NOS1-ADAPTOR PROTEIN DYSFUNCTION IN THE NUCLEUS TRACTUS SOLITARII CONTRIBUTES TO THE NEUROGENIC HEART DAMAGE AND QT INTERVAL PROLONGATION

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Variants of the Nitric Oxide Synthase 1 Adaptor Protein (NOS1AP) locus are strongly related to QT interval prolongation and sudden cardiac death (SCD) in human. Neurogenic cardiac damage due to subarachnoid hemorrhage, stroke, epilepsy and myocardial infarction is known to contribute to sudden death in most cases. Our aim was to study the role of NOS1AP in the neurogenic cardiac damage by silencing NOS1AP expression in the Nucleus Tractus Solitarii (NTS) area of the brainstem using lentiviral vector-mediated NOS1AP shRNA (Lv-NOS1AP-shRNA). Real time PCR data showed NOS1AP mRNA levels were expressed in the NTS 3-fold higher than other organs such as kidney and heart in Sprague Dawley (SD) rats. Microinjection of Lv-NOS1AP-shRNA in the NTS caused significant reduction in NOS1AP expression in SD rats. NOS1AP knockdown in NTS did not alter blood pressure (BP), heart rate (HR) recorded by radiotelemetry. However, ECG analysis revealed heart rate variability (HRV) was significantly reduced (SDNN, 51.2±5.6 vs 5.0±1.3ms, $P< 0.001$, n=6) and QTc interval was markedly prolonged (72.4±4 vs 105±11 ms, $P< 0.05$, n=6) in NOS1AP knockdown rats. Myocardial damage was also observed with the downregulation of NOS1AP in the NTS of SD rats due to the presence of contraction band necrosis. To study the cellular mechanisms underlying NOS1AP action, we investigated the effect of NOS1AP knockdown on NMDA-induced neurotoxicity in primary cultured neuronal cells from the brainstem. Treatment of cells with Lv-NOS1AP-shRNA significantly reduced NOS1AP expression, and was associated with increased NO production and NMDA-induced neurotoxicity, suggesting a protective effect of NOS1AP. Co-immunoprecipitation studies revealed that the association between neuronal Nitric Oxide Synthase (nNOS) and NMDA Receptor (NMDAR) was significantly increased in neurons

treated with Lv-NOS1AP-shRNA, suggesting that NOS1AP might compete with NMDAR in binding to nNOS. Therefore, knockdown NOS1AP expression results in increased association between NMDAR and nNOS, leading to elevated glutamate-induced NO production and neurotoxicity.

In summary, all results indicate that NOS1AP plays an important role in the protection of neurons from glutamate-induced neurotoxicity. NOS1AP dysfunction in the NTS might increase the risk of neurogenic cardiac damage, leading to QT interval prolongation, even sudden cardiac death.

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There are many people to whom I owe a debt of thanks. This dissertation would not have been possible without the kind and generous support of the people I am about to acknowledge. As a foreign student, I appreciate all the faculty, staff and students from the Department of Pharmaceutical Sciences at North Dakota State University. The harmonious environment and warm family-like atmosphere in the department made my stay happy and wonderful.

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I thank each and every one of them for making this possible for me!

DEDICATION

This dissertation is dedicated to the memory of *my loving Grandparents*- people who I admire the most. They continue to inspire me till today with their love, wisdom and knowledge, showered unconditionally on me. Thank you for instilling in me, the values and virtues that have guided me to come this far and will continue to do so.

Here's to hoping that I continue to do you all proud-even in times to come!

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LIST OF ABBREVIATIONS

µg/ml…….....Microgram per milliliters

µm…..…….Micro moles

µl…….....liter

nm……..Nano meter

nM…….….Nano mole

AAV ……..Adeno-Associated Viral vectors

ACTH…….…Adrenocorticotropin hormone

AMPA……....α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ACC……...Anterior cingulate cortex

ANS……...Autonomic Nervous System

ARC……β-cytosine arabinoside

AU……..……Arbitrary Units

BBB……...Blood Brain Barrier

BP…….……Blood Pressure

Ca^{2+}…….…...Calcium ion

cDNA……..…….Complementary Deoxyribonucleic Acid

CAPON……..Carboxy-terminal PDZ ligand of nNOS

CHF…….....Congestive Heart Failure

CHD……...Coronary heart disease

CNS…….……...Central nervous system

CVD…….……..Cardiovascular Diseases

CVM……..Cardiac Vagal Motoneurones

CVLM……....Caudal Ventrolateral Medulla

DAR-4M…….……...Diaminorhodamine-4M

DMEM……...Dulbecco's minimal essential medium

DMSO……....Dimethyl Sulfoxide

DNA……...Deoxyribonucleic Acid

DRG…….....Dorsal root ganglia

dsRNA……Double-stranded RNA

ECG…….…..….Electro Cardio Gram

ECL……...…....Enhanced Chemiluminescence

EDTA…… ….Ethylenediaminetetraacetic acid

EF……..Ejection Fraction

FACS.....…… .....Fluorescence Activated Cell Sorter

FBS….....Fetal Bovine Serum

GABA……...……γ-aminobutyric acid

GFP……..…..Green Fluorescence Protein

GWAS……....Genome-wide association study

H&E……...Hematoxylin and Eosin

HF…….….Heart Failure

HR…….....Heart rate

HRV……...Heart Rate Variability Analysis:

HPA……...……Hypothalamic Pituitary Adrenocortical axis

HS…….....Horse Serum

ICP……..…Intracranial pressure

IL1-β……..….Interleukin 1-β

IML……Intermediolateral

I.P. …….……Intra Peritoneal

LF……..…….....Low frequency

LVEDD …….....Left ventricular end-diastolic diameter

LVESD ……..….....Left ventricular end-systolic diameter

Lv-NOS1AP-shRNA …...…Lentivirus containing NOS1AP shRNA

Lv-SCR ……..….....Lentivirus containing scrambled shRNA

Mg^{2+}……..…….....Magnesium ion

MAP……..Mean arterial pressure

MAPK……Mitogen-activated protein kinase p38

MI……..Myocardial infarction

mRNA……messenger Ribonucleic Acid

Na^{+} .....Sodium ion

nNOS……..neuronal Nitric Oxide Synthase

NA……..Nucleus Ambiguus

NG……..Nodose ganglion

NK1-R……....Neurokinin-1 receptor

NMDA……n-methyl-d-aspartate receptor

NOS1AP…….Nitric oxide Synthase 1 Adaptor Protein

NO.....Nitric oxide

NOS……..…….Nitric oxide synthase

NTS……...…Nucleus Tractus Solitarii

NU……..Normalized units

$ONOO^-$……..…Peroxynitrite

PBS……..…...Phosphate Buffered Saline

PCR…….…….....Polymerase Chain Reaction

PDZ……....PSD/Discs-large/ZO-1 homologous

PDE……....Phospho-Diesterase

PKA……....Protein Kinase A

PI.…….…...Propidium Iodide

PID…….……Phosphotyrosine interaction domain

PSD95…….……...Post-synaptic density 95

PTB……..…..…….Phosphotyrosine-binding

PTSD ……...…….….Posttraumatic stress disorder

PVN……Paraventricular nucleus

qRT-PCR……quantitative Real Time PCR

RISC……..….RNA-induced silencing complex

RNA……...Ribonucleic Acid

RNAi…….….Ribonucleic Acid interference

ROS……...…….Reactive oxygen species

RT-PCR…….…….…….……...Reverse Transcription Polymerase Chain Reaction

RVLM……..……..……...Rostral ventrolateral medulla

SAH…….……..Subarachnoid hemorrhage

S.C. ….…….....Subcutaneous

SCA……..……..Sudden cardiac arrest

SCD……..……..Sudden cardiac death

SD…….……...…...Sprague Dawley

SDS-PAGE……...…..Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis

siRNA…….……....small interfering Ribonucleic Acid

shRNAs…….…….short hairpin RNAs

TH…….……..Tyrosine Hydroxylase

TNF-α ……....Tumor Necrosis Factor - α

TU……..……..…….....Transducing units

UV…….....…...Ultraviolet

VLF……..…..……Very low frequency

WST……....…..…...…Water soluble Tetrazolium

CHAPTER I. INTRODUCTION

Cardiovascular Diseases (CVD), in the present times, pose moderate to high levels of risk to large parts of the world and its impact is believed to increase in future owing to the cumulative behavioral, biological, and social risks (Yusuf S *et al*., 2001). In United States alone, it is the leading cause of death in both men and women, with 2014 Heart and Stroke Statistics showing that, whereas an estimated 83.6 million American adults (>1 in 3) have ≥1 types of CVD. Mortality data show that CVD was the listed underlying cause of death accounting for 31.9% of all deaths in 2010 in the United States (Go AS *et al*., 2013). Sudden cardiac death (SCD) and cardiac arrhythmias remain one of the major causes of cardiovascular mortality among adults over the age of 40 in the United States and other countries. In the U.S. alone, approximately 359,400 people of all ages experience Emergency Medical Services assessed out-of-hospital non-traumatic Sudden cardiac arrest (SCA) in 2013 with a survival rate of 9.5% (Table 1).

Table 1: Statistical Update on Incidence and Survival rate of Sudden Cardiac Arrest. (American Heart Association, 2014)

Statistical Update	Out-of-Hospital Cardiac Arrest			In-Hospital Cardiac Arrest		
	Incidence	Bystander CPR (overall)	Survival rate (overall)	Incidence	Survival rate	
					Adults	Children
2013	359,400	40.1%	9.5%	209,000	23.9%	40.2%
2012	382,800	41.0%	11.4%	209,000	23.1%	35.0%

SCA usually results from sudden cessation of cardiac activity with hemodynamic collapse, typically due to sustained ventricular tachycardia/ ventricular fibrillation (Myerburg RJ,

1997, Chugh SS *et al*., 2000, Wever EF *et al*., 2004). Nearly half of all coronary heart disease (CHD) deaths are sudden, mostly occurring in individuals unrecognized to be at risk and approximately 1/3 of these deaths are the first clinical manifestation of disease. (Go AS *et al*, 2013). Most of the SCA are due to underlying Neurogenic cardiac damage.

1.1. Etiology of SCD: neurogenic cardiac damage

The Neurogenic cardiac diseases that lead to the genesis of the arrhythmia resulting in sudden death are varied, and the association with sudden death in some cases is poorly understood (Kannel WB *et al*., 1985). Identification of the patient at risk for sudden death and identification of the factors that precipitate the fatal arrhythmia continue to represent a major challenge.

Subarachnoid hemorrhage (SAH), stroke, epilepsy and Myocardial infarction (MI) have been associated with the underlying etiology of neurogenic cardiac damage. Described below is the contribution of each of them towards the pathophysiology of neurogenic cardiac damage (Figure 1).Cardiac injury may occur following many types of brain injury; although the most extensively investigated form of neurocardiogenic injury is SAH. Brain damage after transient global ischemia involves similar pathways to those activated after SAH (Harakuni I *et al*., 2006). The mechanism of early brain injury and consequent neuronal death after the SAH has been reported to be primarily due to cellular apoptosis (Sabri M *et al*, 2013). Studies focused on large cerebral arteries found endothelial cell apoptosis after SAH (Aoki K *et al*., 2002; Cahill J *et al*., 2006). Although, in humans, Neuronal apoptosis in the cortex and hippocampus has been detected post SAH (Nau R *et al*., 2002), the mechanism is not exclusive to neurons and other cell types such as astrocytes, and oligodendroglia also exhibited apoptosis after SAH in animals

(Prunell GF *et al*., 2005). In some studies, there were fewer neurons in the hippocampus and inner cortical layers 5 days after SAH in rats (Guresir E *et al*., 2010).

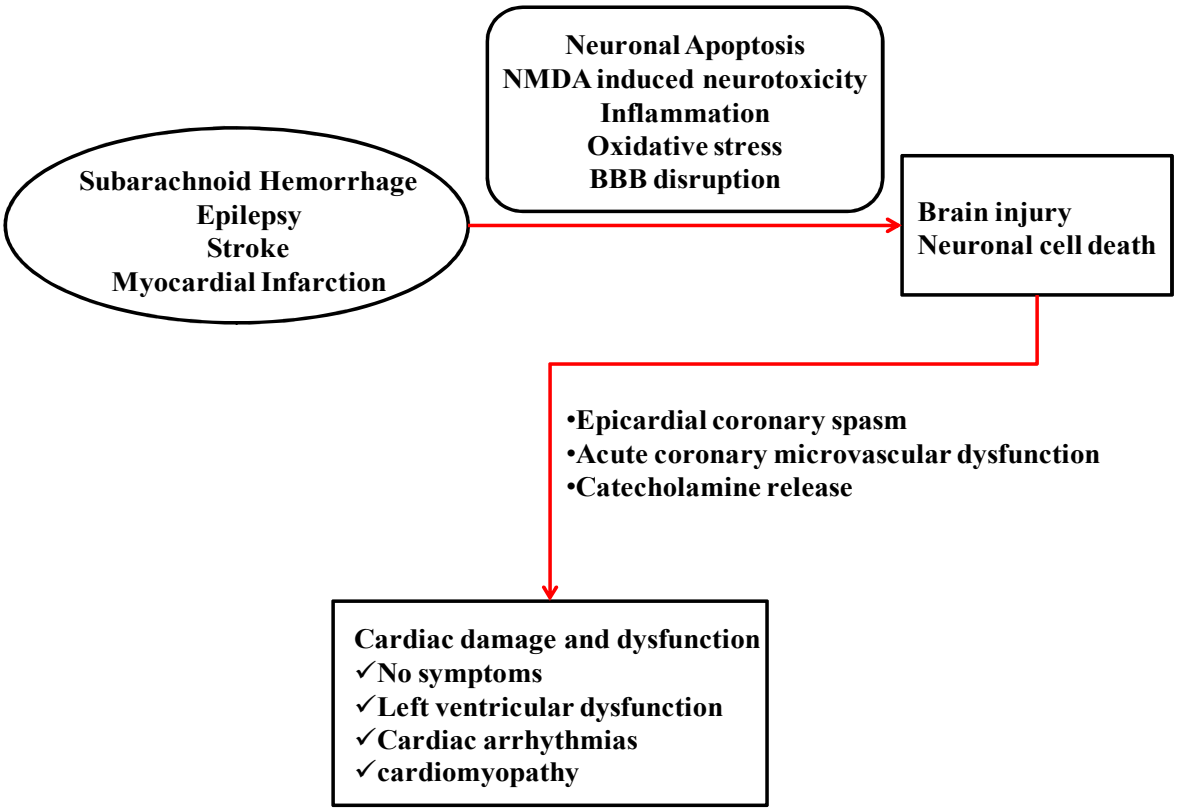

Figure 1: Etiology of Neurogenic cardiac damage

One of the most important pathology associated with SAH and stroke that leads to neuronal death and consequent myocardial damage is Ischemia. Ischemia caused by increased intracranial pressure (ICP) is probably the first process that activates apoptosis within minutes of SAH and persisted for a minimum of 24 hours as reported by studies in a rat endovascular perforation model of SAH (Hasegawa Y *et al*., 2011; Friedrich V *et al*., 2012) Another mechanism through which ischemia exerts its cytotoxic effect is via glutamate mediated excitotoxicity manifested due to the efflux of the amino acid glutamate caused by neuronal

depolarization. Following ischemia, glutamate activates the n-methyl-d-aspartate (NMDA) receptor, resulting in an influx of sodium and calcium into neurons and subsequent neuronal death (Dhawan J *et al*., 2011), which has been suggested to cause neuronal apoptosis both *in vitro* and *in vivo* (Choi DW *et al*., 1992). Another important factor in neuronal cell death mechanism underlying SAH, stroke, epilepsy and MI is the release of reactive oxygen species (ROS) after these pathological conditions, both in human and experimental animals. (Ersahin M *et al*., 2010; Imperator C *et al*., 2010; Lin CL *et al*., 2006, Macdonald RL *et al*., 1994; Marzatico F *et al*., 1998; Sakaki S *et al*., 1986). The generation of ROS leads to enhanced inflammation and oxidative stress after SAH by increasing lipid peroxidation, causing direct DNA damage and protein oxidation. These processes activate apoptotic signals and inflammatory cascades leading to consequent brain injury (Lin CL *et al*., 2006).

Another important mechanism mediating neuronal injury and vasospasm after SAH is known to be inflammation (Chaichana KL *et al*., 2010; Dumont AS *et al*., 2003). Studies have shown an acute increase in proinflammatory cytokines, such as TNF-α, interleukin 1-β (IL1-β), and IL6 after experimental SAH, pharmacologic inhibition of which attenuated neuronal injury after SAH (Ersahin M *et al*., 2010; Sozen T *et al*., 2009; Sugawara T *et al*., 2009). NF-κB, a transcription factor in endothelial cells, is also one of the mediators of the pro-inflammatory cascade. An increase in TNF-α, IL1-β and adhesion molecules was reported upon activation of NF-κB in the arterial wall; which was reduced by Pyrrolidine dithiocarbamate, an inhibitor of NF-κB. (Zhou ML *et al*., 2007) Leukocytes have also shown to play a role in the immune response following SAH by activating cytokines such as endothelin-1, a power vasoconstrictor that becomes elevated in experimental and clinical SAH (Fassbender K *et al*., 2000). Lastly,

Blood Brain Barrier (BBB) disruption also contributes to neurogenic heart damage after SAH (Cahill J *et al*., 2006). Blood breakdown products such as oxyhemoglobin and oxidative stress caused by hemoglobin can contribute to BBB disruption (Meguro T *et al*., 2001). Proinflammatory cytokines such as TNF-α could also cause endothelial cell apoptosis and contribute to BBB dysfunction (Gao Y *et al*., 2000).

