et al., 2007; Chen et al., 2003). Injection or overexpression of apelin in brain elevates blood pressure, increases sympathetic nerve activity, and results in cardiac hypertrophy. On the other hand, elevated sympathetic nerve activity is the major contributor to the development of HF. Thus, in the current study, the role of Apelin/APJ was examined in the PVN of MI-induced cardiac dysfunction. In earlier studies, the

myocardial infarction rat model induced by left coronary artery ligation was characterized. APJ expression was measured in various cardiovascular regulatory regions of the brain including the PVN, NTS, and RVLM and it was found that the APJ receptor was elevated in PVN of the brain. Therefore, in the present study the aim was to construct lentiviral vectors carrying APJ-shRNA transcript to silence the APJ receptor, selectively. Lv-SCR shRNA were packaged using HEK 293T cells as a control. Virus was concentrated by ultracentrifugation and titered using Puromycin selection methods. All the results indicate:

- 1) High titers of viral particles carrying Lv-APJ-shRNA or Lv-SCR-shRNA were prepared and used for *in vivo* and *in vitro* gene transfer.
- 2) Titering viral particles were carried out by transducing the HEK 293T cells with various dilutions of viral particles and Puromycin selection techniques. Results showed that the viral particle concentration in the preparation was up to 5×10^6 TU/mL.
- 3) To determine the efficiency of lentiviral vectors carrying shRNA transcript for APJ receptor to knockdown APJ receptor expression in dividing cells, Cath.a cells were transduced with Lv-APJ-shRNA. The results showed that APJ receptor mRNA levels were significantly reduced in Cath.a cells after transduction with Lv-APJ-shRNA.
- 4) To determine the efficiency of the Lentiviral vector carrying shRNA transcript for APJ receptor to knockdown APJ receptor expression in non-dividing cells, primary cultured hypothalamic neurons were transduced with Lv-APJ-shRNA. The results showed that APJ receptor expression in both mRNA and protein levels were markedly decreased after transduction with Lv-APJ-shRNA.

In summary, this study produced high titer lentiviral vectors carrying Lv-APJ-shRNA and Lv-SCR-shRNA virus. Transduction of dividing Cath.a cell-line and in non-dividing

primary cultured hypothalamic neurons by Lv-APJ-shRNA down regulated APJ expression. All these evidences suggest that we have produced high titer lentiviral vector carrying APJ shRNA or SCR shRNA. Lentiviral vector containing APJ shRNA (Lv-APJ-shRNA) significantly knockdown APJ receptor expression *in vitro* for both dividing and non-dividing neurons.

CHAPTER 5. EFFECT OF LENTI-APJ-shRNA IN THE PVN ON THE HEART IN MI RATS

5.1. Introduction

HF is a serious life threatening complication that is the second leading reason for death and hospitalization in the United States (Lloyd et al., 2012). HF is a disease where the heart is unable to pump enough blood to meet the body's metabolic needs. Pathophysiology of HF is multifactorial. Several different factors contribute to decompensating the capability to pump the blood, including release of arginine vasopressin, other neurohormonal factors, and over excitation of the SNS (Triposkiadis et al., 2009). The central mechanisms that lead to these hyperactive activities in CNS are still not fully understood.

Left ventricular remodeling is a hallmark after MI and includes the expression of molecular, cellular, and interstitial changes in response to cardiac injury manifested by loss of myocardial tissue, cardiac hypertrophy, and fibrosis. Studies show that neurohormonal systems play a pivotal role in left ventricular remodeling including sympathetic nerve activation and norepinephrine, vasopressin, angiotensin II up-regulation (Ira et al., 1996; Chatterjee et al., 2005; Chidsey et al., 1963; Cohn et al., 2000). The central mechanisms underlying increased SNS activity and up-regulated release of endogenous hormones are still not fully understood.

Apelin/APJ system is a novel neurohormonal system involved in the regulation of cardiovascular function. Apelin levels increased after MI or ischemic injury in rats (Atluri et al., 2007). Recent studies indicate that the Apelin/APJ system is expressed in the PVN magnocellular neurons, controls arginine vasopressin release, and regulates body fluid homeostasis (De Mota et al., 2000; Reaux et al., 2001; Brailoiu et al., 2002; O'Carrol et al.,

2003; Roberts et al., 2009). A study from our laboratory shows that injection or overexpression of apelin in the brain of a rat increases SNS activity and induces cardiac hypertrophy (Zhang et al., 2009). Therefore, it would be extremely important to study the role of the brain Apelin/APJ system in pathophysiology of MI.

Data shown in previous chapters demonstrate that APJ receptor expression is significantly increased in the hypothalamic PVN of MI rats. These results lead to hypothesize that enhanced APJ receptor expression contributes to the sympathetic excitation and AVP release, leading to cardiovascular symptoms after MI. This new work will use lentiviral vector-mediated shRNA to silence APJ receptor expression selectively in the PVN to examine the role of APJ receptor in the MI-induced pathologies. To accomplish these goals, the myocardial infarction rat model was developed by ligation of left coronary artery. Left ventricular dysfunction confirmed by measuring LV thermodynamics using a cardiac catheter. Lv-APJ-shRNA and Lv-SCR-shRNA control were constructed. The ability of packaged Lv-APJ-shRNA vectors to specifically knockdown APJ receptor expression was tested in a dividing cell such as the Cath.a and non-dividing primary cultured hypothalamic neurons.

In this present study, we intend to inject Lv-APJ-shRNA virus into the PVN of MI and sham rats to examine the effect of down regulation of this system on left ventricular dysfunction, cardiac remodeling, sympathetic nervous system activation, and AVP release.

5.2. Materials and Methods

5.2.1. Animals

Adult male Sprague-Dawley (SD) rats (Charles River Laboratories) weighing 220–280 g, were used in this study. All animals were housed under controlled conditions with a 12-h

light/dark cycle. Food and water were available to the animals *ad libitum*. The North Dakota State University Institutional Animal Care and Use Committee approved all protocols.

5.2.2. MI Rat Surgery

MI model rats were induced by left coronary arterial ligation as described above. In brief, under anesthesia with isoflurane inhalation (a mixture of 1 L/min O₂ and 3% isoflurane), a left lateral thoracotomy was carried out under aseptic conditions. The left anterior descending coronary artery was ligated 2–3 mm from its origin with a 6-0 polypropylene suture. The thorax was rapidly closed and positive pressure was applied on the chest to expel trapped air. Control (sham) rats underwent the same surgical procedure without coronary artery ligation.

5.2.3. Microinjection of Lenti-APJ-shRNA Viral Vector into the PVN

Sham and MI rats were anesthetized with a mixture of O₂ (1 L/min), and isoflurane (3%) delivered through a nose cone. The rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Skin overlying the midline of the skull was incised, and a small hole was drilled bilaterally on the dorsal surface of the cranium according to the following coordinates: 1.8 mm posterior, 0.4 mm lateral to the bregma, and 7.8 mm ventral to the dura. A 200 nl of either Lenti-APJ-shRNA virus or Lenti-SCR-shRNA virus were injected into the PVN. After the injection, the wound was sutured.

5.2.4. Detection of Efficacy of Lenti-APJ-shRNA *In vivo*

To confirm the knockdown of APJ receptor in sham and MI rats that received PVN microinjection of Lv-APJ-shRNA or Lv-SCR-shRNA, the APJ receptor protein levels were examined using western blot analysis. Tissues from rat PVN that were injected with Lv-APJ-

shRNA or Lv-SCR-shRNA were isolated at 0, 2, and 4 weeks after PVN microinjection of virus. Proteins were isolated by homogenizing in a 2 x lysis buffer. Extracted proteins were separated on a SDS-PAGE gel and transferred to a membrane. The membrane was incubated with rabbit anti-APJ primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), washed with PBST, and incubated with an anti-rabbit peroxidase-conjugated secondary antibody. The primary antibody used in this study is raised against amino acids 1-300 at the N-terminus of the APJ. Immunoreactivity was detected by enhanced chemiluminescence autoradiography Pierce ECL western blotting detection kit(Thermo Fisher, Rockford, IL), and the film was developed and analyzed with Quantity One Software (Bio-Rad, Hercules, CA).

5.2.5. Assessment of LV Hemodynamics

Four weeks after the microinjection of Lv-APJ-shRNA or Lv-SCR-shRNA, rats were anesthetized with a mixture of 2% isoflurane and 98% oxygen. The right carotid artery was cannulated with a catheter-tip pressure manometer (SPR-320, Millar instruments, Houston, TX) for the recording of arterial pressure and HR. After that, the micromanometer was advanced into the LV for measuring ventricular function. The output signal from the Millar catheter was collected and analyzed with Power Lab data-acquisition system (AD Instrument, Colorado Spring, CO).

5.2.6. Measurement of Plasma Norepinephrine Levels

Sham and MI rats were anesthetized with a mixture of O₂ (1 L/min) and isoflurane (3%). Blood was drawn from the tail vein and plasma separated by centrifuging blood for 15 minutes at 1000 X g. An ELISA was performed using the Noradrenalin/Dopamine Elisa kit (Rocky Mountains Diagnostics, Colorado Springs, CO). In brief, the derivatized standards, controls, and

samples were added to 90-well pre-coated microtiter plate and antiserum was added, incubated for 2 hours at 2-8°C, washed four times using a wash buffer, and dried. Enzyme conjugate solution was added after drying and incubated at 20 – 25°C for 30 minutes on a shaker. After incubation, wells were washed four times with wash buffer. The plate was dried by inverting the plate on an absorbent material. Substrate solution was added and incubated for 30 min at 20-25°C avoiding direct contact with light. After 30 minutes of incubation, stop solution was added. The absorbance was read at 450 nm. Quantification of unknown samples was achieved by comparing absorbance with a reference curve prepared with known standard concentrations.

5.2.7. Measurement of Cardiac Hypertrophy

Cardiac hypertrophy is one of the important complications associated with HF. The goal of this study is to determine whether down regulation of the Apelin/APJ system attenuates cardiac hypertrophy. Eosin haematoxylin staining was used to determine whether PVN microinjection of Lv-APJ-shRNA reduced cardiac hypertrophy in MI rats.

Four weeks after PVN microinjection of lentivirus carrying scrambled shRNA or APJ shRNA, the animals were anesthetized with sodium pentobarbital and the hearts were removed. Hearts were fixed in 10% formalin/PBS and embedded in paraffin. Heart sections (10 µm) were cut using a cryomicrotome and placed on a slide. Paraffin sections were deparaffinized and rehydrated by incubating in 3 X 3 min in xylene, 3 x 3 min in 100% ethanol, 1 x 3 min in 95% ethanol, 1 x 3 min in 80% ethanol, and 1 x 5 min in deionized H₂O. Rehydrated tissues were stained by incubating with haematoxylin for three minutes. Excessive and unbound stain was removed by running tap water over the section. Slides were washed by dipping in Acid-ethanol followed by deionized water. Excessive water was removed by blot paper and air-dried. Air dried

slides were incubated in eosin stain for 45 seconds and washed for 3 X 5 min in 95% ethanol, 3 X 5 min and 3 X 15 min in xylene. Slides were covered with a cover slip using a mounting medium. Morphology and cellular dimensions were evaluated by using Image Pro Plus 6.0 (Media Cybernetics, Bethesda, MD).

5.2.8. Measurement of Cardiac Fibrosis

Interstitial fibrosis is a hallmark for cardiac remodeling during HF. The goal of this study was to determine whether injection of Lenti-APJ-shRNA virus attenuated cardiac modeling. Sirius Red staining also known as F3B staining is the most accepted technique for studying interstitial fibrosis. This analysis stained sections derived from sham and MI rats that received PVN microinjection of Lv-APJ-shRNA virus or Lv-SCR-shRNA to determine whether down-regulation of Apelin/APJ system attenuated cardiac remodeling.

Four weeks after left coronary artery ligation, the animals were anesthetized by i.p injection of 200 mg/kg sodium pentobarbital and euthanized by decapitation. Hearts were removed. After the heart weight and cardiac morphology were examined, hearts were fixed in 10% formalin/PBS and embedded in paraffin. Heart sections (10 µm) were cut using a cryomicrotome stained and placed on a slide. Sections were incubated in clearing agent 3 X for 5 minutes by changing the clearing agent after every 5 minutes. Slides were incubated in 100% ethanol for 3 times for 3 minutes each, in 95% ethanol for 3 times for 3 minutes each, in 80% alcohol for 3 minutes and finally wash tap water for 3 minutes. 0.1% Sirius Red was added and incubated for 60 minutes. The slide was washed with acidified water and air-dried. After drying, the slides were washed for 1 minute in 80% alcohol, 2 minutes in 95% ethanol, 2 times in 100% ethanol for 10 minutes each time, and finally 3 times for 3 minutes in clearing solution. Slides

were covered with a cover slip. Digital photographs were taken at a magnification of 400X. The histology, morphology, and cross-sectional area of cardiomyocytes were examined and analyzed by the image analysis software (Image Pro Plus 6.0, Media Cybernetics, Bethesda, MD). The external diameter of the arteries measured was no more than 100 μ m and the number of arterial cross-sections measured was more than 50, from five rats in each group.

5.2.9. Immunohistochemistry

To determine the localization of lentiviral vector-mediated gene transfer within the PVN, immunohistochemistry was performed on the brain sections containing the PVN in the rat that received Lv-SCR-shRNA or Lv-APJ-shRNA injection. Rats were perfused transcardially with 50 ml of saline followed by 50 ml of 4% paraformaldehyde solution for 1 h. The brain was removed, post fixed in 4% paraformaldehyde solution for 24 h, and transferred to PBS containing 20% sucrose. Frozen brain tissues were embedded in OCT medium and cut into 10-micron coronal sections on Leica CM1950 cryostat (Leica Microsystems, Buffalo Grove, Illinois). The PVN sections, identified with a rat brain atlas, were incubated with PBS plus 0.5% tween 20 (PBS-T) containing 5% goat serum for 60 min at room temperature to quench the non-specific binding. Slices were incubated with primary antibodies (anti-Neun monoclonal antibody (Millipore, Billerica, MA) 1:500 and rabbit anti-GFP polyclonal antibody (Abcam, Cambridge, MA.) 1:500 overnight at 4°C. After being washed with PBS-T, the sections were incubated with secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies Grand Island, NY) diluted at 1:1000 and Alexa Fluor 594 goat anti-mouse IgG (Life Technologies Grand Island, NY) diluted at 1:1000 for 2 h. The sections were then washed with PBS-T and staining was detected with an Olympus FluoView FV300 fluorescent microscope (Olympus, Melville, NY) connected to a computer to capture and analyze images with Flow View Software.

5.3. Results and Data Analysis

5.3.1. Statistical Analyses

All data are presented as means \pm SE. Statistical significance was evaluated by 1- or 2-way ANOVA, as appropriate, followed by either a Newman–Keuls or Bonferroni post hoc analysis when indicated. Differences were considered significant at $P < 0.05$, and individual probability values were noted in the figure legends.

5.3.2. Efficacy of Lenti-APJ-shRNA *In vivo*

To confirm the efficiency of Lv-APJ-shRNA in silencing APJ receptor expression in the PVN of MI and sham rats, western blot analysis was performed to determine whether protein levels of the APJ receptor in the PVN was reduced in the rats that received PVN microinjection of Lv-APJ-shRNA as compared with those that received Lv-SCR-shRNA. The APJ receptor protein levels in the PVN were examined using regular western blot analysis after 0, 2, and 4 weeks of injection of the virus. The results are presented in figure 23 showing that APJ protein levels were significantly decreased in the PVN of MI rats that received Lv-APJ-shRNA injection as compared with those that received Lv-SCR-shRNA ($n=8$, $p < 0.01$). Additionally, APJ receptor protein levels was also decreased in sham rats that received Lv-APJ-shRNA injection as compared to those that received the Lv-SCR-shRNA injection ($n=8$, $p < 0.01$). Taken together, these results confirmed APJ receptor expression was down-regulated in MI and sham rats after injection of lentivirus carrying shRNA transcript against APJ receptor.