The neuronal damage in the brain as a result of the SAH, epilepsy, stroke and MI would consequently lead to myocardial damage either in the form of cardiac pathology or cardiac arrhythmias. The 3 main proposed mechanisms for neurogenic cardiac damage might be due to the following (Bybee KA *et al*., 2008): (1) epicardial coronary spasm; (2) acute coronary microvascular dysfunction; and (3) catecholamine-mediated direct myocardial injury.

Neurogenic cardiac damage is caused due to local myocardial adrenergic toxicity resulting from a centrally mediated sympathetic surge. Experimental stroke models have shown that occlusion of the right middle cerebral artery results in changes in brain neurotransmission, which leads to enhancement of sympathetic outflow to the heart, resulting in an increase in synaptic norepinephrine levels (Cechetto DF *et al*., 1997). Neuronal damage in those with SAH, leads to enhanced sympathetic outflow, and experimental hypothalamic stimulation can induce cardiac changes such as those observed in acute cerebrovascular syndromes. The myocardial injury as a result of neurogenic damage is manifested by contraction band necrosis also known as contraction bands. It occurs due to the norepinephrine hyperstimulation of postsynaptic cardiac catecholamine receptors which increase myocardial 3′,5′-cAMP production, leading to pathological myocyte calcium influx, which prolongs actin-myocin interaction and ultimately results in depletion of energy stores with resultant reduction in ATP generation (Samuals MA *et*

al., 1987). Another contributor to myocardial toxicity is enhanced peripheral sympathetic activity with increased plasma catecholamine levels (Masuda T *et al*, 2002; Benedict TR *et al*., 1978). Patients with SAH and neurogenic cardiac damage exhibit abnormal cardiac sympathetic activity indicative of functional sympathetic denervation (Banki NM *et al*., 2005) Treatment with propranolol and phentolamine prevents the development of contraction band necrosis in those with SAH, further supporting the catecholamine induced cardiac damage (Neil Dwyer G *et al*., 1978).

Most well recognized and common secondary complication of neurogenic damage due to SAH is acute left ventricular systolic dysfunction which is a reversible condition (Bybee KA *et al*., 2008). Approximately 20% to 30% of patients with SAH manifest a secondary cardiomyopathy and/or regional wall motion abnormality, which is usually reversible in the absence of underlying obstructive CAD (Bankee N *et al*., 2006). Independent predictors of neurogenic cardiac damage include severity of neurological injury, troponin enzyme elevation and elevated brain natriuretic peptide among others (Mayer SA *et al*., 1999; Tung P *et al*., 2004; Lee VH *et al*., 2006). Histological analysis of myocardial tissue in cases of neurogenic myocardial damage demonstrates contraction band necrosis, also known as myocytolysis, without ischemic necrosis. This finding is consistent with catecholamine-mediated myocardial injury.

Lesions of the CNS could cause detrimental effects either alone or cooperatively with respect to two conditions: Cardiac pathology and cardiac arrhythmias. This triggering neurogenic cardiac damage in patients could originate from 3 sources: directly from heart, from peripheral autonomic nervous system and from central nervous system or from a combination of some or all

of these factors (Lathers CM *et al*., 1978; Lathers CM *et al*., 1977). Most of the victims had been active and apparently healthy before dying suddenly, for example, while doing heavy exercise or sleeping at night. SCD in such cases presents a particularly difficult problem for physicians as there were no triggering factors or predictive symptoms before death occurred. Also victims had no evident structural heart disease. Therefore, it is vital to study and interpret the underlying central regulation of Sudden death in such cases. Clinical and experimental observations demonstrate direct influences of cortical and brainstem activity upon the myocardium and cardiac electrophysiology (Surges R *et al*., 2010). Clinical reports have shown that SAH is associated with higher rates of ECG abnormalities and arrhythmias (Oppenheimer SM *et al.,* 1991, Rogers MC *et al*., 1973, Billman GE *et al*., 1982, La Rovere MT *et al*., 1988), with normal heart and coronary arteries. Thus the ECG changes seen in the above context are a manifestation of autonomic dysregulation, caused by a lesion that affected the cortical representation of the autonomic nervous system as opposed to ischemic heart disease. Abnormal brain activity during seizures is associated with abnormalities in cardiac repolarization and centrally triggered arrhythmia is the likely cause of SCD in epilepsy (Taggart P *et al*., 2011). Evidence shows that focal stimulation of a discrete set of brain regions may produce changes in heart rate, blood pressure, ECG and arrhythmias (Greenshot JH *et al*., 1969). Large body of evidence suggests myocardial necrosis upon hypothalamic stimulation. Such cardiac lesions attributed by myocardial necrosis are characterized by (Baroldi G, 1975) (1) coagulation necrosis : the cell loses its capacity to contract and dies in an atonic state with no myofibrillar damage; (2) colliquative myocytolysis : edematous vacuolization with dissolution of myofibrils without hypercontraction occurs in the low-output syndromes; and (3) coagulative myocytolysis: also

referred as myofibrillar degeneration and contraction band necrosis caused by catastrophic levels of catecholamine exposure to the heart, in which the cell dies in a hypercontracted state with early myofibrillar damage and anomalous irregular cross-band formations. These myocardial necrotic patterns have been established by extensive clinical and experimental researches that have simulated central lesions in the form of intracranial haemorrhage by injecting blood intracranially into mice, which thereby produced the characteristic myocardial lesions that could be reduced but not obliterated by pretreatment with adrenalectomy and the use of either atropine or reserpine or propranolol. These suggest that neurological influences via sympathetic (catecholamines) and/or parasympathetic overactivity may be partly responsible for necrosis of the myocardium. Recent studies conducted on rodents have implicated specific brain region known as the Nucleus Tractus Solitarii (NTS), lesions in which lead to cardiac damage (Nayate A *et al*., 2008).

1.2. Central neural control of cardiovascular system

Neurocardiology or the brain-heart connection (Martin AS, 2007) could be divided into three major categories: the heart's effects on the brain (e.g., cardiac source embolic stroke), the brain's effects on the heart (e.g., neurogenic heart disease), and neurocardiac syndromes (Samuels MA, 1987). The present section provides a review of the central control of the heart and how lesions in the central nervous system predominantly the brain, could cause cardiac damage.

The central nervous system (CNS) is critical in setting up the autonomic responsiveness of the heart at rest and during physiological and pathophysiological stress (Herring N *et al*., 2008). Cardiac activity and vasomotor tone are under the control of the central nervous system

that modulates the excitatory and inhibitory influences on autonomic discharge. Central lesions that lead to a disturbance in autonomic activity tend to cause electrocardiographic and pathological evidence of myocardial damage, cardiac arrhythmias, and disturbances of arterial blood pressure regulation. Such cardiovascular disturbances result from alterations in sympathetic activity that can also occur under stress and lead to cardiac arrhythmias that can cause SCD (Talman WT, 2004). High cardiac sympathetic drive is pro-arrhythmic, increases myocardial oxygen demand and reduces coronary perfusion time (Herring N *et al*., 2008). CNS also indirectly affects the circulatory system through its effects on fluid and electrolyte balance.

Neural control of the heart is integrated at all levels of the neuraxis (Figure2) (Palma J *et al*., 2014). Different regions of the forebrain, including the insular cortex, anterior cingulate cortex (ACC), central nucleus of the amygdala, and several hypothalamic nuclei project to medullary and spinal nuclei controlling cardiac function. Sympathetic activation is triggered by neurons of the rostral ventrolateral medulla (RVLM), which send excitatory projections to preganglionic sympathetic neurons of the intermediolateral (IML) cell columns of the spinal cord. These neurons activate noradrenergic neurons of the stellate and other paravertebral ganglia, which send axons that contribute to the cardiac plexuses innervating the heart.

Parasympathetic output is mediated primarily by vagal neurons located in the nucleus ambiguus. These neurons send preganglionic axons that synapse on cholinergic and noncholinergic neurons located in the cardiac ganglia. Inputs from cardiac receptors are conveyed by spinal afferents that follow the trajectory of spinal nerves and have their cell body in the dorsal root ganglia (DRG), or by vagal afferents with cell bodies in the nodose ganglion (NG). Cardiac vagal afferents, together with carotid baroreceptor afferents, provide inputs to the

nucleus of the solitary tract (NTS). The NTS initiates a variety of cardiovascular reflexes and also conveys cardiovascular receptor information to the thalamus and parabrachial nucleus. The parabrachial nucleus is a site of integration of spinal and brainstem afferents and conveys this information to the thalamus, amygdala, and hypothalamus. Several research studies are under way to try to find the exact causes of neurogenic cardiac damage and how to prevent them. One of the most important, intriguing and foremost causes of the cardiac arrhythmia leading to Sudden death is related to neurohumoral and CNS alterations triggering the electrocardiographic abnormalities either alone or in concert with myocardial damage (Mann DL *et al*., 2014).

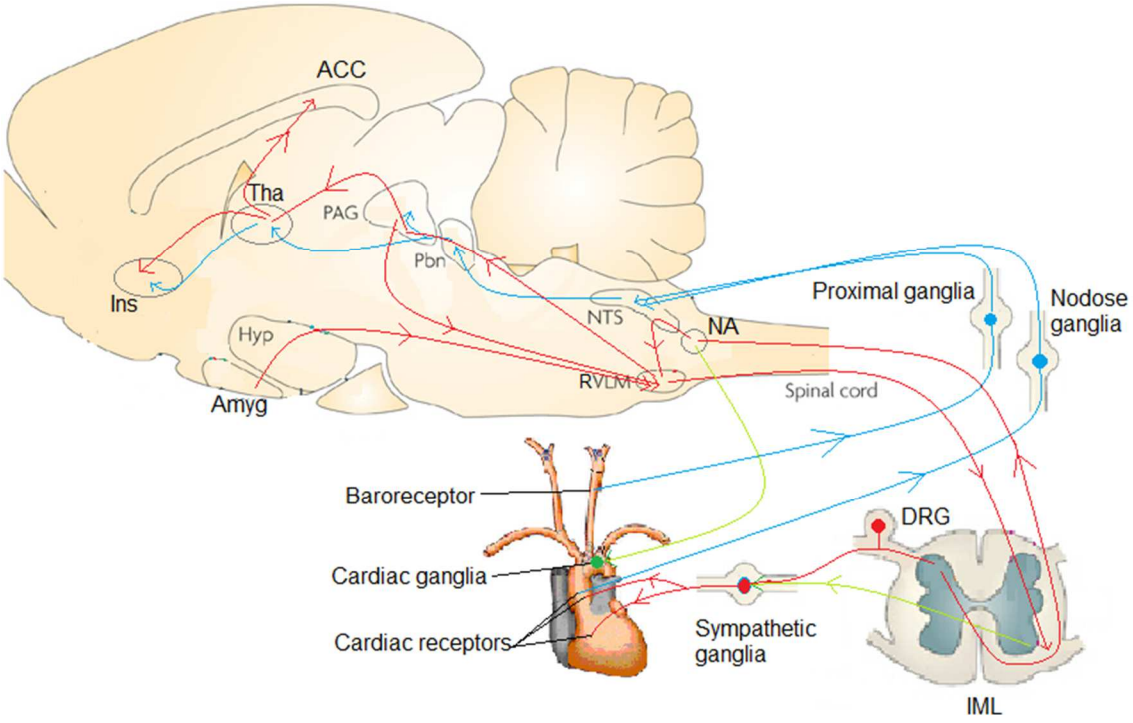

Figure 2: Neural control of cardiac function is integrated at all levels of the neuraxis. (Palma J et al., 2014: Adopted and modified). ACC- Anterior Cingulate Cortex, Amyg- Amygdala, DRG- Dorsal Root Ganglia, Hyp- Hypothalamus, IML- Intermediolateral nucleus, Ins- Insular cortex, NA- Nucleus Ambiguus, NTS- Nucleus Tractus Solitarii, PAG- Periaqueductal Gray, Pbn- Parabrachial nucleus, RVLM- Rostral Ventrolateral Medulla.

1.3. Brain NTS in neurogenic cardiac damage and SCD

NTS is a major integrative center for circulatory control, with inputs from cardiopulmonary afferents (such as arterial baroreceptors) and polysynaptic inputs from many sympathetic and somatic afferents (Paton JFR, 1999, Potts JT *et al*. 2003, Blessing WW *et al*. 1997). Abnormalities of baro- or chemoreceptor afferent input, or of their processing in the NTS, could contribute to several forms of neurogenic hypertension (Guyenet PG, 2006). Lesions in the NTS have been experimentally performed by either selectively destroying Neurokinin-1 receptor (NK1-R) neurons in the NTS or ablation of the NTS catecholaminergic neurons expressing tyrosine hydroxylase (TH). Both cases lead to chronic lability of arterial pressure, a finding consistent with loss of baroreflex responses, pathological changes in the myocardium demonstrated by hypereosinophilic myocytes and the evolvement of ECG from a normal sinus rhythm through arrhythmia, asystole, and death of some of the animals. Thus, the lesions in the NTS interrupting the baroreflex may induce cardiac arrhythmias and myocardial changes similar to those seen in humans with sustained central lesions (Talman WT *et al*., 1993, Nayate A *et al*., 2008). The altered baroreflex transmission was associated with sudden death in approximately one third (33%) of the experimental animals (Nayate A *et al*., 2008). Also, the cardiac and cardiovascular effects of lesions directed toward catecholamine neurons of the NTS are similar to those following damage directed toward NK1 receptor-containing neurons, believed to be due to the sympathetic overactivity. Although researchers have pointed out that lesions in the NTS could contribute to the Sudden death in some cases, intracellular mechanisms linking these two are yet to be elucidated.

1.4. NOS1AP gene variants in prolonged QT interval and SCD

Familial history provides important evidence in the form of a host of demographic and epidemiological genome wide association studies presenting a broad influence of genetic factors on the susceptibility of SCD in common forms of cardiac diseases in various populations across the world. Such genetic specifically allelic variants cause this susceptibility by affecting either one or more of the listed 3 mechanistic pathways responsible for the electrophysiological functioning of heart including sympathetic neural activation of the heart beat (Arking DE *et al.*, 2004).

The multiple pathways that contribute to neurogenic cardiac damage leading to sudden death risk can be broadly classified into three categories, as shown in Figure 3, (Spooner PM *et al*., 2001) leading to: 1) atherosclerosis and thrombosis – manifested through allelic variants affecting cholesterol metabolism, plaque formation and stability, proteins involved in the clotting cascade, inflammatory mediators or vascular factors; 2) arrhythmia initiation and propagation involving variants modulating ion channels (Albert CM *et al.*, 2010), connexins, gap junctions and energetic including redox factors; and 3) initiating aberrant influences and triggers for sustained arrhythmias caused due to the central neural modulation of the sympathetic and/or parasympathetic control of the heart function.

The electrocardiographic (ECG) QT interval duration, a non-invasive measure of ventricular repolarization, is one of the quantitative predictors of arrhythmogenesis. Variation of the QT interval in the general population is ~35% heritable and prolonged QT intervals are associated with increased cardiovascular morbidity and mortality. (Newton CC *et al*., 2005, Hong Y *et al*., 2001, Straus SM *et al*., 2006).

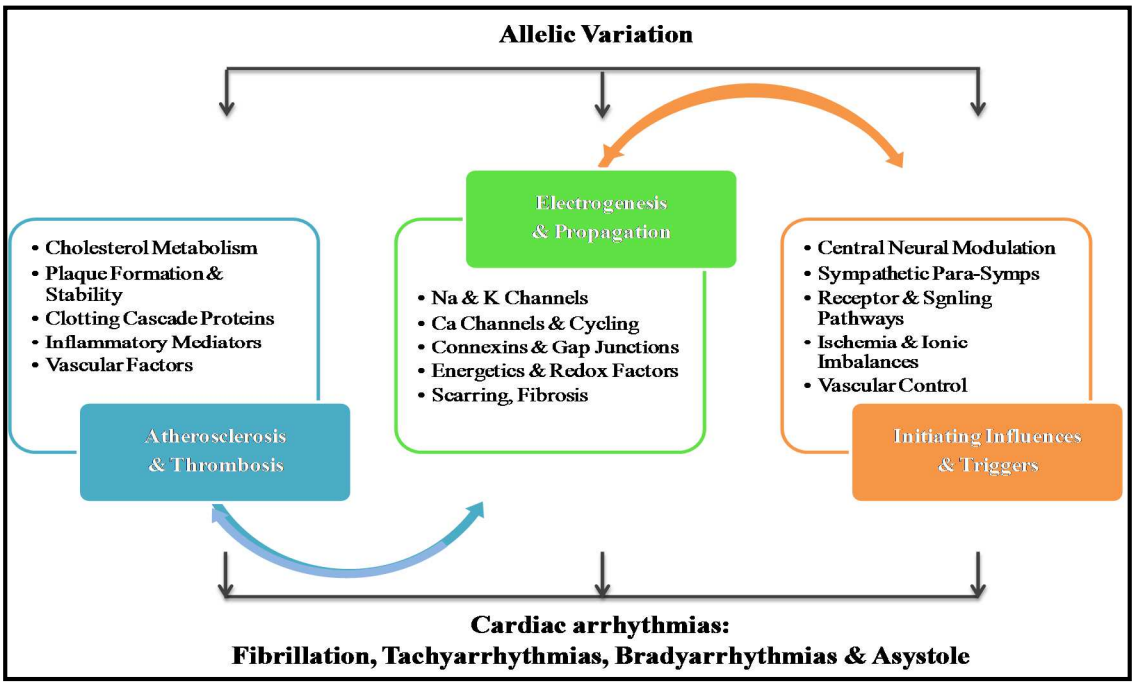

Figure 3: Genetic mechanisms of cardiac arrhythmias (Spooner PM et al., 2001: Adopted and modified). Genetic mechanisms by which variant alleles could affect initiation and propagation of cardiac arrhythmias.