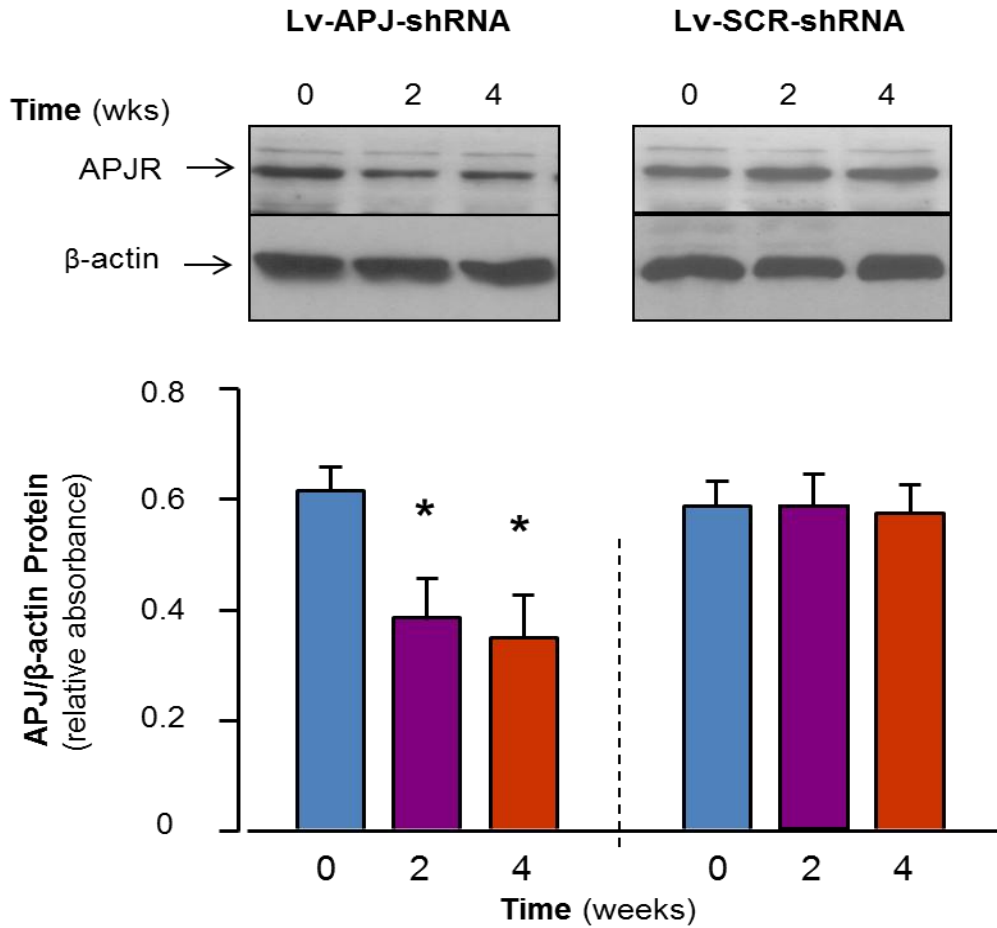


Figure 23. APJ receptor protein levels in the PVN of MI rats receiving lentiviral vector-mediated transduction of APJ-shRNA. Upper panel showing representative blots of APJ receptor protein levels in the PVN of MI rats. APJ receptor protein levels were measured using Western Blot analysis in the PVN micropunches from the brain section of rats treated with Lv-APJ-shRNA or Lv-SCR-shRNA at the time points indicated in the figure. Bar graphs in lower panel summarizing the quantitation of APJ receptor protein levels in the PVN of MI rats at the time points after receiving Lv-APJ-shRNA or Lv-SCR-shRNA. Data are means \pm SE normalized to β -actin (n=4 rats in each group). * $P < 0.05$ versus the control rats without viral vector injection (blue bars).

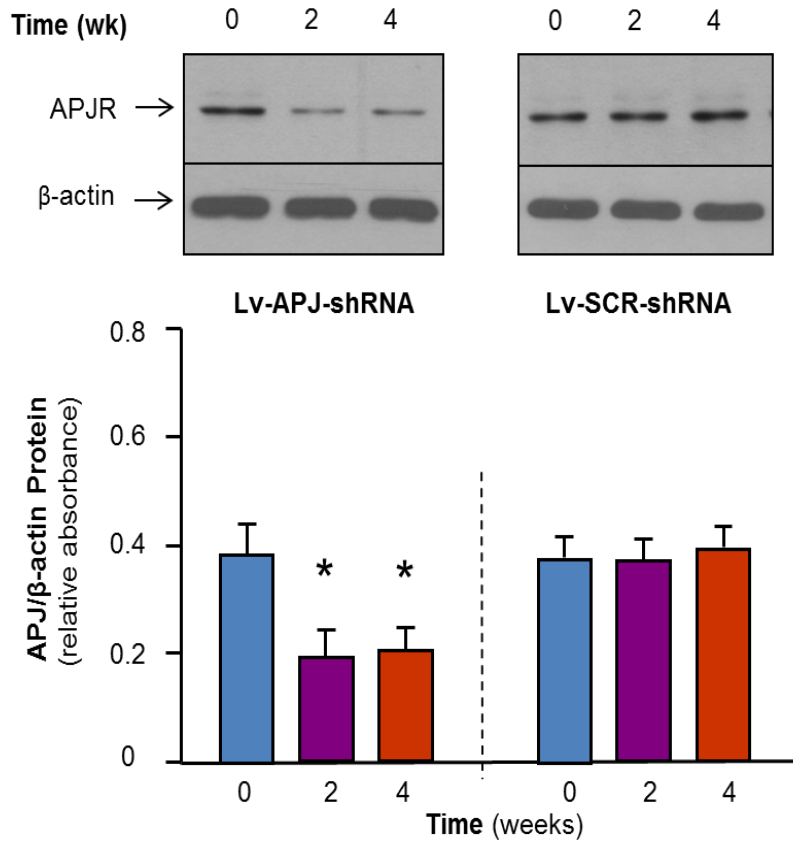


Figure 24. APJ receptor protein levels in the PVN of sham rats receiving lentiviral vector-mediated transduction of APJ-shRNA. Upper panel showing representative blots of APJ receptor protein levels in the PVN of sham rats. APJ receptor protein levels were measured using Western Blot analysis in the PVN micropunches from the brain section of rats treated with Lv-APJ-shRNA or Lv-SCR-shRNA at the time points indicated in the figure. Bar graphs in lower panel summarizing the quantitation of APJ receptor protein levels in the PVN of sham rats at the time points after receiving Lv-APJ-shRNA or Lv-SCR-shRNA. Data are means \pm SE normalized to β -actin (n=4 rats in each group). * $P < 0.05$ versus the control rats without viral vector injection (blue bars).

5.3.3. Effect of Lenti-APJ-shRNA Microinjected into PVN on Cardiac Hemodynamics

To determine the effect of down regulation of APJ receptor in the PVN on cardiac hemodynamics, lenti-APJ-shRNA virus and lenti-SCR-shRNA was injected into the PVN of sham and MI rats. Left ventricular function was measured using cardiac catheter inserted through

the left carotid artery as described above. Results demonstrated that PVN microinjection of Lenti-APJ- shRNA did not alter the HW/BW ratio in sham rats. PVN microinjection of Lenti-APJ-shRNA significantly attenuated MI-induced increases in HW/BW (Data not shown), suggesting a reduction in cardiac hypertrophy induced by MI.

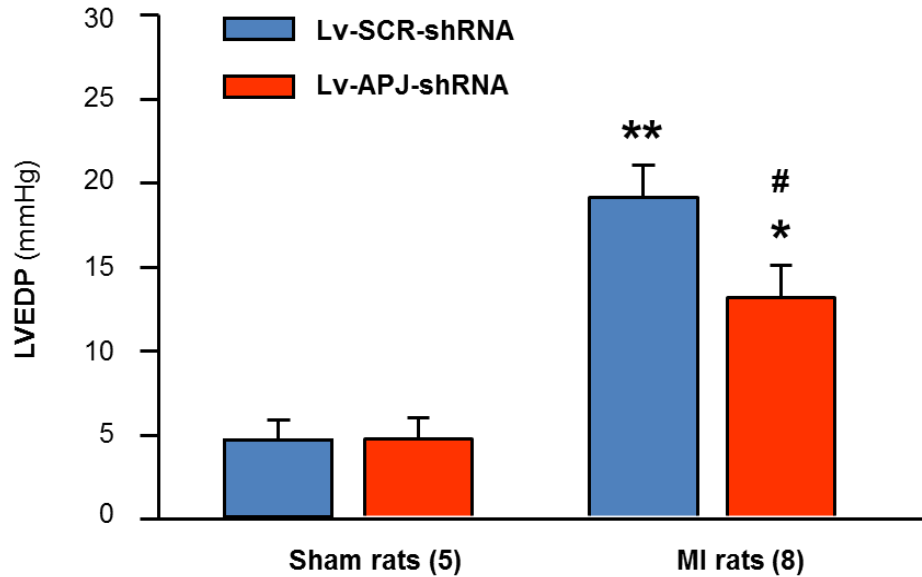


Figure 25. Effect of lentivirus-mediated delivery of short-hairpin small-interference RNA for APJ receptors on left ventricular end-diastolic pressure (LVEDP). LVEDP was recorded in sham or myocardial infarction (MI) Rats four weeks after PVN microinjection of Lv-APJ-shRNA or scramble control (Lv-SCR-shRNA) viral vector. Data are means \pm SE (n=5 or 8 rats). *P<0.05 compared with respective sham rats. **P<0.01 compared with respective sham rats. #P<0.05 compared with MI rats received Lv-SCR-shRNA.

The LV end-diastolic pressure (LVEDP) indicates the blood volume remaining after ventricular contraction; thus, is a very important parameter for left ventricular contractile function. This work examined the effect of microinjection of Lv-APJ-shRNA or Lv-SCR-shRNA into the PVN on the LVEDP in MI and sham rats. The results are presented in figure 25, showing that MI induced a significant increase in LVEDP. PVN microinjection of Lv-APJ-shRNA did not alter LVEDP in sham rats (4.67 ± 1.22 mmHg in Lv-SCR-shRNA and 4.70 ± 1.27 mmHg in Lv-

APJ-shRNA, n=5, p>0.05). However, PVN microinjection of Lv-APJ-shRNA attenuated MI-induced increases in LVEDP by 42% as compared with rats that received Lv-SCR-shRNA (n=5 or 8 rats, P<0.05).

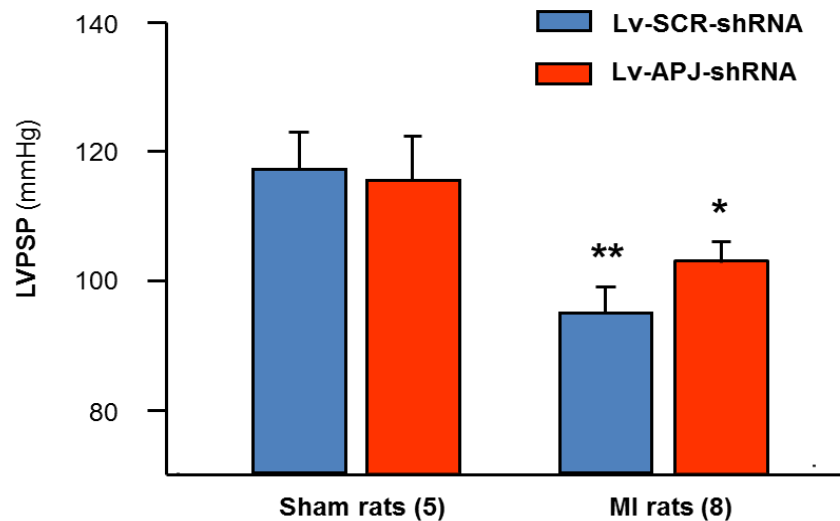


Figure 26. Effect of lentivirus-mediated delivery of short-hairpin small-interference RNA for APJ receptors on left ventricular peak systolic pressure (LVPSP). LVPSP was recorded in sham or myocardial infarction (MI) Rats four weeks after PVN microinjection of Lv-APJ-shRNA or scramble control (Lv-SCR-shRNA) viral vector. Data are means \pm SE (n=5 or 8 rats). *P<0.05 compared with respective sham rats. **P<0.01 compared with respective sham rats.

The effect of microinjection of Lv-APJ-shRNA or Lv-SCR-shRNA into the PVN on the left ventricle peak systolic pressure (LVPSP) was also examined in MI and sham rats. The result is presented in figure 26, demonstrating that MI induced a significant reduction in LVPSP. PVN microinjection of Lv-APJ-shRNA did not alter LVSP in sham rats (117.3 \pm 5.7 in Lv-SCR-shRNA and 115.5 \pm 6.9 in Lv-APJ-shRNA, n=5 in each group, P>0.05). However, PVN microinjection of Lv-APJ-shRNA attenuated MI-induced decrease in LVPSP by 24% compared with Lv-SCR-shRNA.

Left ventricular pressure rising rate (dp/dt, including LV dp/dt max and Lv dp/dt min) was the most important parameter, which reflects left ventricular contractility independently from ventricular pressure alteration. This research examined the effect of microinjection of Lv-APJ-shRNA or Lv-SCR-shRNA into the PVN on the LV dp/dt max and LV dp/dt min in both MI and sham rats. The results are presented in figure 27 and demonstrate that MI induced significant decreases in LV dp/dt max. PVN microinjection of Lv-APJ-shRNA did not alter the LV dp/dt max in sham rats (7635.6 ± 234.7 mmHg/s in Lv-SCR-shRNA and 7592.2 ± 223.1 mmHg/s in Lv-APJ-shRNA, $n=5$ rats in each group, $P>0.05$). However, PVN microinjection of Lv-APJ-shRNA attenuated MI-induced decreases in LV dp/dt max by 45% compared with Lv-SCR-shRNA ($n=5$ or 8 rats, $P<0.05$). The same alteration was also observed for LV dp/dt min as shown in figure 27.

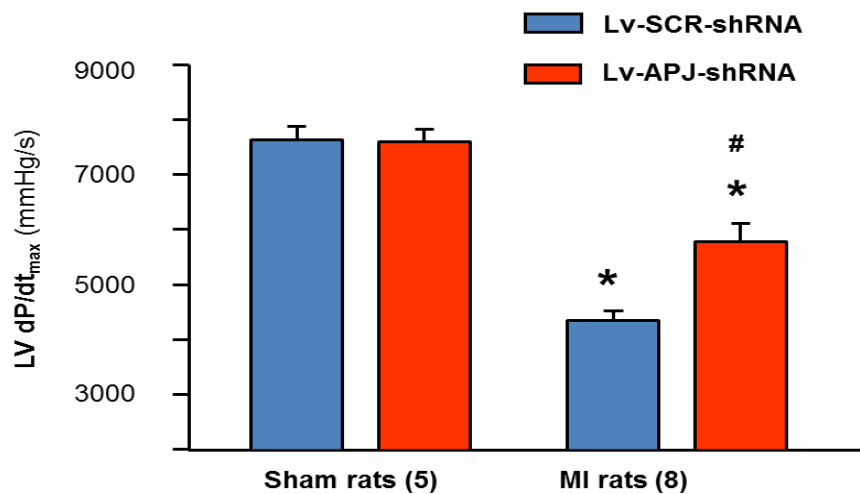


Figure 27. Effect of lentivirus-mediated delivery of short-hairpin small-interference RNA for APJ receptors on left ventricular maximum rate of rise of left ventricular pressure (LV dp/dt_{max}). LV dp/dt_{max} was recorded in sham or myocardial infarction (MI) Rats four weeks after PVN microinjection of Lv-APJ-shRNA or scramble control (Lv-SCR-shRNA) viral vector. Data are means \pm SE ($n=5$ or 8 rats). * $P<0.05$ compared with respective sham rats. ** $P<0.01$ compared with respective sham rats.