Genetic variants play a significant role in the risk stratification of sudden death due to cardiac arrhythmias particularly by modulating the QT interval in healthy subjects. Common genetic variants may modulate both disease and response to drugs and the effect is variable in different individuals. The variability is attributed to the genes that encode the receptors for drug targets, or affect the overall activity of any complex signaling pathway through which the drug acts and also genes that encode proteins responsible for drug metabolism within the system (Arking DE *et al.*, 2004). Thus gene variants could affect any one or more of those to cause a different degree of response from different individuals for the same drug. One such gene that has been extensively studied across various populations is Nitric oxide Synthase 1 Adaptor Protein

(NOS1AP) (Arking DE *et al.,* 2006, 2009; Eigelsheim M *et al.*, 2009; Crotti L *et al.*, 2009, Kao WH *et al.*, 2009). NOS1AP gene variants have been found to be associated with prolonged QT interval syndrome in various populations including healthy young population, older population, diabetic subjects, population with a familial history of Sudden death and subjects on anti-arrhythmic drugs. A genome-wide association study (GWAS) examining the relation between the variant rs10494366 T>G (NOS1AP gene, n=1,842) and QT interval duration in a population of healthy young adults aged 24-39 years (Aarnoudse AJ *et al*., 2007) found that the G allele was associated with a 3.2 ms (95% confidence interval (CI) 1.7-4.6 ms, P<0.0001) increase in QTc interval duration for each additional copy. The population was adjusted for age, sex, systolic blood pressure, body mass index, alcohol use, and smoking. These findings provide evidence from a population of healthy young adults that within the normal physiological range, a common variation in the NOS1AP gene influences cardiac repolarization (Aarnoudse AJ *et al*., 2007). Another GWAS (Post W *et al*., 2007) was conducted on healthy subjects from two different cohorts- 2,646 subjects from Germany and 1,805 subjects from the US Framingham Heart Study. Approximately 60% of subjects of European ancestry carry at least one minor allele of the NOS1AP genetic variant, which explains up to 1.5% of QT interval variation. Older subjects (≥55 years of age) from the Rotterdam Study (n=7983; 58% female, 98% white) were evaluated for the statistical evidence supporting the association of rs10494366 ($P<10^{-19}$) and rs10918594 ($P<10^{-16}$) with QTc interval. The rs10494366 G allele (36% frequency) was associated with a 3.8-ms (95% confidence interval, 3.0 to 4.6; $P=7.8\times10^{-20}$) increase in QTc interval duration for each additional allele copy, and the rs10918594 G allele (31% frequency) was associated with a 3.6-ms (95% confidence interval, 2.7 to 4.4; $P=6.9\times10^{-17}$) increase per additional allele copy,

confirming a genuine association. This study examined the relationship of genetic variation, present at birth, in an elderly cohort and the results showed that genetic factors continue to play a role even at older age. In another demographic study (Lehtinen AB *et al*., 2008), two single nucleotide polymorphisms (SNPs) in NOS1AP were genotyped in 514 European Americans and 115 African Americans from 400 pedigrees enriched for type 2 diabetes, after excluding individuals taking QT-altering medications from the analyses. In diabetic patients, the magnitude of the estimated effect of NOS1AP SNPs on QT interval duration was larger than that observed in the total sample of diabetic and nondiabetic subjects. Also, a test comparing diabetic and nondiabetic individuals suggested that there is a significant difference in QT interval duration between the two groups (two-sided P values of 0.029 and 0.088 for rs10494366 and rs10918594, respectively) in the study population. No association between the NOS1AP SNPs and QT interval duration was observed in the limited number of African Americans. The rationale behind the association of NOS1AP gene variants with QT-interval duration can be attributed to a recent study demonstrating that the NOS1AP protein is expressed in the heart and has been shown to interact with nNOS to accelerate cardiac repolarization by inhibition of L-type calcium channels. NOS1AP is also regarded as a candidate gene for schizophrenia (Hwu HG *et al*., 2003), autism spectrum disorders and obsessive-compulsive disorder (Buxbaum AD *et al*., 2004). Thus, the genetic variants of NOS1AP are not only associated with prolonged QT interval or ventricular arrhythmias but also a host of psychiatric disorders too, prompting one to speculate the role of NOS1AP at the cellular level in the context of central control of the cardiac functions.

Although studies performed in the heart have shown that NOS1AP over-expression in ventricular myocytes leads to the inhibition of the L-type calcium channels and an increase in the

delayed rectifier potassium current that result in hastening of the action potential (Chang KC *et al*., 2008). These studies could serve as a peripheral mechanism for arrhythmogenesis. The effect of NOS1AP alteration in the brain at a cellular level, particularly the NTS, influencing arrhythmia and cardiac pathology manifesting myocardial damage remains to be elucidated and would provide a probable mechanistic approach towards understanding of neurogenic cardiac damage leading to SCD.

1.5. NOS1AP protein: molecular structure and domains

NOS1AP also known as CAPON (carboxy-terminal PDZ ligand of nNOS) was first identified in rat brain neurons (Jaffrey SR *et al*., 1998, Jaffrey SR *et al*., 2002) and is a highly conserved protein with 92% sequence homology between rat and humans. The NOS1AP is present in two isoforms: long isoform- NOS1AP-L and the short isoform- NOS1AP-S. The long isoform of NOS1AP is a 506 amino acid protein made from all 10 exons of the gene. The short isoform is made from the last two exons of NOS1AP and produce a short protein of 210 amino acids. The NOS1AP-L protein contains two distinguishable domains, the C-terminal PSD/Discs-large/ZO-1 homologous (PDZ)-binding domain (Jaffrey SR *et al*., 1998), which is responsible for interaction of NOS1AP with nNOS and a phosphotyrosine-binding (PTB) domain (Figure 4).

The domain structure of NOS1AP is described in Table 2. Proteins encoding PTB domains function as adaptors or scaffolds to organize the signaling complexes involved in wide-ranging physiological processes including neural development, immunity, tissue homeostasis and cell growth. PTB domains are divided into three groups represented by phosphotyrosine-dependent IRS-like, phosphotyrosine-dependent Shc-like, and phosphotyrosine- independent

Dab-like PTBs. The last two PTBs have been named as phosphotyrosine interaction domain (PID or PI domain).

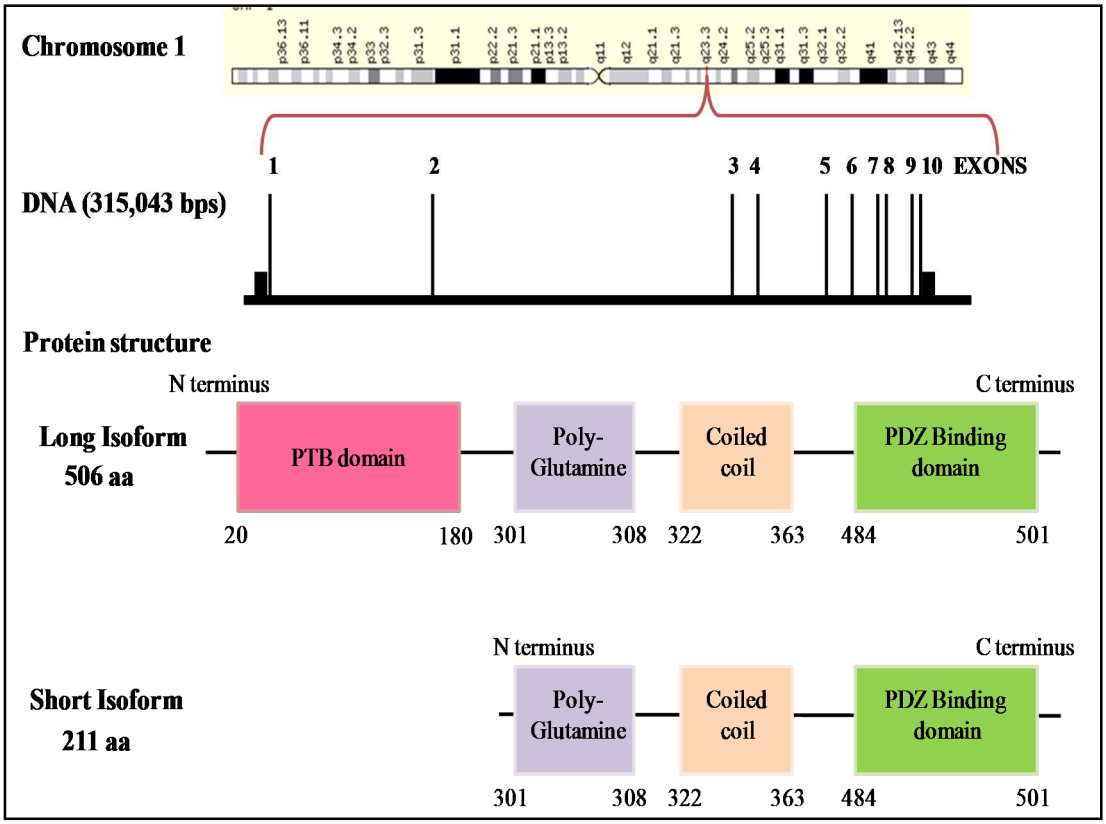

Figure 4: Schematic diagram of DNA and Protein structure of Nitric oxide synthase 1 adaptor protein (NOS1AP).

The Shc-like PID specifically binds to the Asn-Pro-Xaa-Tyr(P) motif found in many tyrosine-phosphorylated proteins including growth factor receptors. On the other hand the Dab-like PID domain binds to non-phosphorylated tyrosine residue or even a phenylalanine at the same position (Bork P *et al*., 1995). Most of the ligands for Shc-like PID domains are RTK or cytokine, whereas phosphotyrosine independent Dab-like PID domains seems to mediate other types of signaling pathways, like endocytosis/processing or exocytosis. This domain binds both

peptides and headgroups of phosphatidylinositides, utilizing two distinct binding motifs to mediate spatial organization and localization within cells (Uhlick MT *et al*., 2005; Kavanaugh WM *et al*., 1994). Through our web database search we have found out that the PTB domain of NOS1AP is a phosphotyrosine independent Dab-like PID domain and is located at the N terminal of the long isoform between 20-180 amino acids.

Table 2: Domain Structure of NOS1AP protein: Conserved domain analysis of NOS1AP protein sequence performed by Uniprot and NCBI web Conserved Domain search tool.

Type of arrangement	Amino acid	Length	Name
Domain	26-196	171	PTB domain
Compositional bias	301-308	8	Poly-glutamine
Coiled coil	322-363	42	Coiled coil
Region	494-506	13	Interaction with NOS1 by similarity
Motif	488-501	3	PDZ binding domain

We performed a conserved domain search for NOS1AP protein sequence using Uniprot and NCBI web CD search tool (Table 2). Based on the conserved domain of protein structure analysis we found out that the NOS1AP also consists of 3 smaller but specific regions. These are 1). A small 13 amino acid stretch that shows complementarity to neuronal NOS and might be involved in further binding of NOS1AP to nNOS strengthening the interaction of NOS1AP with nNOS. This interaction could provide another level of binding and interaction of nNOS to

NOS1AP enhancing or streamlining their functions. 2). A polyglutamine stretch of approximately 8 amino acid forming a compositional bias. Compositional bias for a subset of residues is a widespread phenomenon in protein sequences (Harrison PM, 2006); and has been linked to proteins having a structural role, or displaying some intrinsic protein disorder (Rammazzotti M *et al*., 2012). Polyglutamine repeats are associated to specific sequence biases that are evolutionarily conserved among eukaryotes. Nine human neurodegenerative diseases, including Huntington's disease and several spinocerebellar ataxia, are associated to the aggregation of proteins comprising an extended tract of consecutive glutamine residues once it exceeds a certain length threshold; and are characterized by a progressive motor and cognitive degeneration that ultimately lead to death (Bracken C *et al.*, 2004, Fink AL 2005, Dyson HJ *et al.*, 2005). Thus, NOS1AP with this polyglutamine stretch could be an important neuronal functional protein mutations in which could lead to neurogenic disorders. 3). Coiled coil motif- The presence of a coiled coil motif provides structural and functional diversity to NOS1AP. Due to the presence of coiled coil motif, NOS1AP could be oligomerizing and/or binding with other proteins with similar motif to provide functional diversity.

The PTB domain of NOS1AP-L binds to Dexras1 and synapsin (Fang M *et al*., 2000; Jaffrey SR *et al*., 2002). Interestingly, Dexras1 activity is regulated by nNOS-elicited nitrosylation. The NOS1AP-S shorter isoform has a truncated PTB domain and contains the PDZ-binding domain. Only the full-length form of NOS1AP could serve as an adaptor protein between nNOS and its targets. A physiologic role of the short form likely would be limited to the competitive inhibition of binding of other ligands to the PDZ domains of nNOS and PSD93/PSD95.

Thus, we were able to characterize the molecular structure and different domains of the NOS1AP protein through conserved domain analysis of NOS1AP protein sequence. This information would lead us towards a better understanding of NOS1AP interactions with other intracellular proteins and subsequently will help to dissect out its function in various physiological conditions.

1.6. Stresses and glutamate mediated neurotoxicity

Stress is a condition defined by the endocrinologist, Selye (Selye H, 1936), as "the non-specific response of the body to any demand placed on it." The body's principal physiological responses to stressful stimuli are mediated by the sympathoadrenal system and the hypothalamic pituitary adrenocortical (HPA) axis, mediated by the hippocampus (Hoschl C *et al*., 2001; Tanzi RE *et al*., 2005; Green KN *et al*., 2006). Stress stimulates the release of corticotropin-releasing factor (CRF), from the hypothalamic paraventricular nucleus (PVN), into the hypophysial-portal circulation, where it induces the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary and glucocorticoids (cortisol in humans; corticosterone in rodents) from the adrenal glands (Owens MJ *et al*., 1991). Neuronal and hormonal mechanisms limit the magnitude of the HPA stress response elicited by PVN neurons and maintain the glucocorticoid levels within tolerable limits (Keler-Wood ME *et al*., 1984). Psychological stress, from mild to severe posttraumatic stress disorder (PTSD), has been reported to impair cognition (Alkadhi K, 2013) by elevating excitatory amino acid such as glutamate and glucocorticoid levels, which subsequently cause excitotoxicity and hippocampal atrophy (McEwen BS, 1989).

The mechanisms underlying stress-induced neuronal dysfunction are largely unknown. Recent advances in animal and human studies have resulted in the understanding of two primary

mechanisms involved in stress mechanism. (Sapolsky RM, 1999). First mechanism is the glucocorticoid hypersecretion that is correlated with memory impairment and reduced hippocampal volume in aged rats (Issa AM *et al*., 1990) as well as adult rats exposed to high levels of glucocorticoids. Thus, prolonged exposure to glucocorticoids reduces the ability of neurons to resist insults, thus increasing the rate at which they are damaged. The second mechanism is excessive release of glutamate and repeated activation of glutamate NMDA receptors (Magarinos AM *et al*., 1996; Christian KM *et al*., 2011), modified interneuronal GABA inhibitory tone (Magarinos AM *et al*., 1999), and increased serotonergic tone (Mckittrick CR *et al*., 2000). The elevated glutamate release is due to the activation of the sympathetic nervous system, which increases the levels of circulating norepinephrine and epinephrine and elevates the levels of norepinephrine in the brain. This will consequently lead to peripheral vasoconstriction, increase in heart rate and vagal withdrawal, and increased energy mobilization (Steptoe A *et al.*, 2012). The prolonged and sustained activation of NMDA receptors in response to elevated sympathetic nervous system activity consequently leads to neurotoxicity with severe implications on cardiac function. Described below is the cellular mechanism of Glutamate induced neurotoxicity.

Excitotoxicity refers to excessive exposure to the neurotransmitter glutamate or overstimulation of its membrane receptors (Olney JW *et al*., 1972), and has been implicated as one of the key factors contributing to neuronal injury and death in a wide range of both acute and chronic neurologic disorders (Lipton SA *et al*., 1994). Glutamate, an excitatory amino acid, play a significant role in the brain as they have been shown to activate different types of ion channel forming receptors (ionotropic) and G-protein-coupled receptors (metabotropic) to develop their

essential role in the brain (Michaelis EK, 1998). However, high concentrations of glutamate or neurotoxins acting at the same receptors, cause excessive activation of these receptors and hence excessive Ca^{2+} influx through the receptor's associated ion channel leading to excitotoxic cell death (Lipton SA *et al*., 1994, Lipton SA *et al*., 1998). Glutamate receptors are subdivided into three receptor classes that are named by their selective agonists: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, kainate receptors and NMDA (N-methyl-D-aspartic acid) receptors. AMPA and kainate receptors trigger rapid excitatory neurotransmission in the CNS by promoting entry of Na^{+} into neurons. NMDA receptors are associated with a high-conductance Ca^{2+} channel that in resting, non-depolarizing conditions is blocked by Mg^{2+} in a voltage-dependent manner. NMDA receptors are composed of a tetramer of different subunits: NR1 (mandatory), NR2A-D, and, in some cases, NR3A or B subunits. The difference in the subunit composition as well as the alternative splicing of some subunits, contributes to the pharmacological properties of the receptor-ion channel complex. Also, it has been reported that the subunits are differentially expressed both regionally in the brain and temporally during development (Kemp JA *et al*., 2002).