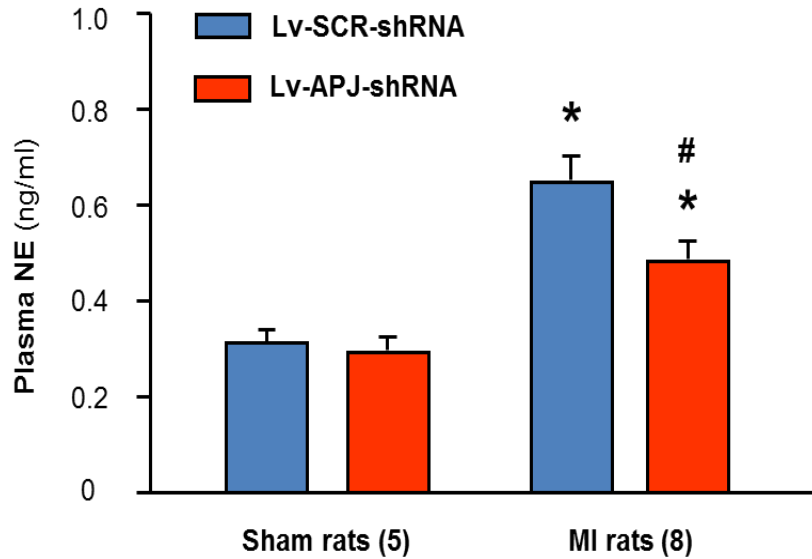


Figure 28. Effect of lentivirus-mediated delivery of short-hairpin small-interference RNA for APJ receptors on plasma norepinephrine (NE) levels. Plasma NE levels were measured as described in Methods in sham or myocardial infarction (MI) Rats four weeks after PVN microinjection of Lv-APJ-shRNA or scramble control (Lv-SCR-shRNA) viral vector. Data are means \pm SE (n=5 or 8 rats). *P<0.05 compared with respective sham rats. #P<0.05 compared with MI rats received Lv-SCR-shRNA control.

5.3.4. Effect of Lenti-APJ-shRNA on Plasma Norepinephrine Levels

Increased SNS activity is a compensatory mechanism in HF. Hyperactive SNS increases cardiac output by increasing HR thus releasing the neurotransmitter NE. Increased circulating plasma NE levels are a hallmark pathophysiology of HF (Pepper et al., 1999). Then the effect of PVN microinjection of Lv-SCR-shRNA or Lv-APJ-shRNA on the plasma NE levels in MI and sham rats was examined. The plasma NE levels were examined four weeks post injection of virus into the PVN using an ELISA kit. Results are presented in figure 28, demonstrating that plasma NE levels increased in MI as compared with sham rats. PVN microinjection of Lv-APJ-shRNA did not alter the plasma NE levels; however, significantly attenuated MI-induced increases in NE level by 40%. NE elevation is a significant indicator for the chronic sympathetic

nerve activation. Thus, this result suggests microinjection of Lv-APJ-shRNA into the PVN may attenuate NE plasma levels and sympathoexcitation in MI rats.

5.3.5. Effect of Lenti-APJ-shRNA on Cardiac Hypertrophy

Cardiac Hypertrophy is an important morphological marker in HF. Decreased cardiomyocyte diameter and cardiac hypertrophy is an important manifestation for attenuation of HF (Triposkiadis et al., 2009). The effect of PVN microinjection of Lv-SCR-shRNA or Lv-APJ-shRNA on the cardiomyocyte hypertrophy was examined in sham and MI rats. Hearts from each group were cut using a freezing microtome and stained with eosin and haematoxylin. Results are presented in figure 29 demonstrating that MI induced a significant increase in cardiomyocyte diameter. However, PVN microinjection of Lv-APJ-shRNA did not significantly alter the size of cardiomyocytes as compared with Lv-SCR-shRNA in sham rats. Microinjection of Lv-APJ-shRNA into the PVN significantly attenuated MI-induced increases in cardiomyocyte diameter by 48% (n=5, P<0.05) as compared with Lv-SCR-shRNA. All together, these results suggest that down regulation of APJ receptor in the PVN attenuates MI-induced cardiac hypertrophy.

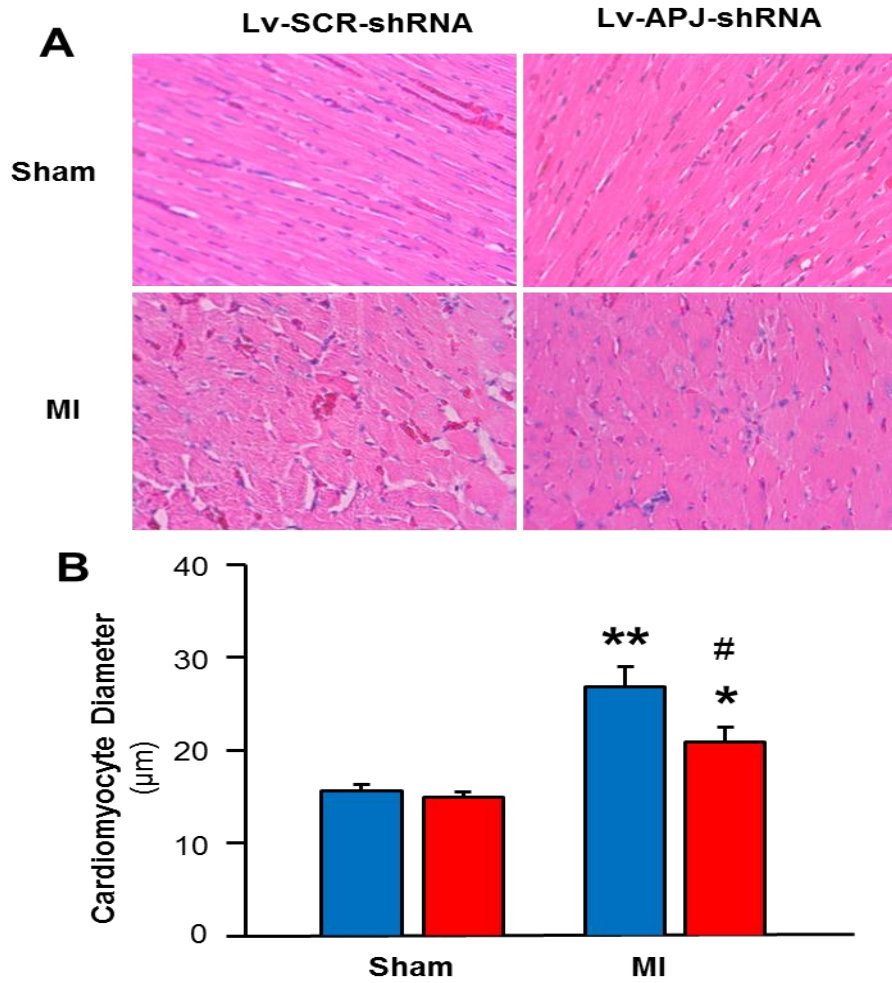


Figure 29. Effect of lentivirus-mediated delivery of shRNA for APJ receptors on myocardial hypertrophy in the peri-infarct area of left ventricle. The left ventricular sections were stained with hemotoxylin and eosin (HE) to determine the size of cardiomyocytes of sham or MI rats at four weeks after microinjection of viral vectors into the PVN. Upper panel showing representative photomicrographs (X40 magnification) of HE-stained sections . B, Quantitative analysis of myocyte diameters in sham and MI rats at four week after microinjection of viral vectors into the PVN. Data are means \pm SE (n=5 rats in each group). *P<0.05 vs. sham; **P<0.01 vs. sham; #P<0.05 vs. MI rats treated with Lv-SCR-shRNA.

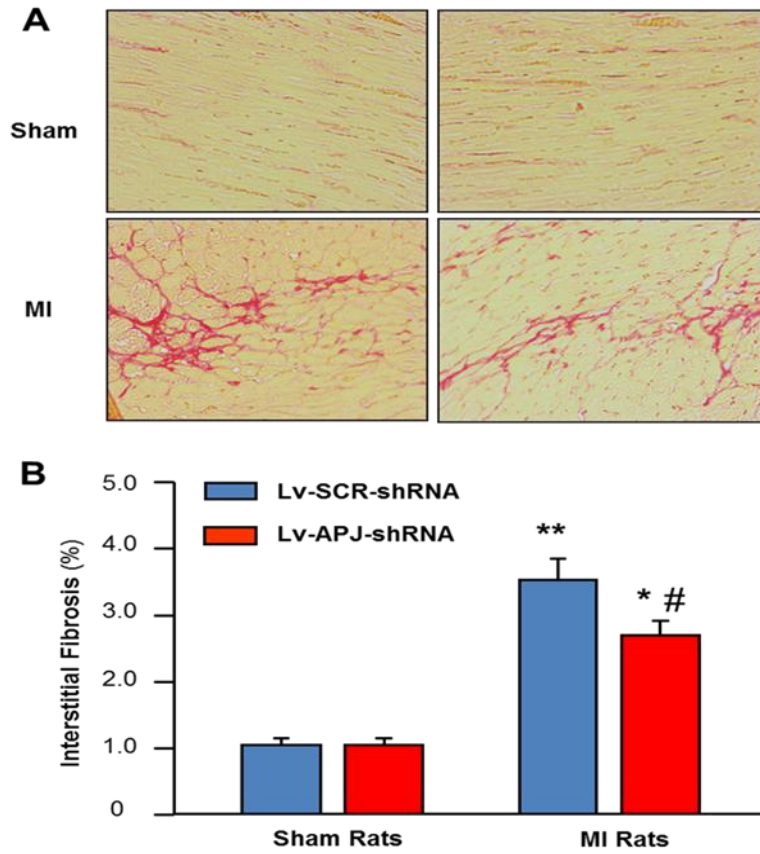


Figure 30. Effect of lentivirus-mediated delivery of shRNA for APJ receptor on interstitial fibrosis in the peri-infarct area of left ventricle. The left ventricular sections from sham or MI rats were stained with Sirius red to determine the interstitial fibrosis four weeks after microinjection of Lv-APJ-shRNA or its scramble control Lv-SCR-shRNA viral vector into the PVN. A, Representative photomicrographs (X40 magnification) showing Sirius red-stained myocardial sections in the peri-infarct area of left ventricle. B, Quantitative analysis of interstitial fibrosis four weeks after microinjection of viral vectors into the PVN in sham and MI rats. Data are means \pm SE (n=5 rats in each group). *P<0.05 versus sham rats; **P<0.01 versus sham rats; #P<0.05 versus MI rats treated with Lv-SCR-shRNA control.

5.3.6. Effect of Lenti-APJ-shRNA on Cardiac Fibrosis

Cardiac fibrosis is one of the main characteristic of cardiac remodeling during HF. Thus, this work examined the effect of PVN microinjection of Lv-APJ-shRNA or Lv-SCR-shRNA on the cardiac fibrosis in MI and sham rats. The fibrosis in the heart was detected by histological staining of heart section with Sirius red. Results are presented in figure 30 demonstrating that MI

induced a significant interstitial fibrosis. PVN microinjection of Lv-APJ-shRNA did not alter fibrous content in the heart. However, this injection significantly attenuated MI-induced cardiac fibrosis by 28% (n=5 rats in each group, $P < 0.05$). These results demonstrate that down regulation of APJ receptor in the PVN attenuate MI-induced cardiac fibrosis.

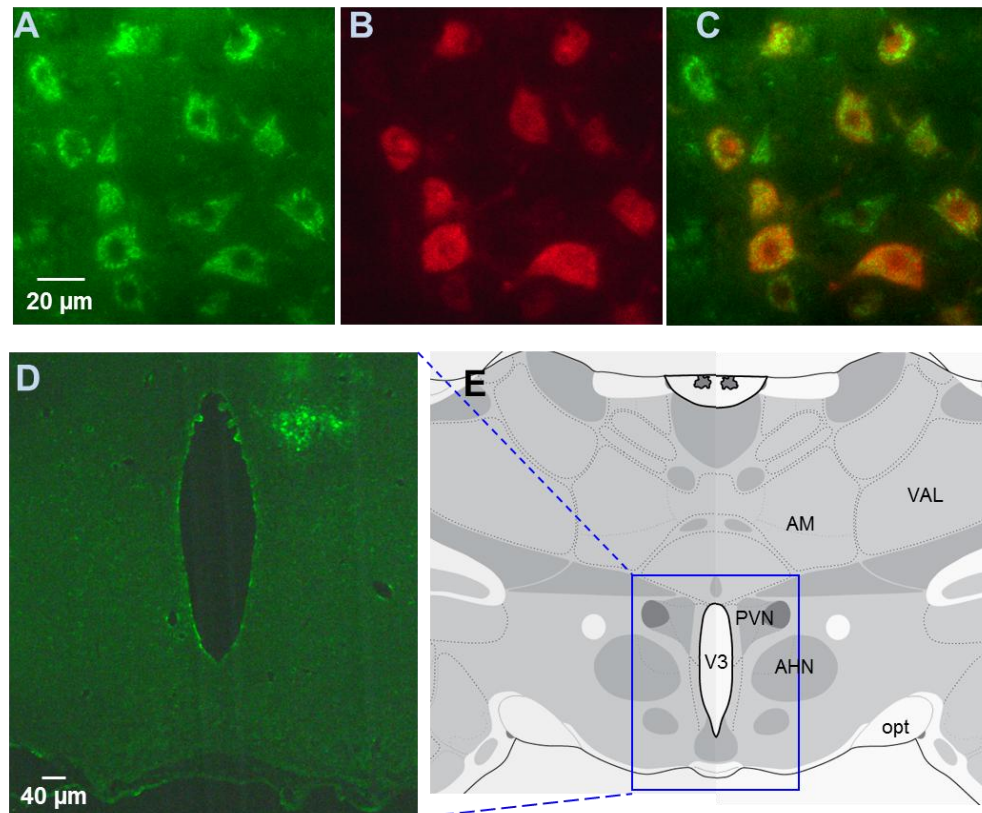


Figure 31. Lentiviral vector-mediated gene transfer in neurons within the PVN of rats. Immunofluorescence images showing the location of gene transfer in the PVN of rats using lentiviral vector. A, fluorescence micrograph (X40 magnification) demonstrating location of GFP in PVN neurons (green). B, Same field of cells as in panel A, immunostained with anti-NeuN antibodies (red). C, Overlap of A and B, showing neurons with GFP gene transfer. D, Fluorescence micrograph (X10 magnification) demonstrating localization of GFP within the PVN after gene transfer. E, Location of the stained PVN brain section shown in A-D, based on the rat brain atlas of Swanson. AHN, anterior hypothalamic nucleus; AM, anteromedial nucleus thalamus; PVN, paraventricular hypothalamic nucleus; opt, optic tract; VAL, ventral anterior-lateral complex thalamus; V3, third ventricle.

5.3.7. Detection of Lentiviral Gene Transfer

To demonstrate efficiency of lentiviral gene transfer into the neurons within the PVN of rats after microinjection of Lv-APJ-shRNA or Lv-SCR-shRNA. Here, immunohistochemistry analysis was performed on rats that received PVN microinjection of Lv- SCR-shRNA or Lv-APJ-shRNA. The double immunostaining technique was used to detect GFP localization in the neurons of the PVN with GFP specific antibody and Anti-NeuN antibody as a neuronal marker. The results are shown in figure 31 and clearly demonstrate that viral vector-mediated gene transduction is localized within the PVN and that neurons in this brain area have significant GFP gene expression.