Studies have shown that potential neuroprotective agents that block virtually all NMDA receptor activity will very likely have unacceptable clinical side effects indicating that physiological NMDA receptor activity (Lees KR *et al*. 2000, Kemp JA *et al*., 1999, Sacco RL *et al.*, 2000), is essential for normal neuronal function. Increased activity of the enzyme nitric oxide synthase (NOS) is also associated with excitotoxic cell death (Lipton SA, 2004). NMDAR and nNOS are tethered together by a scaffolding protein PSD-95 at excitatory synapses and assembles into a macromolecular signaling complex (Aarts M *et al*., 2002, Cao J *et al*., 2005).

NMDAR-mediated calcium influx is thus responsible for nNOS activation and increased levels of nitric oxide (NO) have been detected in animal models of stroke and neurodegenerative diseases. It is known that nNOS contributes to glutamate-induced neuronal death (Dawson VL *et al*., 1996). Role of NMDAR in pathophysiology of excitotoxicity has been extensively studied. In some areas of the brain, NMDAR induced Ca^{2+} influx is important for long-term potentiation (LTP), considered as a cellular/electrophysiological correlate of learning and memory formation. During normal synaptic transmission, the NMDAR is blocked by Mg^{2+} blocking in the channel. Under pathological conditions, overactivation of the receptor causes an excessive amount of Ca^{2+} influx into the neurons leading to Ca^{2+} overload of mitochondria, resulting in the generation of reactive oxygen species and activation of caspases, Ca^{2+}-dependent activation of neuronal NOS, leading to increased NO production which reacts with ROS to form toxic peroxynitrite ($ONOO^-$), and stimulation of mitogen-activated protein kinase p38 (MAPK p38) signaling pathway (Okamoto SI *et al*., 2005), thus activating transcription factors that trigger apoptosis (Figure 5) (Dawson VL *et al*., 1991, Dawson VL *et al*., 1993, Budd SL *et al*., 2000, Yun HY *et al*., 1998, Tenneti L *et al*., 1998, Lipton SA *et al*., 1993).

NMDAR-PS95-nNOS has been implicated in a variety of neurodegenerative disorders but directly blocking NMDAR function and inhibiting nNOS activity could produce undesirable psychomimetic or cardiovascular side-effects. Thus, disrupting the NMDAR-PSD95-nNOS interaction may be a useful strategy to prevent neuronal excitotoxicity in pathological cases.

1.7. NO in glutamate mediated neurotoxicity

Neuronal NOS (nNOS, also known as: NOS1) was the first NO synthase (NOS) was isolated from mammalian brain and named due to its localization in neurons (Bredt DS *et al*.,

1990; Bredt and Snyder, 1990). nNOS activity is primarily regulated by increases in intracellular Ca^{2+}, which activate nNOS through calmodulin binding (Bredt and Snyder, 1990). NO formed in the CNS by nNOS is involved in the central regulation of blood pressure (Togashi H *et al*., 1992, Sakuma I *et al*., 1992, Elkarib AO *et al*., 1993). nNOS activity blockade in the medulla and hypothalamus causes systemic hypertension (Toda N *et al*., 2009). NO in the CNS is also responsible for the autonomic regulation of the cardiovascular system. Disrupted NO cyclic nucleotide signaling due to increased oxidative stress has been implicated in impaired autonomic control in a number of chronic cardiovascular diseases. Recent evidence suggests that a reduction in nNOS-derived NO may contribute to dysfunctional neural control of the myocardium that is associated with several cardiovascular diseases (as reviewed by Förstermann U *et al*., 2012).

One of the major receptor proteins for nNOS is the glutamatergic receptor known as the NMDA receptor (NMDAR) binding with which nNOS is shown to exert its action. NMDAR and neuronal Nitric Oxide Synthase (nNOS encoded by NOS1), one of the isoforms of NOS, are linked through an intermediary adaptor protein PSD95 (post-synaptic density 95) for NMDA receptor-mediated calcium influx and nNOS activation (Jaffrey SR *et al*., 1998, Jaffrey SR *et al*., 2002) (Figure 5). This interaction of nNOS with NMDAR through PSD 95 protein occurs through the PDZ domain of PSD 95. It is to be noted that NOS1AP also has a PDZ binding domain thus enabling it to bind with several PDZ domain containing proteins such as nNOS. Thus, interaction of the PDZ domains of nNOS and PSD95 leads to calcium influx from NMDA receptors and consequently activation of nNOS due to binding of Ca^{2+}. Under pathophysiological conditions involving high glutamate neurotransmitter release, the NMDA receptors in the neurons become activated, causing more Ca^{2+} influx leading to overactivation of nNOS causing

high NO production. This excessive NO production might be responsible for the neurotoxic effects underlying various pathophysiological manifestations.

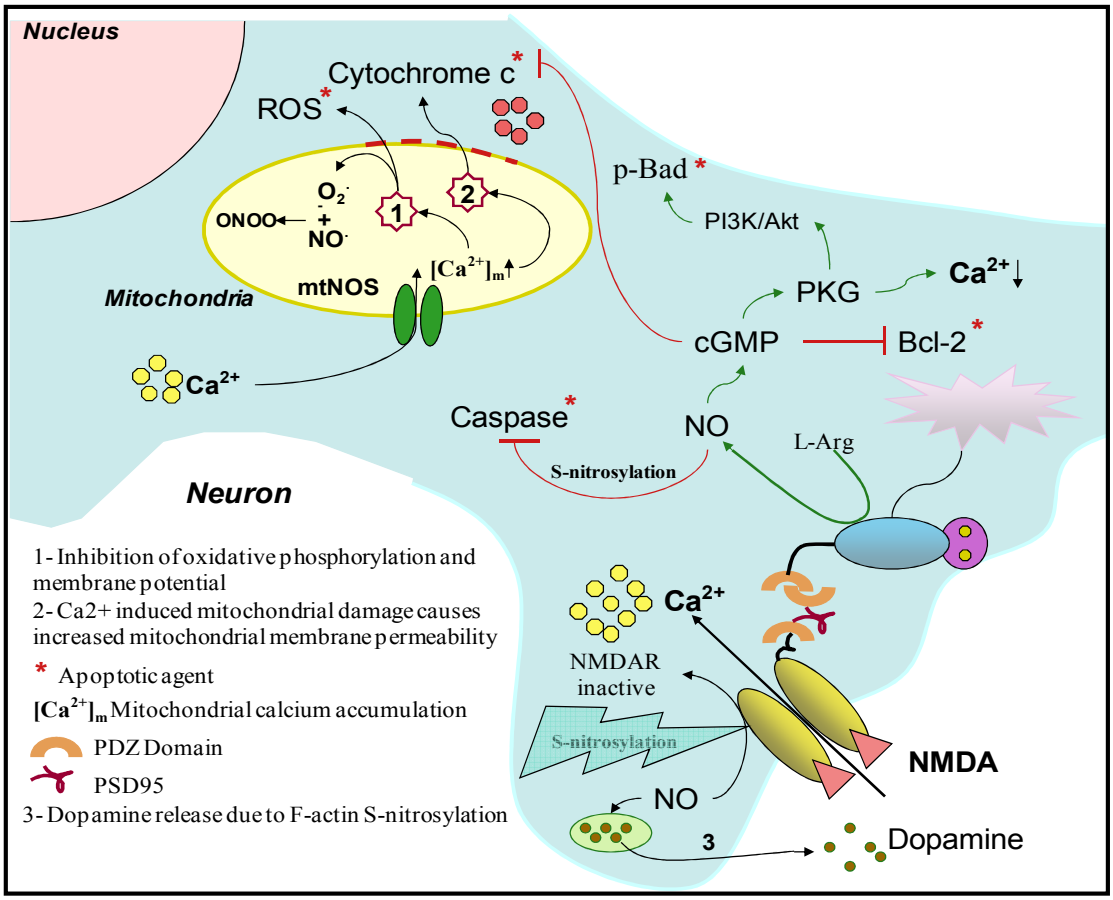

Figure 5: Schematic representation of intraneuronal mechanisms involved in NMDA mediated neurotoxicity.

Several pathophysiological conditions are manifested by changes in the expression levels of the nNOS protein (Dawson TA *et al*., 2008). Studies show that heart failure is associated with decreases in nNOS gene expression in at least three regions of the brain and with increased sympathetic outflow to the periphery. The decreased NO production that is likely associated with the decreases in nNOS gene expression may lead to the increased sympathetic drive seen in

chronic heart failure (Patel KP *et al*., 1996). These studies indirectly signify the importance of neuronal NO as a neuromodulator. Pharmacological disruption of NO production by chronic nitric oxide synthase (NOS) inhibition in genetically normal animals lead to hypertension (Hu L *et al*, 1994).Since NOS1AP contains a PDZ binding domain; it could interact with the PDZ domain of nNOS causing its functional disruption. Thus, further understanding the role of nNOS NOS1AP interaction in the cardiac pathology by selectively targeting regions in the brain may lead to the proper understanding of the molecular mechanisms involved in the central neurogenic control of cardiac functions.

1.8. Hypothesis of the current study

Despite recent advances in preventing SCD due to cardiac arrhythmia, it remains a leading cause of death in the United States, claiming an estimated 325,000 lives each year. Neurogenic cardiac damage has been found to be the most important contributing factor to sudden death. This neurogenic cardiac damage might be caused due to SAH, epilepsy, stroke or MI. The cardiac damage associated with central lesions might occur in the form of cardiac arrhythmia and/or damage to the myocardium. Two major factors determine an individual's risk for fatal cardiac arrhythmias– heritable genetic disorders such as Long QT interval Syndrome and genetic variations along with cardiac pathology. Recent studies have identified NOS1AP gene variants and provide evidence for the association between these variants and SCD through prolonged QTc interval. The specific mechanism(s) by which this effect is exerted remains to be elucidated. NOS1AP acts as a negative regulator of NO production and its downstream signaling events by competing with PSD95 for interaction with nNOS. Thus, overexpression of NOS1AP prevents NMDA–nNOS interaction. Our results demonstrated Nucleus tractus solitaries (NTS) in

the brain of Sprague Dawley (SD) rats have the most abundant NOS1AP mRNA. This finding is consistent with a recent study showing damaging neurons in NTS causes increased rate of SCD in rats, making it an excellent additional target to study neurogenic cardiac damage due to cardiac arrhythmia or myocardial damage. However, the nature and fundamental neuronal mechanisms linking NOS1AP in the NTS to arrhythmogenesis and/or myocardial damage are largely unknown. Based on recent evidences and our preliminary data, we hypothesized that NOS1AP knockdown in the NTS leads to more NMDA-PSD95-nNOS interaction causing overproduction of NO which causes neurotoxicity. This neurotoxicity in the NTS might induce arrhythmogenesis and/or cardiac damage (Figure 6).

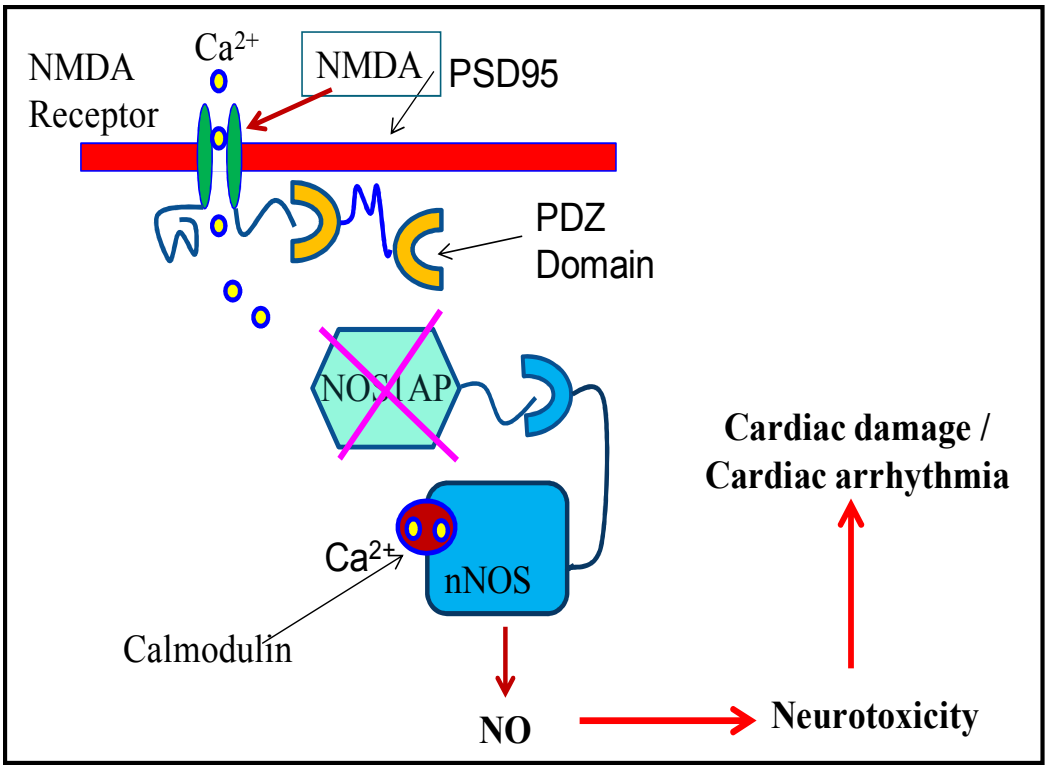

Figure 6: Project Hypothesis. NOS1AP knockdown in the NTS leads to more NMDA-PSD95-nNOS interaction causing overproduction of NO which causes neurotoxicity. This neurotoxicity in the NTS might induce arrhythmogenesis and/or cardiac damage.

We tested our hypothesis through the following specific aims:

1.1 To identify the role of NOS1AP in NMDAR mediated neuronal excitotoxicity and the downstream intracellular mechanisms in cultured neurons from SD rats. In this specific aim we would investigate whether NOS1AP downregulation in primary rat brain neuronal cultures lead to neurotoxicity due to overproduction of NO (Figure 6) due to change in functional NMDA receptor/nNOS coupling.

1.2 To detect the effect of NOS1AP knockdown in the NTS on the cardiovascular functions and electrophysiology of SD rats. The purpose of this specific aim was to assess the biological function of NOS1AP by genetic knockdown in the NTS.

Thus, our major objective was to provide evidences to support/or refute the above hypothesis. The combinations of *in vitro* and *in vivo* techniques with cellular, molecular and physiological approaches were used to accomplish the objective.

Completion of the proposed aims will provide new information indicating if 1) NTS is an important site contributing to fatal cardiac arrhythmias due to altered NOS1AP levels; and 2) NOS1AP induced NO neurotoxicity in the NTS is an important pathway contributing to central control of cardiac function. Both of these are critical for defining SCD risk markers. The results from these specific aims could fill the gap in knowledge regarding the intracellular mechanisms involved in NOS1AP-induced neurotoxicity, both *in vitro* and *in vivo* and help in establishing NOS1AP as a potential target for the prediction, prevention, and therapy of neurogenic cardiac damage underlying various cardiovascular diseases.

CHAPTER II. NOS1AP EXPRESSION PROFILING IN THE CARDIOVASCULAR REGULATORY REGIONS IN SD RATS

2.1. Introduction

Recent studies have implicated genetic variants of NOS1AP in prolonged QT interval, associated with increased risk for sudden cardiac death (SCD) (Newton CC *et al*., 2005, Hong Y *et al*., 2001, Straus SM *et al*., 2006). Also, NOS1AP is shown to sequester nNOS from binding to PSD-95. Thus, NOS1AP could help stability and localization and in turn directs and enhances the specificity of NO signaling (Jaffery SR *et al*., 1998, Sharma NM *et al*., 2011). Since nNOS and NO have been studied for their role in various cardiovascular pathophysiological conditions involving sympathetic activation, it would be interesting to unravel a role of NOS1AP under such conditions. The main cardiovascular regulatory regions involved in majority of pathophysiological processes are the PVN, RVLM and the NTS.

The Nucleus Tractus Solitarii (NTS) along with the Caudal Ventrolateral Medulla (CVLM) and the Rostral Ventrolateral Medulla (RVLM) comprises the central baroreflex arc. Both primary baroreceptor afferents and spinal dorsal horn neurons which transmit somatosensory input, project to and synapse within the NTS. During an interaction between the sensory pathways in the NTS, central baroreceptor activity is transmitted to other central nuclei involved in baroreceptor transmission. (Barraco RA *et al*., 1994). Therefore, there is compelling evidence to think that the NTS is involved in mediating the central interaction between baroreceptor and somatosensory receptor inputs. Other medullary regions, such as the RVLM, may also participate in this central interaction. Briefly described is the role of different brain regions in baroreflex resetting.