5.4. Data Summary and Conclusion

This work observed that APJ receptor expression in the PVN increased in MI compared to sham rats. To examine the role of the APJ receptor in the PVN in the MI-induced cardiovascular pathologies, a lentiviral vector-carrying transcript for APJ shRNA was constructed. In the present study, Lv-APJ-shRNA or Lv-SCR-shRNA was microinjected into the PVN of MI and sham rats. The results are summarized:

- 1) Immunohistochemistry was performed to demonstrate efficiency of lentiviral vector-mediated gene transfer in the neurons within the PVN after microinjection of Lv-SCR-shRNA or Lv-APJ-shRNA into the PVN. Results confirm that a successful gene transfer into the neurons of PVN as evidenced by co-localization of GFP and NeuN, a neuron marker. The results also confirmed the precise injection site.
- 2) The successful knockdown of APJ receptor expression in the PVN was confirmed in MI and sham rats after microinjection of Lv-APJ-shRNA by western blots and RT-PCR.

Microinjection of Lv-APJ-shRNA significantly reduced APJ receptor expression in both protein and mRNA levels. However, Microinjection of Lv-SCR-shRNA did not alter the APJ receptor expression.

- 3) Microinjection of Lv-APJ-shRNA into the PVN significantly attenuated MI-induced decrease in cardiac contractility. This conclusion was demonstrated by several evidences showing that PVN microinjection of Lv-APJ-shRNA in MI rat's significantly attenuated MI-induced elevation in LVEDP, MI-induced increase in LVdp/dt max and LV dp/dt min, and MI-induced decrease in LVSP.
- 4) PVN microinjection of Lv-APJ-shRNA significantly attenuated MI-induced increase in plasma NE level and in HR, which may be mediated by attenuation of sympathoexcitation in MI rats.
- 5) PVN microinjection of Lv-APJ-shRNA significantly attenuated MI-induced cardiac hypertrophy and fibrosis. This was demonstrated by microinjection of Lv-APJ-shRNA showing markedly reduced MI-induced increase in HW/BW and in size of cardiomyocyte in the MI rats.

In summary, all results verify that selective silencing of APJ receptor using Lv-APJ-shRNA in the PVN improves left ventricular contractility, reduces sympathoexcitation, and attenuates cardiac remodeling. Those are the most common pathophysiological alterations in HF. Those evidences strongly support the hypothesis that elevated action or expression in the Apelin/APJ system in the PVN contributes to the development of MI-induced cardiac injury or failure.

CHAPTER 6. DISCUSSION

6.1. Major New Discoveries in the Current Study

APJ receptor is a novel G-protein coupled receptor (GPCR) and its ligand, apelin, was discovered recently (O'Dowd et al., 1993). Apelin/APJ system participates in many physiological functions including energy metabolism, obesity, glucose metabolism, and cardiovascular homeostasis (Lee et al., 2005; Cheng et al., 2003; Reaux et al., 2001). In the brain, apelin-immunoreactive cell bodies, fibers, and mRNA for apelin and the APJ receptor are distributed predominantly in neurons of the hypothalamus and brainstem, including cardiovascular regulatory regions such as the paraventricular nucleus (PVN), supraoptic nucleus (SON), circumventricular organs, nucleus tractus solitarius (NTS), and rostral ventrolateral medulla (RVLM) (De Mota et al., 2000; O'Carroll et al., 2000; Reaux et al., 2002). Peripheral or central administration of Apelin-13 elevates blood pressure and cardiac hypertrophy (Tatemoto et al., 2001; Charles et al., 2006; Kagiya et al., 2005). Acute injection or chronic overexpression of apelin in the brain increases HR, mean arterial pressure, sympathetic overexcitation, and cardiac hypertrophy (Kagiya et al., 2005, Zhang et al., 2008). Intracerebral injection of apelin increased the C-fos activity in the PVN of the brain (Kagiya et al., 2005). More interestingly, apelin in the PVN regulate AVP excretion and water balance.

Based on all these evidences, elevated expression or activity of Apelin/APJ in the PVN was hypothesized to contribute to the pathophysiological cardiac damage induced by MI. To examine this hypothesis, APJ receptor expression in the cardiovascular regulatory brain areas, such RVLM, PVN, and NTS in MI rats was assessed. The results indicate that myocardial infarction results in an increased expression of APJ receptor in the PVN of the brain and down

regulation of this receptor attenuated the pathophysiology of HF, decreases SNS activity, and cardiac remodeling. All the above results confirm that down regulation of Apelin/APJ system in the PVN area of the brain improves the function of the heart.

6.2. Myocardial Infarction Induced Congestive Heart Failure

A MI rat model created by using ligation of the left coronary artery. Function of the heart was determined by using cardiac catheter inserted through the left carotid artery. Results showed the body weight and heart weight ratio was significantly increased in MI vs. sham rats.

Left ventricular systolic pressure and dP/dtmax were dramatically diminished and the left ventricular end-diastolic pressure was significantly elevated in MI compared with sham rats. These results indicate that MI results in a severe left ventricular dysfunction. Histological analysis by eosin haematoxylin staining confirmed myocardial infarction in MI rats.

Further work determined that the expression of APJ receptor in several cardiovascular regulatory areas of the brain by RT-PCR and western blot analysis showed increased expression of APJ receptor in the PVN of the brains of MI as compared to sham rats confirming with earlier studies (O'Carroll et al., 2003; Zhang et al., 2009). Immunohistochemistry was performed on PVN sections of brain of MI rats and results showed localization of APJ receptor in the neurons of the PVN corroborating earlier studies (De Mota et al., 2004; Roberts et al., 2009; Kagiya et al., 2005).

6.3. Mechanisms of Lenti-APJ-shRNA in APJ Gene Silencing

We created Lenti APJ shRNA and Lenti SCR shRNA viral vectors to study whether down regulation of Apelin/APJ system would attenuate the pathophysiology of HF. The efficiency of

the virus to specifically knockdown the APJ receptor was determined by transducing a Cath.a cell line and rat primary neuronal cell culture with Lenti APJ-shRNA virus and analyzed by RT-PCR and western blot analysis that showed knockdown of APJ receptor *in vitro*.

To study whether down regulation of APJ receptor in PVN of the rat brain Lenti-APJ-shRNA and Lenti-SCR-shRNA virus was injected into the PVN of the MI and sham rat brains and successful knockdown was confirmed using western blot analysis. Results showed that injection of virus decreased body weight and heart weight ratio in MI rats injected with Lenti APJ-shRNA virus as compared to MI rats injected with Lenti SCR-shRNA virus. Left ventricular systolic pressure and dP/dtmax were increased and the left ventricular end-diastolic pressure was significantly decreased in MI rats Lenti APJ-shRNA virus compared to MI rats injected with Lenti SCR-shRNA virus and no change was observed in cardiovascular hemodynamics of Sham rats that received Lenti-APJ-shRNA virus and Lenti-SCR-shRNA virus. In the earlier studies conducted (Zhang et al., 2008; Kagiya et al., 2005; Seyedabadi et al., 2002) it was reported that injection or over expression of apelin in brain increases blood pressure, HR, and cardiac hypertrophy and in other study (Atluri et al., 2007) it was shown that myocardial infarction increases circulating APJ levels. The current study is the first to show that APJ expression is increased in the hypothalamic nuclei of the brain during the time of HF and down regulation of APJ receptor in the PVN of the brain attenuates the pathophysiology of heart failure.

These results indicate that enhanced activity of Apelin/APJ system in the PVN of the rat brain plays an important role in pathophysiology of HF and down regulation of this system attenuates pathophysiology of HF.