Resting heart rate (HR) and sympathetic nerve activity (SNA) is under the tonic control of cardiac vagal motoneurones (CVM) located in the nucleus ambiguus (NA) and inhibitory GABA neurons in the caudal VLM that inhibit sympathetic premotor neurons in the RVLM. The NTS sends excitatory glutamatergic fibers to the CVLM, activating the CVLM which then sends inhibitory GABAergic neurons to the rostral ventrolateral medulla (RVLM), thus inhibiting the RVLM. The RVLM is the primary regulator of the sympathetic nervous system, sending excitatory glutamatergic neurons to the sympathetic preganglionic neurons located in the intermediolateral nucleus of the spinal cord. Physiologically, the level of neural activity involved in the central pathways is modulated by the degree of excitatory drive from barosensitive NTS neurons that are dynamically controlled by input from aortic and carotid sinus baroreceptors which continuously respond to changes in arterial pressure (Sagawa, 1983).Thus, during a pathological condition characterized by an increase in blood pressure, the baroreceptors are activated, the neurons in the NTS activates the CVLM, which in turn inhibits the RVLM, thus inhibiting the sympathetic branch of the autonomic nervous system, leading to a decrease in blood pressure. This negative feedback control system results in the classic baroreflex function curve, components of which determine the balance between baroreceptor input and basal cardiovascular variables (such as HR and sympathetic nerve activity) .

Another level of sympathetic control is reported to occur at the level of paraventricular nucleus (PVN). It is well established that another central nuclei in the hypothalamus, PVN, also influences the regulation of sympathetic outflow through the interactions of excitatory and inhibitory neurotransmitters within. The afferent neural projections from the nucleus tractus solitarius (NTS) are received by PVN (Swanson LW *et al*., 1983). After receiving the vagal

cardiac and baroreceptor afferent information, the NTS transfers these cardiac sensing signals to the PVN (Lovick TA *et al*., 1988; Lovick TA *et al*., 1989). The PVN sends efferent projections to the pituitary gland, the RVLM, and the spinal cord (Pyner S *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 (Swanson LW *et al*., 1980; Swanson LW *et al*., 1979). These projections give rise to sympathetic efferent fibers to the heart (Wurster RD *et al*., 1977) and kidneys (Taylor RB *et al*., 1992), respectively. Latest evidence indicates that parvocellular neurons in the PVN are involved in the mediation of the neural component of cardiovascular reflexes via influencing sympathetic nerve discharge (Lovick TA *et al*., 1993; Haselton JR *et al*., 1994). However, dorsolateral portion of PVN contains magnocellular neurons. Magnocellular neurosecretory cells are differentiated in the following two types, oxytocin producing, and vasopressin producing. It is well proven that by controlling vasopressin release, the magnocellular neurons of the PVN are accountable for the humoral component of the regulation of fluid (Poulain DA *et al*., 1982). Most of the major central neurotransmitters and neuromodulators have been found in the PVN. Among the major neurotransmitters and neuromodulators that have been found in the PVN, glutamate (Hermes ML *et al*., 1996) and angiotensin II (Zucker IH *et al*., 2004) generally exert excitatory effects on cardiovascular reflexes. γ-aminobutyric acid (GABA) and NO (Stern JE *et al*., 2002) act as PVN inhibitors to influence sympathetic activity. The balance of these interactions might play important roles in the sympathetic dysfunction. The balance of these interactions might play significant roles in the sympathetic dysfunction.

Recent study has shown a decreased nNOS expression in the PVN of rats with heart failure, (Li YF *et al*., 2003, Zheng H *et al*., 2004) due to a decrease in NO production, which contributes to an increase in sympathetic outflow. nNOS gene transfer studies in the NTS have shown that although decreased expression of nNOS in the NTS inhibits sympathetically mediated baroreflex responses in rat, augmentation of nNOS in the same region did not have any effect on the baroreflex (Lin LH *et al*., 2012). Also nNOS augmentation in the RVLM is shown to normalize the impaired baroreflex function in CHF (Wang Y *et al*., 2003).

Studies have suggested that NOS1AP acts as a negative regulator of NO production and its downstream signaling events by competing with PSD95 for interaction with nNOS. Thus overexpression of NOS1AP prevents NMDA–nNOS interaction (Jaffery SR *et al*., 1998). It is important to elucidate the role of NOS1AP in the cardiovascular pathology, considering its involvement in the nNOS-NO pathway which is vital in the sympathoexcitation underlying various cardiovascular pathophysiological conditions.

Thus, the objective of the current chapter is to determine the NOS1AP expression in different cardiovascular regulatory regions of the brain and heart of SD rats. Also, the cellular localization of NOS1AP within some of the regions will be studied. These studies would help us in unraveling the specific regions of interest where NOS1AP expression could be different than others suggesting a role of NOS1AP in those regions. Lastly, we also determined the NOS1AP expression levels in myocardial infarction SD rat models to determine whether there is any change in NOS1AP levels in pathological condition underlying sympathoexcitation.

2.2. Materials and methods

2.2.1. Animals

Twelve-week-old Sprague-Dawley (SD) rats were obtained from Charles River Farms (Wilmington, MA). Rats were housed at 25 ± 2°C on a 12:12-h light-dark cycle and provided with food and water ad libitum. All animal protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee.

2.2.2. Induction of Myocardial infarction

CHF was induced by ligation of the left coronary artery, as described previously (Zhang K *et al*., 1998). Briefly, rats were ventilated at a rate of 60 breaths/min with 2–3% isoflurane during the surgical procedure. A left thoracotomy was performed through the fifth intercostal space, the pericardium was opened, the heart was exteriorized, and the left anterior descending coronary artery was ligated. Left ventricular dysfunction was assessed using hemodynamic and anatomic criteria. Echocardiograms were performed to measure left ventricular end-systolic diameter (LVESD), left ventricular end-diastolic diameter (LVEDD), and ejection fraction (EF). Left ventricular end-diastolic pressures (LVEDP) were measured using a Mikro-Tip catheter (Millar Instruments, Houston, TX, USA) inserted into the left ventricle via the right carotid artery. To measure infarct size, the heart was dissected free of adjacent tissues, and atria were removed. The right ventricle was opened with a lengthwise incision such that the heart was flattened with the left ventricle lying in the middle with the right ventricle on either side of it. The right ventricle was removed and the remaining left ventricle laid flat. A digital image of the left ventricle was captured using a Kodak DC290 digital camera (Kodak, Rochester, NY, USA) and the infarcted area and total left ventricular area quantified using SigmaScan Pro. Infarct size

(%) was determined by dividing the size of the infarcted area by the total size of the left ventricle. Rats with elevated LVEDP (≥15 mmHg) and infarct size >30% of the total left ventricular wall were considered to be in CHF.

2.2.3. Micropunch of the NTS, PVN and RVLM

A separate group of SD rats was euthanized by overdose of pentobarbital (65 mg/kg ip) and the brains were removed and quickly frozen on dry ice. Six serial coronal sections (100 μm) of the brain were cut using a cryostat. The PVN, NTS and RVLM were bilaterally punched using the Palvokits technique (Palvokits M. *et al*., 1983).

2.2.4. Real-time RT-PCR

We used real-time PCR to detect changes in the expression of NOS1AP in the different cardiovascular regulatory regions of SD rats. Total RNA was isolated from the tissue micropunches of three cardiovascular regulatory regions of the brain – the RVLM, NTS and PVN, and from the kidney heart and aorta with RNeasy Mini Kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA purity and concentration was determined spectrophotometrically using Nanodrop. For each Reverse Transcription PCR reaction, 100 ng of total RNA was converted into cDNA with reverse transcriptase kit (Promega catalogue no. 3500). Genomic DNA was eliminated by treatment with DNase I. For negative controls, RNA without the Reverse Transcriptase enzyme was used. For real-time PCR, the reactions were conducted by placing 5 μl of cDNA into 96-well plates with the 10μl of TaqMan qPCR Master Mix (Applied Biosystems). NOS1AP Probe (1μl) (Rn00356918_m1) and GAPDH probe (1μl) (Rn0000576699_m1) as an internal control, obtained from Applied Biosystems, CA, were used for each sample and the final volume was made to 20 μl by addition of Nuclease free water. A

no-template control was also run for every experiment with nuclease free water instead of cDNA template. Real Time-PCR was performed in an Applied Biosystems 7500 real time PCR system for 40 cycles (50°C, 2 min; 95°C, 10 min; 94°C 15 sec; 60°C, 36 sec) according to the protocol from the manufacturer. C_t values were obtained and relative change in gene expression was calculated using 2^-ΔΔCt method. In each experiment, samples were analyzed in triplicate.

2.2.5. Immunohistochemistry

To determine the tissue immunolocalization of NOS1AP on the neurons within the PVN and NTS, immunofluorescence staining was performed on the brain sections containing the PVN as well as NTS. Rats were perfused transcardially with 50 ml of saline followed by fixation in 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 and NTS containing transverse brain 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 NOS1AP polyclonal antibody (Santa Cruz Biotech) 1:500 overnight at 4°C. After being washed with Phosphate buffered saline with tween 20 (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)

2.2.6. Mosaic imaging of rat brain sections

The scanning stage was controlled by the AxioVision 4.6.3 software (Carl Zeiss), using the MosaiX module. Image acquisition was carried out with the AxioVision multichannel fluorescence module and the AxioCam MRm camera. For the single complete image of the whole section, multiple single adjacent images were acquired in a continuous series, with green, red and blue excitation and assembled automatically by stitching the images together using the MosaiX module of the AxioVision software (Zeiss, Germany). The composite image was 20 by 30 single images and covered an area of approximately 1 cm^2. Images were stored in the TIFF format. This kind of imaging would give a complete single image of the whole section under the same exposure and laser intensity that is advantageous when comparing several different regions with that particular transverse section. Also, specific regions could be zoomed in and analyzed separately.

2.2.7. Data analysis

Results are expressed as means ± SE. Statistical significance was evaluated with the use of a one-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at $P < 0.05$.

2.3. Results and data analysis

2.3.1. NOS1AP expression in cardiovascular regulatory regions

Before addressing the biological function of NOS1AP, it is important to investigate the NOS1AP expression pattern in the different cardiovascular regulatory areas of normotensive

Sprague-Dawley (SD) rats. For this NOS1AP expression profiling was performed by detection of mRNA levels using real time PCR in the heart, kidney, aorta and brain cardiovascular regulatory regions (NTS, PVN and RVLM). Results are shown in Figure 7. The NOS1AP expression in the aorta and heart are comparable (1.0+0.3 AU and 0.7+0.1 AU respectively, n=5, $p>0.05$) to that in kidney. However, NOS1AP expression was significantly elevated in the NTS, PVN and RVLM as compared with the kidney (2.8+0.6 AU, 1.8+0.6 AU and 1.4+0.2 AU respectively, n=5, $p<0.05$). Therefore, results showed that NOS1AP expression levels were significantly higher in the brainstem region of NTS of SD rats (Figure. 7) when compared with other cardiovascular regulatory areas such as RVLM, PVN in the brain, heart and aorta.

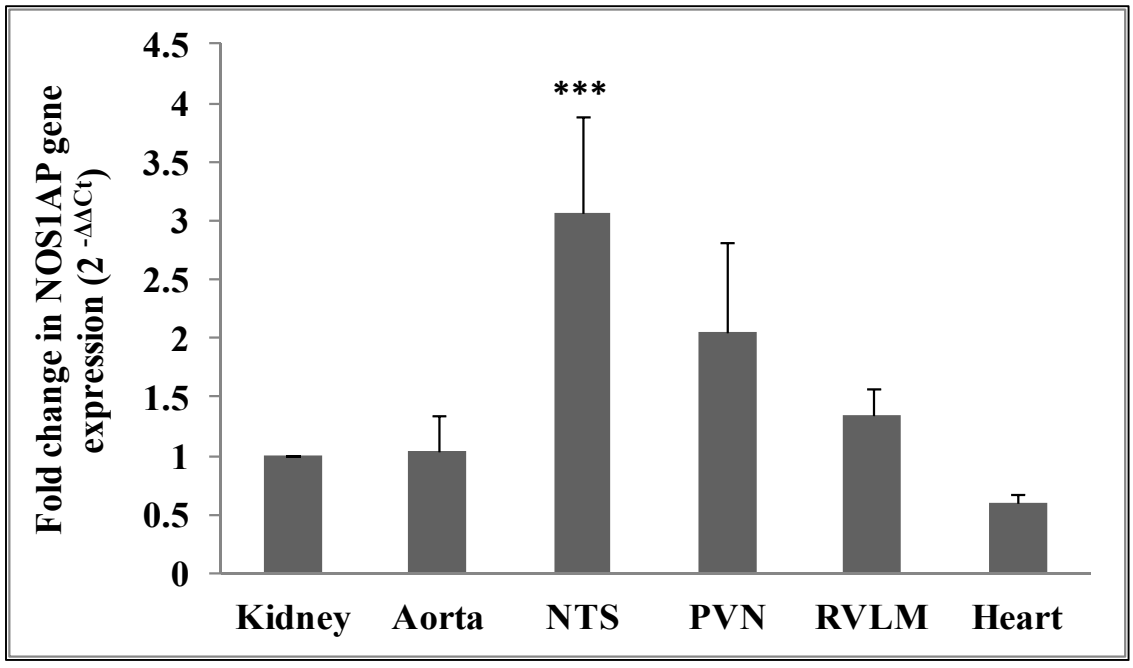

Figure 7: NOS1AP expression profiling in SD rats. NOS1AP mRNA expression in various cardiovascular regulatory regions of SD rats measured by real-time PCR. Values are mean ± SD of triplicate analyses of four to five rats in each group. Changes in NOS1AP mRNA are presented in folds with reference to the kidney. ***$P < 0.005$.n=5

2.3.2. NOS1AP immunolocalization in specific cardiovascular regulatory regions of the brain

To corroborate the expression data that there is an increase in NOS1AP expression in the NTS relative to other central cardiovascular regulatory regions such as PVN, transverse sections of the NTS and PVN were stained for NOS1AP, in situ, in SD rats. As assessed visually, NOS1AP immunostaining was more intense in the NTS region (Figure 8) of the brain as compared to the PVN (Figure 9).

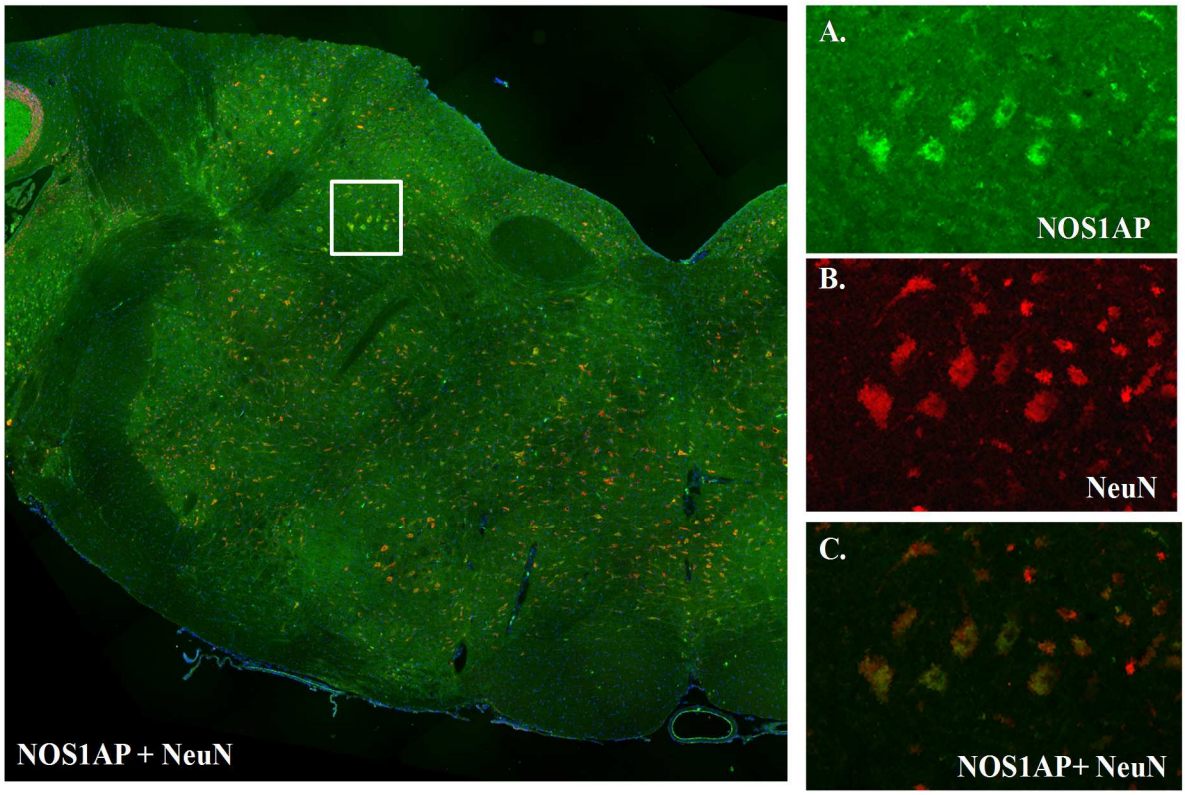

Figure 8: NOS1AP immunolocalization in the NTS of SD rats. Immunofluorescent photomicrographs from the transverse section of the brainstem containing the NTS region of SD rat after immunostaining with anti-NOS1AP (A) and with anti-NeuN antibody (B) antibody. Images in the right panel correspond to magnified area of the mosaic image showing the NTS region. (C)Overlap of A (NOS1AP) and B (NeuN) indicates that NOS1AP is localized in neurons in the NTS.