6.4. Possible Signaling Pathway Underlying Enhanced APJ Expression in the PVN of Congestive Heart Failure

Apelin/APJ system is a novel system with significant cardiovascular functions. Apelin/APJ levels are elevated during HF or myocardial ischemia MI in rats and mice. However, the mechanisms behind the enhanced expression of Apelin/APJ system are an enigma. CHF by definition is a disease associated with decreased heart contractility and increased fluid buildup in the body causing pulmonary edema in the lungs. CHF patients have low oxygen tensions in tissues and cells and difficulty in breathing due to fluid buildup in the body leading to a condition known as hypoxia (Jessup et al., 2003; Costa et al., 2006).

Hypoxia leads induction of transcription in various genes involved in iron metabolism, glucose metabolism, and cell proliferation and survival. The main factor mediating such a response is the hypoxia-inducible factor-1 (HIF-1), an oxygen-sensitive transcriptional activator. In hypoxia, the HIF-1 subunit becomes stable and interacts with co-activators such as cAMP response element-binding protein binding protein/p300 and regulates the expression of target genes (Costa et al., 2006). HIF-1 factor is reported to be activated during ischemia and coronary artery occlusion.

A recent study (Ronkainen et al., 2007) showed that pharmacological activation of HIF-1 in cardiomyocyte culture increased apelin expression and at the same time inhibition of HIF-1 lead to a decrease in apelin expression. In a similar study (Sheikh et al., 2006) in which human coronary arteries were kept under hypoxic conditions, data showed increased expression of Apelin/APJ system. In another study it was shown that apelin was a HIF-1 target gene and demonstrates that on hypoxia, HIF-1 binds to the first intron of apelin leading to up regulation of

apelin expression (Eyries et al., 2008). All these evidences indicate that enhanced APJ receptor expression in the PVN of the brain of the MI rat may be due to activation of HIF-1 factor that in turn induces APJ receptor expression in MI rats.

6.5. PVN Apelin/APJ System in the Regulation of Sympathetic Nerve Activity

Increased SNS activity is an important pathophysiological manifestation in HF. In HF, the heart compensates its inability to pump the blood by employing several mechanisms with increased SNS activity among them (Triposkiadis et al., 2009). Increased SNS activity direct the release of endogenous neurohormones, such as NE, resulting in an increase in myocardial contractility, venous blood flow return, and revival of normal cardiac function. However, this adaptive response is short term and harmful. Chronic elevation of SNS activity causes arrhythmias, activation of rennin angiotensin system (Hogg et al., 2005; Grassi et al., 2009; Todd et al., 1985; Mann et al., 1992). All of these factors cause alterations in myocardial cell phenotype, myocardial cell death, cardiac remodeling, and other symptoms.

In a recent study (Zhang et al., 2009) it was shown that administration of apelin increases RSNA in the rats and in another study it was shown that APJ knockout mice showed abnormal fluid homeostasis and vasopressinergic activity and in the same study c-fos analysis showed decreased neuronal activity in the hypothalamic nuclei of the brain indicating the importance of Apelin/APJ system in SNS and vasopressinergic activity of the body (Roberts et al., 2009). In a recent study it was demonstrated that super fusion of neurons cultured from neonatal rat brainstem with apelin-13 resulted in a two-fold increase in neuronal firing rate(Sun et al., 2007). All these evidences suggest the role of apelin in the activation of SNS. Increased SNS activity is one of the major compensatory mechanisms employed by the heart during HF. This study

assessed the SNS activity by measuring plasma NE levels and results showed injection of Lenti APJ-shRNA virus reduced sympathetic nerve activity in MI rats. These results indicated that down regulation of Apelin/APJ system in PVN attenuates increased SNS activity MI rats.

6.6. *In Vitro* Studies

Interstitial fibrosis and cardiac hypertrophy are biomarkers for cardiac remodeling (Jessup et al., 2003). Earlier studies showed that i.c.v. injection or overexpression of apelin in the brain (Zhang et al., 2000; Kagiya et al., 2005) resulted in increased HR and cardiac hypertrophy. We performed histological analysis to determine whether down regulation of Apelin/APJ system decrease the symptoms of HF. Results from siruis red staining shows decrease fibrosis in MI rats that are injected with Lenti APJ-shRNA virus as compared to MI rats injected with Lenti SCR-shRNA virus. Eosin haematoxylin staining showed a decrease in cardiac hypertrophy of MI rats injected with Lenti APJ-shRNA virus. These results showed that down regulation of Apelin/APJ alters cardiac remodeling of HF. In summary, all these studies showed that APJ receptor expression was increased in PVN of brain in conditions of HF and selective silencing of APJ receptor in the PVN of rat attenuates the pathophysiology of HF and prevents cardiac remodeling.

CHAPTER 7. CONCLUSIONS

7.1. Conclusions

This study we created a MI rat model by ligating coronary artery and left ventricular dysfunction was confirmed by measuring hemodynamics using a cardiac catheter inserted through the left carotid artery. This work measured the level of APJ receptor expression in various cardiovascular regulatory regions using RT-PCR, western blot, and immunohistochemical analysis and found that APJ receptor expression was enhanced in the PVN of the brain and this enhanced APJ Receptor Expression in the PVN was associated with exacerbating the pathophysiology of HF.

Lentiviral vectors were created to knockdown the APJ receptor in order to determine whether down regulation of APJ receptor attenuates the pathophysiology of HF. Lentiviral vectors carrying APJ-shRNA and lentiviral vectors containing SCR-shRNA were injected into the PVN of MI and sham rats. Results showed that down regulation of the APJ receptor improved the left ventricular function in MI rats and no change was observed in LV hemodynamics of sham rats injected with Lenti-SCR-shRNA virus; hence, it can be concluded that down regulation of APJ receptor in the PVN of the rat brain in MI rats alleviated the cardiac dysfunction associated with MI.

Cardiac hypertrophy and interstitial fibrosis are hallmarks of failing heart (Jessup et al., 2003; Triposkiadis et al., 2009). This research injected lenti-APJ-shRNA and lenti-SCR-shRNA virus into MI and sham rats and observed the effect of knockdown of APJ receptor on cardiac hypertrophy and fibrosis. Results showed that knockdown of APJ receptor attenuated myocardial fibrosis and cardiac hypertrophy. Increased SNS activity to compensate for the inability of the

heart to pump the blood is an important compensatory mechanism in HF. This study found that the APJ receptor was over expressed in PVN of the brain of MI rats and this over expression was associated with increased SNS activity in MI rats. Lenti-APJ-shRNA virus injected into PVN of the MI rats showed down regulation of APJ receptor decreased the plasma NE levels thereby decreasing SNS activity. Thus, the Apelin/APJ pathway in the PVN plays an important role in sympatho excitation and pathophysiology of CHF.

7.2. Future Research Directions

In the light of evidence from the current study regarding the role of the Apelin/APJ system in the pathophysiology of HF, it becomes extremely important to understand the molecular mechanisms and pathways this receptor and ligand employ to bring about a physiological change in the body. It is evident from earlier studies that Apelin stimulates neurons the respective firing rate. Recent studies from this lab showed that apelin-13 directly increases neuronal activity via stimulation of NAD(P)H oxidase-derived superoxide (Sun et al., 2007; Yao et al., 2011) hence it would be interesting to study the role of this pathway in the pathophysiology of HF. Hypoxia is one of the important symptoms in heart failure. HIF-1 is known to up regulate the expression of Apelin/APJ expression in the conditions of hypoxia. Therefore further studies should be carried out to further understand the molecular mechanisms behind hypoxia and Apelin/APJ expression in pathophysiology of HF.

7.3. Clinical Significance

This is the first study to show that hyperactive Apelin/APJ system activates SNS activity in HF and exacerbates the pathophysiology of the condition. In this study the data showed that the APJ receptor is overexpressed in PVN of the brain after MI and down regulation of this

system by RNAi technology attenuated the pathophysiology of HF, This study showed that targeting the Apelin/APJ system by developing pharmacological agents or gene therapy in the patients with HF may attenuate the pathophysiology of the condition. Hence, future research should be carried out in developing targeting agents against apelin and the APJ receptor.

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