For imaging, we used the advanced Mosaic Imaging technique in order to get a complete fluorescent image of the complete transverse section of the rat brain to confirm the relative fluorescence in specific regions under same exposure conditions. Immunolocalization of NOS1AP within the NTS was also performed with neuron specific marker, NeuN. Results showed that NOS1AP is localized within the neurons of the NTS.

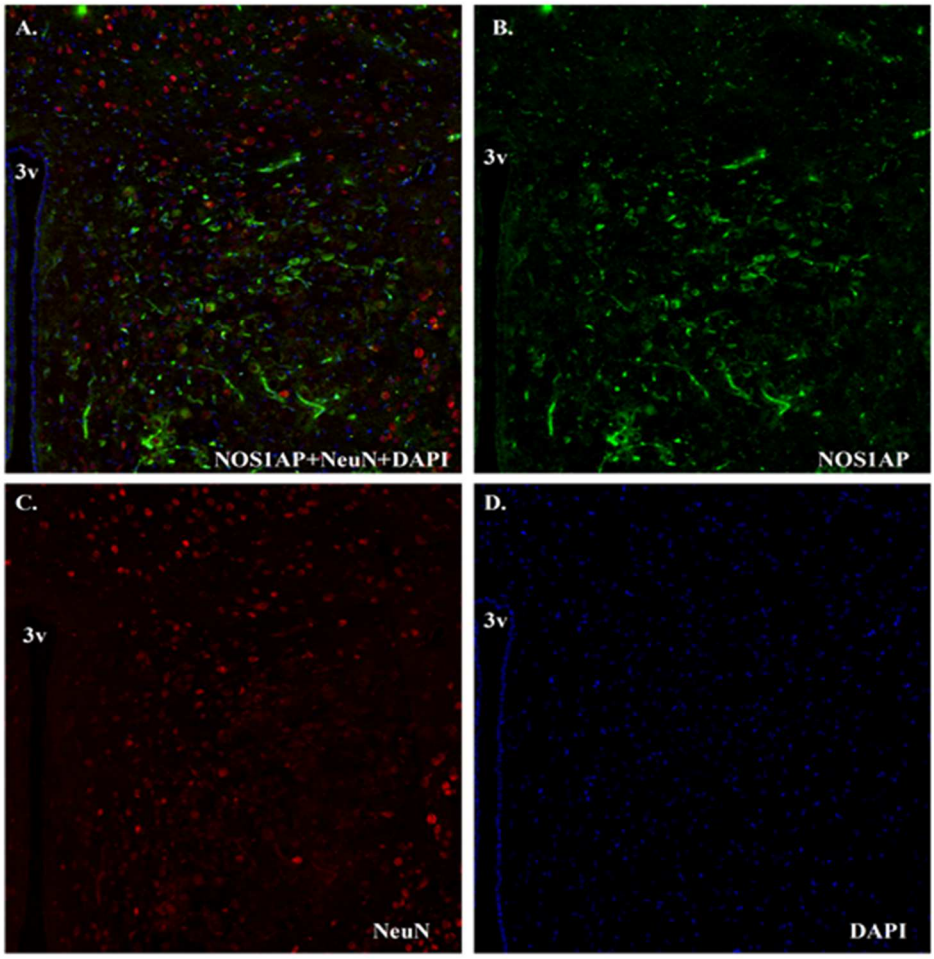

Figure 9: NOS1AP expression in the PVN of SD rats. Immunofluorescent photomicrographs from the transverse section of the brain containing the PVN region of SD rat after immunostaining with anti-NOS1AP (B) and with anti-NeuN (C) antibody and DAPI (D). The image A. corresponds to the merged overlap of NOS1AP with NeuN, indicating that NOS1AP is localized in neurons in PVN.

2.3.3. NOS1AP expression in cardiovascular regulatory regions under pathological condition

To determine whether NOS1AP expression is altered in cardiovascular pathophysiological conditions, we performed real time PCR experiment to investigate the NOS1AP expression pattern in the different cardiovascular regulatory areas of Myocardial Infarction SD rat models as compared to normotensive Sprague-Dawley (SD) rats.

For this, RNA was extracted from the NTS, RVLM and PVN micropunches and the right ventricle of the heart from the MI rat models and normal SD rats. qPCR was performed for detection of NOS1AP mRNA levels in these tissues. The NOS1AP mRNA levels in MI rat models were compared with the respective tissues from the normal SD rats. Results were expressed as the fold change in NOS1AP expression in MI rat models with respect to controls. NOS1AP expression in the RVLM of the MI rats were comparable (1.07±0.1 fold vs control, n=4, $p>0.05$) to that in control SD rats. However, NOS1AP expression was significantly elevated in the NTS (1.7±0.2 fold vs control, n=4, $p<0.01$), PVN (1.3±0.1 fold vs control, n=4, $p<0.05$) and heart (1.5±0.1 fold vs control, n=4, $p<0.01$) as compared with the control SD rats. Therefore, results demonstrated that during acute MI, there is an augmentation of NOS1AP expression levels in the NTS, PVN and heart (Figure 10).

2.4. Data summary and conclusion

The present study examined the NOS1AP expression profiling in the major cardiovascular regulatory regions of the brain, kidney, heart and aorta in SD rats. Also, the cellular localization of NOS1AP was confirmed in the cardiovascular regulatory regions of brain. In order to confirm that NOS1AP levels are perturbed in response to a cardiovascular

pathophysiological condition, we assessed the NOS1AP mRNA expression in the NTS, PVN, RVLM and heart in Myocardial Infarction rat models that were compared with normal SD rats.

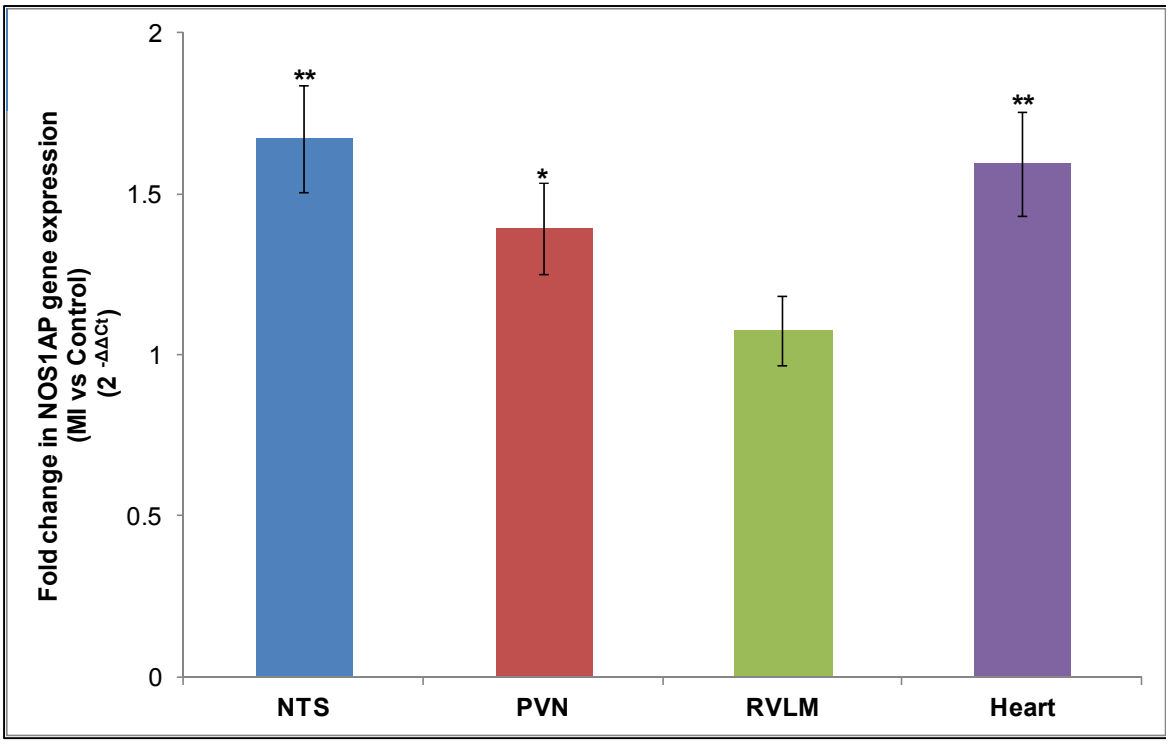

Figure 10: NOS1AP expression in different cardiovascular regulatory regions in Myocardial infarction. mRNA expression of NOS1AP in various cardiovascular regulatory regions from Myocardial infarction SD rat models as compared to Control SD rats measured by real-time PCR. Values are mean ± SE of triplicate analyses of four to five rats in each group. Changes in NOS1AP mRNA are presented in folds with reference to the control group. $^{**}p < 0.01$, *p<0.05 wrt controls. n=4. Data presented as Mean±SD.

The major findings of the present study are:

1. The NOS1AP mRNA expression is enhanced in the brain cardiovascular centers, the RVLM, PVN and NTS. The NOS1AP mRNA expression was comparable among the heart, aorta and the kidney.

2. The NOS1AP mRNA expression was the highest in the NTS of the SD rats as compared to the kidney.
3. The NOS1AP is localized in the neurons of the NTS and the PVN in SD rats, although the expression is augmented in the NTS as compared to other regions such as the PVN or RVLM.
4. Under pathophysiological MI condition, the NTS, PVN and heart showed an increase in NOS1AP mRNA expression as compared to the corresponding tissues in the normal control SD rats. The NOS1AP mRNA expression in the RVLM was similar in both normal and MI rat models.

Thus, through the above results, successful NOS1AP expression profiling was achieved. The results demonstrating that NOS1AP is abundantly expresses in the neurons of the NTS are consistent with recent evidences that NTS lesions interrupting the baroreflex may induce cardiac arrhythmias and myocardial changes similar to those seen in humans with central lesions (Biaggioni I *et al*, 1994). The altered baroreflex transmission was associated with sudden death in 33% of the experimental animals (Talman WT, 1993; Nayate A *et al*., 2008). Based on our results from this chapter and the previous data, we used NTS as the site of NOS1AP knockdown in our proposed experiments to test the effect of NOS1AP dysfunction on cardiovascular and electrophysiological functions. The finding that cardiovascular pathophysiologies involving sympathoexcitation leads to alteration of NOS1AP mRNA expression in major cardiovascular regulatory areas- NTS, PVN and heart. These results are in line to previous studies that have shown decrease in gene expression of neuronal nitric oxide synthase in hypothalamus and brainstem of rats in heart failure (Patel KP *et al*., 1996). This can be explained by our previous

observation and corresponding hypothesis that NOS1AP acts as a negative regulator of NO production and its downstream signaling events by competing with PSD95 for interaction with nNOS. Thus, overexpression of NOS1AP prevents NMDA–nNOS interaction. Therefore, in conditions manifested with sympathetic overactivation, such as heart failure, NOS1AP expression might be altered as a protective mechanism so that augmented NOS1AP will sequester more nNOS rendering them inactive and hence reduced NO production. Reduction in NO might serve as a protective mechanism considering it primarily plays an excitatory role. Also, reduced NO might also lead to a reduction in neuronal damage in the brain and myocardial damage in response to prolonged sympathoexcitation. Hence, the results in figure 10 contribute to an interesting finding.

Taken together, the results from the present study point towards an important role of NOS1AP in the various cardiovascular centers across the brain and heart under normal physiological conditions, which gets augmented with the development of pathophysiologies underlying sympathetic activation.

CHAPTER III. PREPARATION OF LENTIVIRAL PARTICLES FOR SELECTIVELY SILENCING NOS1AP GENE EXPRESSION

3.1. Introduction

In the previous chapter, results established that NOS1AP was highly expressed in the neurons of NTS along with some enhanced expression in the PVN and RVLM also. Moving forward, it would be interesting to know the effect of NOS1AP alteration in the cardiovascular function of these regions. Specifically, we are interested in looking at the effects of NOS1AP downregulation in the NTS as well as cellular systems in order to dissect its function. This would be helpful in gaining insights into the role of NOS1AP in cardiovascular propagation if any. A number of techniques have been implemented 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.

RNA interference (RNAi) was first coined by Fire and Coll in 1998. RNAi is the process by which expression of a target gene is silenced or knocked down by the selective inactivation of its corresponding mRNA by double-stranded RNA (dsRNA). RNAi is proving to be an invaluable research tool, allowing much more rapid characterization of the function of known genes (Fire A *et al*., 1998). Two main approaches to RNAi that have gained much interest for use in gene silencing are the double-stranded small interfering RNAs (siRNAs) and the vector-based short hairpin RNAs (shRNAs). While both siRNAs and shRNAs can be used for protein knockdown, there are differences in their mechanisms of action. siRNA are dsRNA with 2 nt 3’

end overhangs that activate RNAi, leading to the degradation of mRNAs in a sequence-specific manner dependent upon complimentary binding of the target mRNA. Short hairpin RNA (shRNA) that contains a loop structure that is processed to siRNA and also leads to the degradation of mRNAs in a sequence-specific manner dependent upon complimentary binding of the target mRNA. RNAi is result of a multistep process (Figure 11).

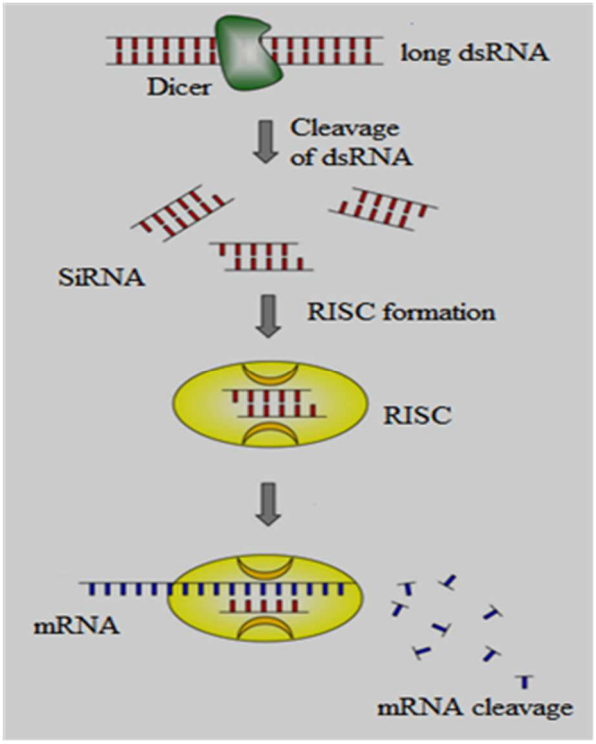

Figure 11: Mechanism of RNA interference (RNAi).

Long dsRNA is introduced to a cell. Once it is in the cytoplasm, the RNAse III enzyme, Dicer cleaves the dsRNA into 21–23 bp fragments with two nucleotide 3' end overhangs, i.e. siRNAs (Kinght SW *et al*., 2001). Following cleavage, RNA-induced silencing complex (RISC) loads and unwinds the siRNA, and binds to the complementary target mRNA which is

subsequently cleaved by the RISC. Following cleavage, the RISC complex disassembles and is ready to load another siRNA for cleavage of additional mRNA (Bernstein E *et al*., 2001).

The exact procedure for delivery of the siRNA or shRNA will depend on the cell type, since different cell types have varying sensitivities to the introduction of nucleic acids, and whether using siRNA- or shRNA-mediated knockdown, as well as the length of the assay. Transfection, electroporation, and certain non-viral delivery methods are transient (short-term/in-vitro study). Viral vectors have become a popular approach for transduction of shRNA because they allow for longer-lived gene silencing effects by stably integrating the shRNA into the cell's genome, allowing for persistent (long-term/in-vivo study) expression. Several viral vectors have been used for this purpose including adenoviral vector, adeno-associated viral vectors (AAV), retroviral vector (Anson DS, 2004), and lentiviral vectors (Lever AM *et al.*, 2004). These vectors have very good efficiency for the transduction of cells, but differ largely in integration into host chromosomal DNA. Adenoviral and retroviral vectors have been reported to produce siRNAs in vivo. (Shen C *et al*., 2003, Stewart SA *et al*., 2003) Adenoviruses are small dsDNA viruses that infect most cell types. DNA delivered using adenovirus-derived vectors are maintained as epichromosomes in the nucleus, making the expression transient, but eliminating the risk of insertional mutations. Adenovirus vectors exhibit extremely broad cell tropism and are safe to work with in laboratory settings. While this is advantageous for experiments in tissue culture, these vectors pose problems in clinical settings owing to several drawbacks that include being immunogenic (Sun JY *et al*., 2002), limited payload capacity to carry transgene, and difficulty in large-scale production. Many lentiviral and retroviral plasmids are suitable for shRNA expression as they transduce both dividing and non-dividing cells and integrate into host

chromosome conferring long-term transgene expression. Specific plasmid and shRNA design may vary slightly depending on the cell type (non-transformed primary cells) the basic shRNA-expressing lentivirus construct contains the Pol III promoter followed by the shRNA, associated enhancer elements, a 5' and 3' LTR, and a packaging sequence. Lentiviral or retroviral plasmid is cotransfected into a packaging cell line (such as 293T) along with packaging plasmids, which encode the enzymatic and packaging proteins required for production of the lentivirus. The replication deficient lentivirus that is developed recently can be used to transduce the target cells. Replication incompetent lentiviral vectors due to their low toxicity and low immunogenicity profile and are considered to be ideal vector for transduction (Park TG *et al*., 2006; Logan AC *et al*., 2002).The lentiviral RNAi technology has provided the ability to quickly generate transgenic animal models. Once the procedure is set up and optimized, the time to generate lentiviral transgenic mice can be very short (Warnock J *et al*., 2011, Devroe E *et al*., 2002).

The goal of the present study is to construct lentiviral particles encoding NOS1AP shRNA in order to selectively knockdown the NOS1AP expression both *in vitro* and *in vivo* and further on, to assess the biological function of NOS1AP by genetic knockdown in the NTS. Lentivirus containing scrambled shRNA was also prepared and used as a negative control.

Thus, objectives of the current study are (1) To construct a lentiviral vector containing shRNA to effectively knockdown the NOS1AP gene expression. (2) Determine viral titer using flow cytometry since the cells would express GFP when transduced and consequently detected by green fluorescence. (3) To check whether the Lv-NOS1AP-shRNA lentiviral preparation is capable of effectively downregulating NOS1AP protein expression in both *in vitro* as well as *in vivo* applications. For in vitro testing of Lv-NOS1AP-shRNA we transduced three different cell

systems- Primary neuronal cell cultured from hypothalamus/brainstem of neonatal rats, a neuron cell-line, Cath.a cells [CRL11179](ATCC CRL11179, Manassas,VA) and HEK293 cells. For *in vivo* testing, we microinjected the Lv-NOS1AP-shRNA into the NTS to evaluate the effect of the lentiviral particle on NOS1AP knockdown.

3.2. Materials and methods

3.2.1. Animals

Twelve-week-old Sprague-Dawley (SD) rats were obtained from Charles River Farms (Wilmington, MA). Rats were housed at 25 ± 2°C on a 12:12-h light-dark cycle and provided with food and water ad libitum. All animal protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee.

3.2.2. Construction of pGIPZ plasmid containing NOS1AP shRNA

To selectively knockdown NOS1AP, *E. coli* (DH5α) bacteria carrying pGIPZ plasmid encoding for NOS1AP shRNA (figure 12) or pGIPZ plasmid containing scrambled shRNA were obtained from Thermo Open Biosystems (Huntsville, AL).

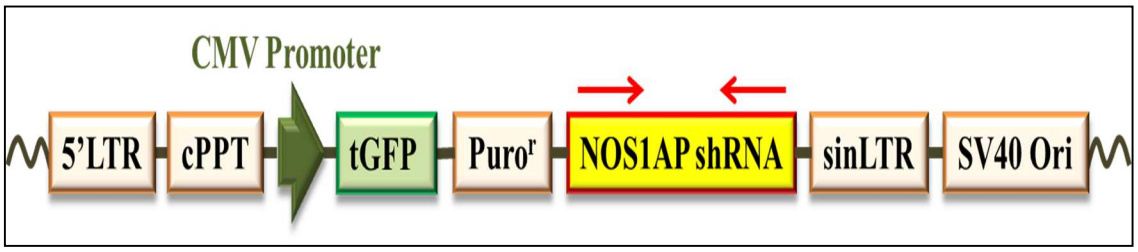

Figure 12: Plasmid design of pGIPZ lentiviral vector/ Lv-NOS1AP-shRNA. The vector elements as shown in figure are: cPPT- Central Polypurine tract help translocation into the nucleus of non-dividing cells; tGFP- turbo GFP Marker to track shRNAmir expression; Puro^r- Mammalian selectable marker; 5'LTR- 5' long terminal repeat; SIN-LTR- 3' Self inactivating long terminal repeat.

E. coli (DH5α) bacteria containing pGIPZ plasmids were supplied as frozen glycerol stocks. *E. coli* from frozen stocks were streaked onto a Lennox broth (LB) agar plate containing 100 μg/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 μg/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 pGIPZ plasmids with NOS1AP-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 μl isolated DNA to 990 μl DNase/RNase free water. The samples were mixed well and pipette into a quartz cuvette. O.D. was measured at 260 nm. DNase/RNase free water was used as blank. Total DNA concentration of the sample was calculated using the following formula.

Unknown DNA concentration (mg/ml) = 50 mg/ml x Measured A260 x dilution factor

The integrity of the pGIPZ plasmid DNA with NOS1AP shRNA isolated from *E. coli* (DH5α) was characterized with enzyme restriction analysis and agarose gel electrophoresis. Restriction enzymes XhoI, NotI, PsiI, SacII and KpnI (New England Bio labs, Ipswich, MA) were chosen based on the plasmid map provided from the manufacturer. Single, double and triple digestions were performed with the restriction enzymes in order to completely verify the pGIPZ plasmids. Reagents for the setup of restriction reaction are indicated in Table 3.

Table 3: Reaction Mixture Set for Restriction digestion of pGIPZ plasmid.

Component	Amount
Sterile, nuclease-free water	Xµl
Restriction enzyme 10X buffer	1µl
BSA (10X, 10mg/ml) if required	1µl
DNA sample 1µg, in water or TE buffer	Xµl
Restriction enzyme 20U	0.25µl
Final volume	10µl

Plasmid DNA without restriction enzyme was used as 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.

3.2.3. Preparation of HEK293T cell cultures

Production of lentiviral vectors carrying shRNA for NOS1AP (Tiscornia G *et al.*, 2006) 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. 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 37ºC 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 resuspended in the medium and added to a T-25 flask containing DMEM supplemented with 5% FBS. Cells were cultured at 37ºC with 5% CO_2. 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.

3.2.4. Lentiviral packaging

We used a Trans-Lentiviral GIPZ Packaging System (Thermo Open Biosystems, Huntsville, AL) to make lentivirus containing NOS1AP shRNA. This system allows creation of a

replication incompetent HIV-1 based lentivirus. 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 and the transfer vector plasmid containing NOS1AP shRNA transcript. Co-transfection of the trans-lentiviral packaging mix and the transfer vector containing the NOS1AP shRNA or scramble control shRNA (SCR shRNA) encoding gene into HEK293T cells produces a replication-incompetent lentivirus that can be used to silence NOS1AP receptor expression.

After preparation of the lentiviral expression vector and packaging plasmids, HEK293T cell 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 x 106 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 µl 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 CO_2 incubator with 5% CO_2. The transfection mixture was aspirated and fresh 12 ml 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.

3.2.5. Titration of lentivirus

After the lentiviral production they need to be assessed for the accurate quantification of functional vector particles within a given vector preparation. Since the lentiviral vector contains tGFP, we can calculate its biological titer by Flow cytometry which gives the titer value based on the GFP positive cells.

In brief, HEK 293T cells were plated in 12 well cell culture plates and grown in a CO_2 incubator until 30-50% confluent. On the day of transduction (Day 2), lentiviral stock was thawed and serial diluted in serum free media ranging from 25X-625X were prepared. For each dilution, the lentiviral stock was diluted with serum free culture medium to a final volume of 500 µl. 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 cells were allowed to incubate at 37°C for 48hrs in a humidified 5% CO_2 incubator. After 48 hrs. We resuspended the pellet in 1 ml of 1× PBS after fixation of the cells in 4% PFA and analyzed the cells for GFP expression using flow cytometer. We calculated the viral titer using the following equation:

Titer of Lv-NOS1AP-shRNA (TU/ml) =

$$\frac{No.of\ target\ cells\ (count\ on\ day\ 1) * \%\ of\ GFP\ positive\ cells}{Volume\ of\ vector\ (ml)}$$

3.2.6. Preparation of Cath-a cell cultures

In order to test the Lenti-NOS1AP-shRNA transduction, Cath.a cell-line was used because of its neuronal origin. Cath.a cells express a variety of pan-neuronal markers, neurofilaments and GPCR 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 37ºC 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 (HS) and 4% FBS. 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% HS and 4% FBS. Cells were grown at 37ºC with 5% CO2 and passaged regularly when the confluence reached 90%. Cath-a cells grow both in suspension as well as adhered to the cell culture dish.

3.2.7. Cath.a cell transduction and imaging of transduced cells

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 5×10^7 transducing units (TU) per ml Lenti-shRNA (Lv-NOS1AP-shRNA) virus was added onto the cells (n=3) and incubated for 18 hours at 37°C in a CO_2 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); Fixed with 4% PFA and imaged for bright field and green

fluorescence detected with a confocal fluorescence microscope. The fluorescent images will be collected and analyzed with computer software.

3.2.8. Preparation of primary neuronal cultures

In the present study, the efficiency of Lv-NOS1AP-shRNA was determined in knocking down NOS1AP expression in non-dividing primary cultured neurons. Primary cultured neuronal cells were prepared from the hypothalamus/brainstem 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 precoated 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 7–14 days before use.

3.2.9. Determination of NOS1AP protein levels in primary cultured neurons after lentiviral transduction

To confirm the efficiency of Lv-NOS1AP-shRNA vector on reducing NOS1AP expression in primary neuronal cultures from the hypothalamus/brainstem of rats, cultured cells were transduced with 2 μl of Lv-NOS1AP-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-NOS1AP 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 NOS1AP. 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).

3.2.10. Microinjection experiment

Lv-NOS1AP-shRNA or Lv-SCR was microinjected bilaterally into the NTS, according to procedures described previously (Ito K et al., 2003, Tsukamoto K et al., 1993). In brief, the anesthetized animals were placed in a stereotaxic frame. A multiple-barrel glass injection pipette (tip size 20–40 μm) was positioned in the NTS. The coordinates for the NTS were determined from the Swanson rat atlas (Swanson LW, 2004), which were 0.5 mm rostral to the caudal tip of the area postrema, 0.5 mm lateral to the midline, and 0.5 mm below the dorsal surface of the brain stem. Proper placement was confirmed by checking for an l-glutamate-induced (200 pmol, in 50 nl) depressor response. This would induce a characteristically abrupt decrease in BP (ΔBP > 35 mmHg) and HR (ΔHR > 50 beats/min), if the needle tip was located precisely in the NTS. After a responsive site was identified by l-glutamate, the probe remained in this site throughout the remainder of the experiment. Lv-NOS1AP-shRNA (1×10^9 genome copy in 50 nL) will be microinjected bilaterally into the NTS over a 25-minute period with a microinjection device. Throughout the experiment, rat body temperature was maintained in the range of 36.5–37.5°C with a heating pad (Gaymar Industries, Orchard Park, NY). After the protocol, the injection

position was also confirmed by microinjection of methylene blue dye (50 nl). To avoid the spread into non targeted area of the brain other than NTS, the volume of each injection would not be more than 100nl and will be microinjected bilaterally into the NTS over a period of 25 minutes.

3.2.11. Determination of NOS1AP protein levels in the NTS of SD rats after microinjection of Lv-NOS1AP-shRNA

To confirm the efficiency of Lv-NOS1AP-shRNA vector on reducing NOS1AP expression in NTS of SD rats, total proteins from NTS micropunch from control and Lv-NOS1AP-shRNA injected SD rats, 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-NOS1AP 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 NOS1AP. 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).

3.2.12. Data analysis

Results are expressed as means ± SE. Statistical significance was evaluated with the use of a one-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at $P < 0.05$.

3.3. Results and data analysis

3.3.1. Validation of pGIPZ plasmid

E. coli bacteria carrying pGIPZ plasmid (11610 bp) that encodes for NOS1AP shRNA was cultured overnight and plasmid DNA was isolated using the Qiagen plasmid isolation kit. Isolated plasmid DNA was restriction digested with XhoI, Not1, KpnI, Sac II and PsiI that have multiple restriction sites within the plasmid.

Results are presented in Figure 13 showing digested plasmid DNA run on a 0.8 % agarose gel. Restriction digests with pGIPZ. Lane 1 shows a 10kb molecular weight DNA ladder. Lane 2 shows XhoI (cleaves at 5391) single digested Linear pGIPZ vector. Lane 3 shows KpnI (cleaves after 4560, 6391) double digested pGIPZ produces 2 bands at 1750bp and 9860bp. Lane 4 shows SacII (cleaves after 4907, 6166, 8668) triple digest produces 3 bands at 1178bp, 2502bp and 7930bp. Lane 5 shows XhoI (cleaves at 5391) and NotI (cleaves at 4100) double digest produces 2 bands at 1210bp and 10400bp. Lane 6 shows PsiI (cleaves after 6726, 7426, 9062, 11406) produces 4 bands at 700bp, 1636bp, 2344bp and 6934bp. Thus through a combination of various restriction enzymes that have multiple cleavage sites we were successfully able to confirm that the plasmid isolated from E. coli bacteria was pGIPZ.

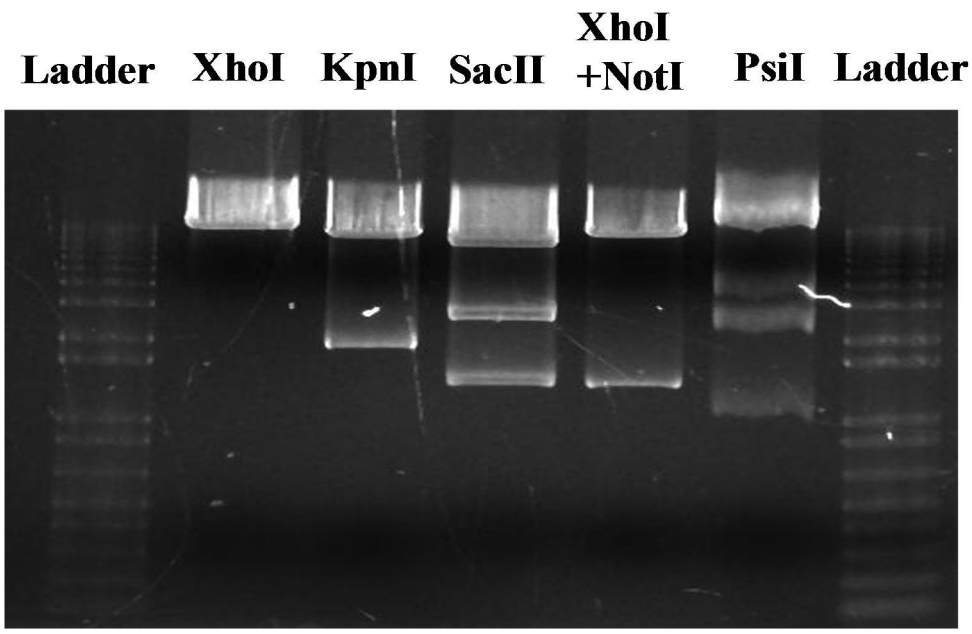

Figure 13: Restriction digests with pGIPZ. Lane 1- 10kb molecular weight DNA ladder. Lane 2 – XhoI single digested linear pGIPZ vector. Lane 3 - *Kpn*I digested pGIPZ produces 2 bands at 1750bp and 9860bp. Lane 4- *Sac*II digest produces 3 bands at 1178bp, 2502bp and 7930bp. Lane 5 - *Xho*I, *Not*I double digest produces 2 bands at 1210bp and 10400bp. Lane 6 - *Psi*I produces 4 bands at 700bp, 1636bp, 2344bp and 6934bp.

3.3.2. Determination of the viral titer

Lentiviral vectors carrying transcripts encoding for NOS1AP shRNA and SCR shRNA were prepared using a lentiviral packaging kit. Viral tittering was performed to determine the number of viral particles in the concentrated viral stock. Flow cytometry which gives the titer value based on the GFP positive cells.

For this, we plated HEK293T cells/well in a 12-well plate and transduced the cells with serial dilutions of the Lentiviral particles as describes previously. After 48-72 hrs, we analyzed the cells for GFP expression using flow cytometer. We calculated the titer using the equation described previously. Applying the formula, total titer of our viral stock of Lv-NOS1AP-shRNA

was found to be 1.5 X 10^9 TU/mL (figure 14). These results indicate that we have produced viral particles with high titer values for transduction of neuronal cells.

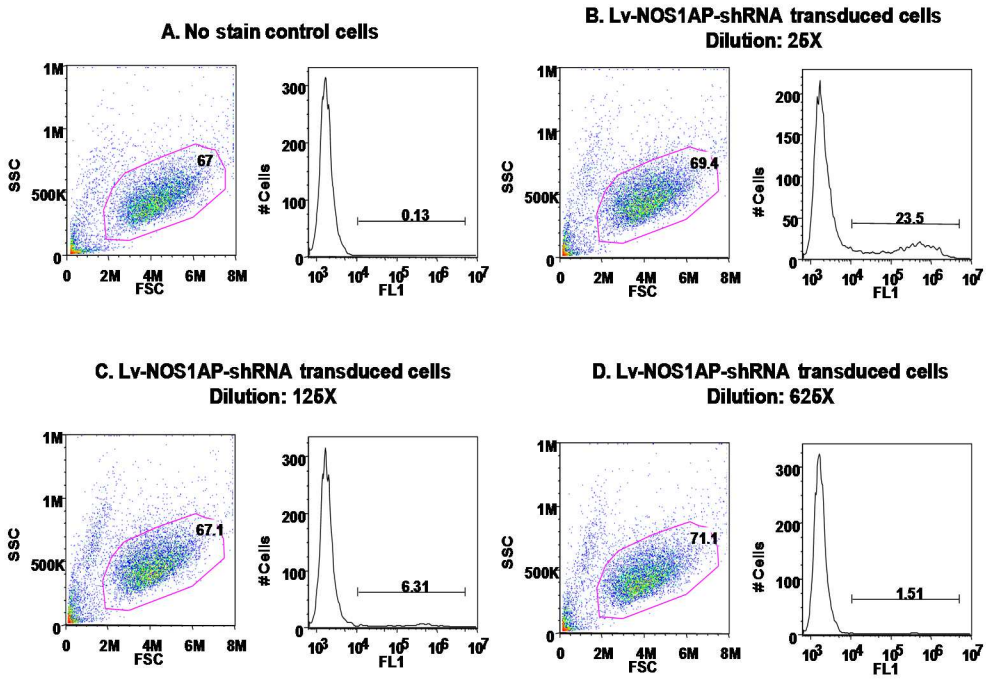

Figure 14: Flow cytometric analysis of titer of the Lentiviral particles. Representative flow cytometric analysis images of HEK293T cells transduced by Lv-NOS1AP-shRNA viral particles for 48hrs. The experiment has been performed on different dilutions of the viral particle and the corresponding number of transduced cells counted by flow cytometric evaluation of GFP expressed by transduced cells. A. represents no stain control consisting of untransduced cells, followed by cells transduced with different dilutions (B. 20X, C. 125X, D. 625X diluted). The results from this flow cytometric analysis were used to calculate the titer of the lentivirus.

Also, as a first line of evidence GFP fluorescence was also measured in three different cellular systems- HEK293T cells, Cath-a cells and primary neuronal culture cells, after lentiviral transduction. Results of fluorescence imaging as shown in figure 15 show an enhanced GFP expression indicating high transduction efficiency in all three cellular systems.

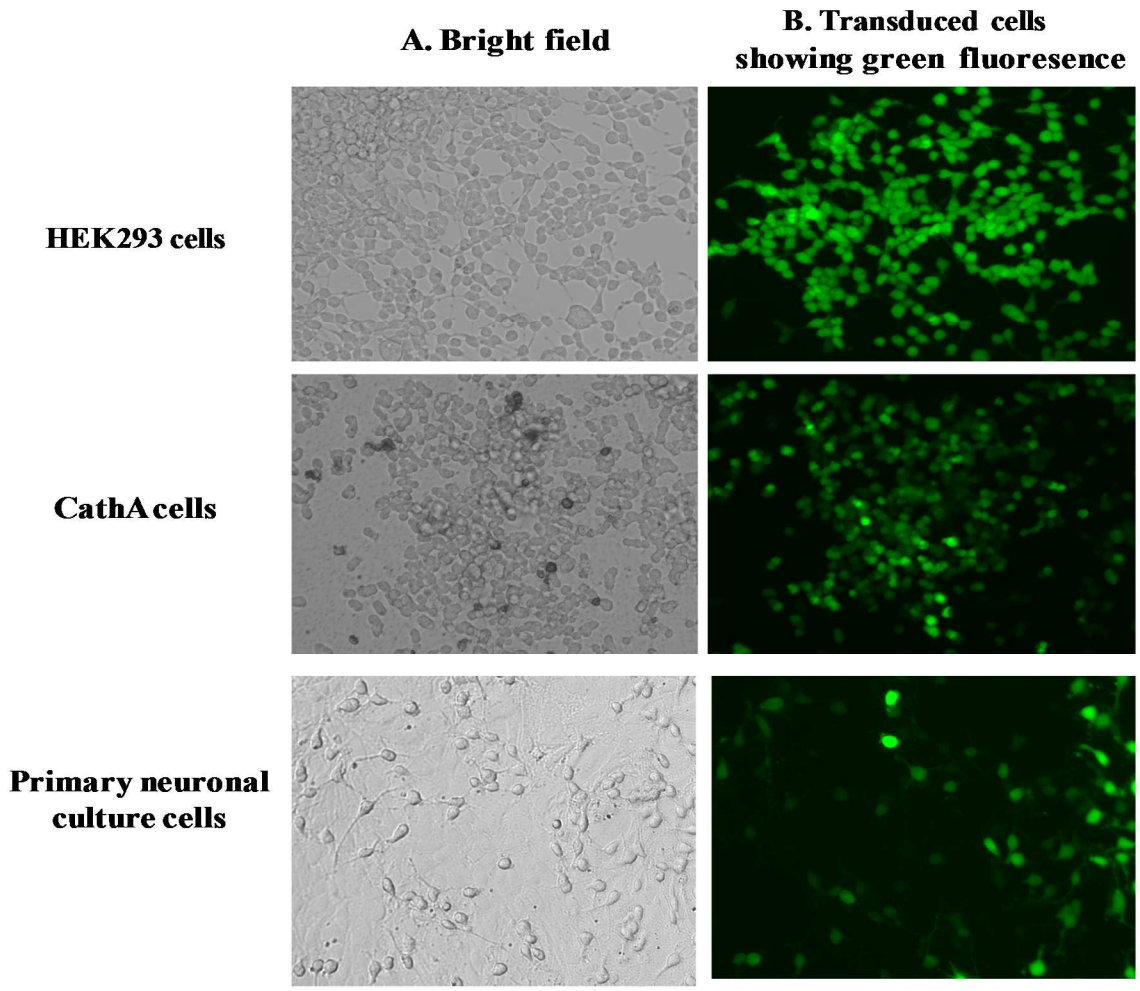

Figure 15: Transduction efficiency of Lv-NOS1AP-shRNA Lentivirus in different cellular systems. Representative images of transduction of HEK293 cells (upper panel), Cath-a cells (middle panel) and Primary neuronal culture cells (lower panel) with Lv-NOS1AP-shRNA. A. Bright field B. GFP expression. High percentage of total cell population is shown to express the NOS1AP shRNA after 48hrs of lentiviral transduction.

3.3.3. Effect of Lv-NOS1AP-shRNA-shRNA on NOS1AP protein levels of neuronal cell culture

To determine whether protein levels of NOS1AP were down-regulated in the neurons transduced with Lv-NOS1AP- shRNA as compared to untransduced control western blot

analysis was performed. The NOS1AP protein levels in the cultured neurons were examined using the regular western blot assay.

The results showed that NOS1AP protein levels were significantly decreased in the neurons transduced with Lv-NOS1AP-shRNA as compared with control (Figure 16) ($P<0.05$, n=3, each incubation). A densitometric analysis was also performed to quantitate the intensity of the respective protein bands in control and experimental conditions using Image J software. Results showed that NOS1AP protein levels were significantly reduced in the neurons transduced with lenti-NOS1AP-shRNA virus by 60%, as compared with control neuronal cells ($P<0.05$). Taken together, these observations demonstrate that NOS1AP expression is downregulated in neuronal cells transduced with lenti-NOS1AP-shRNA virus showing that lenti NOS1AP-shRNA virus are effective in knocking down of NOS1AP in primary cultured neurons.

3.3.4. Effect of Lv-NOS1AP-shRNA-shRNA on NOS1AP protein levels of NTS

In the previous experiment we demonstrated the efficiency of the lenti-NOS1AP-shRNA viral particles in reducing the NOS1AP level specifically in primary neuronal culture cells cultured from the hypothalamus/brainstem. Next, we determined the effect of Lv-NOS1AP-shRNA on the NOS1AP protein levels specifically in the NTS of the brain.

Briefly, Rats will be anesthetized and Lv-NOS1AP-shRNA (1×10^9 genome copy in 50 nL) will be microinjected bilaterally into the NTS over a 25-minute period with a microinjection device. Rats were allowed to heal and then after 3 days the brain was collected and Western blot was performed on the protein isolated from the tissue micropunches from the NTS. The NOS1AP protein levels in the NTS tissue were examined using the regular western blot assay.

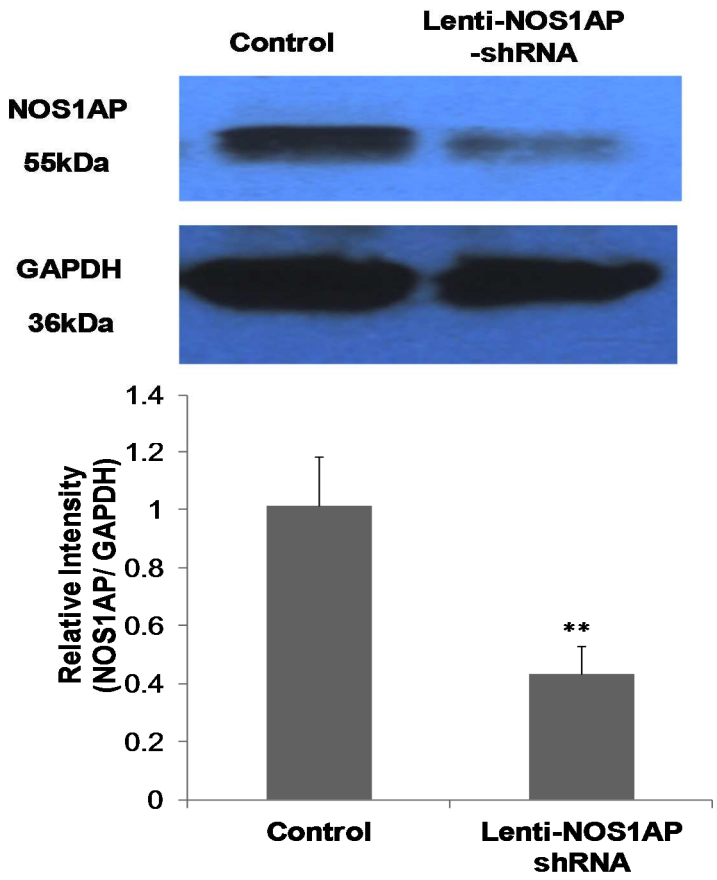

Figure 16: Knockdown of NOS1AP in primary neuronal culture cells with Lv-NOS1AP-shRNA. Upper panel demonstrates western blots showing that NOS1AP levels were significantly decreased in neuronal culture cells 72hrs after Lv-NOS1AP-shRNA transduction. Lower Panel represents a densitometric analysis of western blots performed by ImageJ. Data presented as mean±SE, n=3 in each group. **p <0.01 vs control group.

The results showed that NOS1AP protein levels were significantly decreased in the NTS microinjected with Lv-NOS1AP-shRNA as compared with control (Figure 17) ($P<0.05$, n=6, each group). A densitometric analysis was also performed to quantitate the intensity of the respective protein bands in control and experimental conditions using Image J software. Results showed that NOS1AP protein levels were significantly reduced in the neurons transduced with lenti-NOS1AP-shRNA virus by 60%, as compared with control neuronal cells ($P<0.05$).

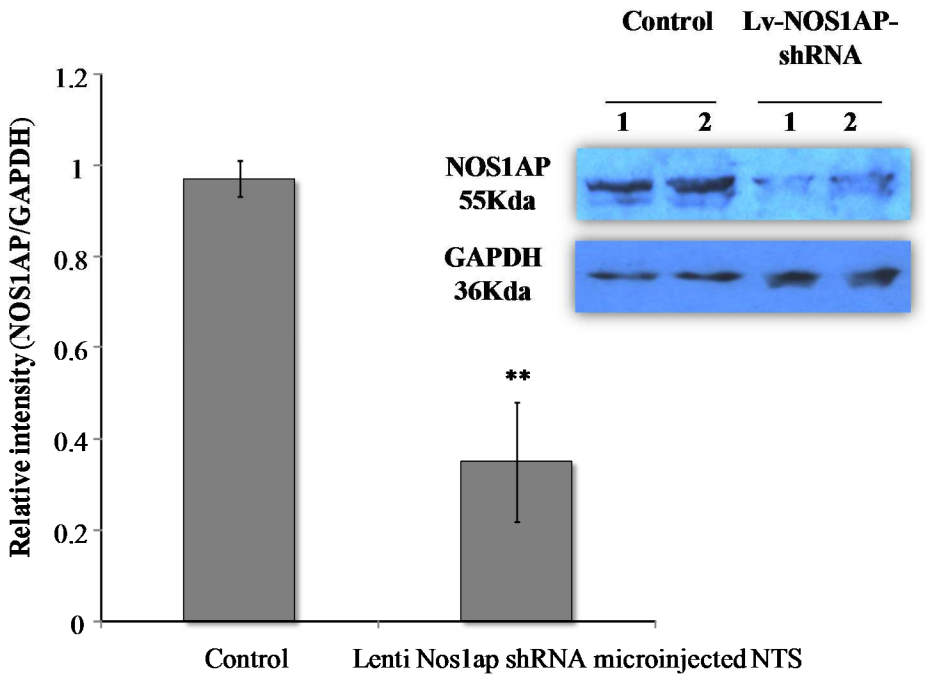

Figure 17: Knockdown of NOS1AP in the NTS of SD rats. Upper panel demonstrates western blots which revealed that NOS1AP levels were significantly decreased in NTS 4 weeks after Lv-NOS1AP-shRNA injection into the NTS of SD rats (n=6). Lower Panel represents a densitometric analysis of western blots performed by ImageJ. Data are presented as mean±SE from all six rats in each group. **p <0.01 vs control group.

Taken together, these observations demonstrate that NOS1AP expression is downregulated in NTS microinjected with lenti-NOS1AP-shRNA virus showing that lenti NOS1AP-shRNA virus are effective in knocking down of NOS1AP in the tissue, thus, establishing the efficacy of lenti NOS1AP shRNA virus to be used for the specific knockdown of NOS1AP expression *in vivo.*

3.4. Data summary and conclusion

The overall objective of the study is to determine the effect of NOS1AP dysfunction in the progression of neurological lesions leading to myocardial damage which might be

accompanied by cardiac arrhythmia. In earlier studies, the NOS1AP mRNA expression was measured in various cardiovascular regulatory regions of the brain including the PVN, NTS, and RVLM and it was found that the NOS1AP was elevated in NTS of the brain. Therefore, in the present study the aim was to construct lentiviral vectors carrying NOS1AP-shRNA to silence the NOS1AP expression in the region of interest thereby imparting selectivity in the NOS1AP expression knockdown. Lv-SCR were packaged using HEK 293T cells as a control. Virus was concentrated by ultracentrifugation and tittered using Flow cytometry analysis.

The major findings from the present study are listed below:

1) High titers of viral particles carrying Lv-NOS1AP-shRNA or Lv-SCR were prepared and used for *in vivo* and *in vitro* gene transfer.

2) Tittering viral particles were carried out by transducing the HEK 293T cells with various dilutions of viral particles and flow cytometry techniques. Results showed that the viral particle concentration in the preparation was up to $1.5 \text{ X } 10^9$ TU/mL.

3) Enhanced Lentiviral vector transduction in the form of high GFP fluorescence was observed after 48hrs of transduction in the 3 different cellular systems - HEK293, Cath.a cells and primary rat brain neuronal culture cells.

4) NOS1AP protein levels were markedly decreased after transduction with Lv-NOS1AP-shRNA in primary neuronal culture cells, thus establishing the lentiviral efficacy in vitro.

5) NOS1AP protein levels were markedly decreased after bilateral microinjection of Lv-NOS1AP-shRNA in the NTS of SD rat brain, thus establishing the lentiviral efficacy in vivo.

In summary, this study produced high titer lentiviral vectors carrying Lv-NOS1AP-shRNA and Lv-SCR viral particles. Lentiviral vector containing NOS1AP shRNA (Lv-NOS1AP-shRNA) significantly knockdown NOS1AP expression *in vitro* for both dividing and non-dividing neurons as well as in NTS *in vivo***.** The use of lentiviral vectors for knockdown in the current study is a promising approach. Apart from stably integrating into the host genome to provide stable knockdown of NOS1AP expression over long term, it can also transduce non dividing cells such as neurons. Both the advantages are a prerequisite for the present study since the experiments involve long term recording of different cardiovascular and electrocardiographic variables. Thus, lesser variability in recordings will be achieved due to stable NOS1AP expression knockdown over a longer duration of time.

Other conventional gene delivery systems such as lipid-mediated gene delivery is based on cationic lipid molecules have made a lot of progress but still lag behind lentiviral vectors with respect to transfection of primary neuronal cells with reduced cytotoxicity. Studies have shown that transfection efficiencies of up to 85% can be achieved with cell lines, however, when used to transfect postmitotic neurons, tend to transfect only 1–5%, with a maximum of up to 30% reported for primary neurons (Dalby *et al*., 2004). These comparatively low transfection efficiencies for primary neuronal cells have limited the application in our current study.

Thus, the Lv-NOS1AP-shRNA prepared is used for the rest of the study to knockdown NOS1AP. Another advantage of using this type of knockdown approach via microinjection to a particular site in the brain is that, it helps to dissect out the function of that specific region. This is opposed to global gene silencing or knockout mice models where region specificity in deciphering a particular protein function is restricted. Thus, further in the study this technique is

successfully implemented in dissecting the effect of NOS1AP dysfunction, caused due to the microinjection of Lv-NOS1AP-shRNA, in the neurological damage in the NTS which might lead to cardiac arrhythmia accompanied by cardiac pathology.

CHAPTER IV. ROLE OF NOS1AP IN GLUTAMATE MEDIATED NEUROTOXICITY IN PRIMARY RAT BRAINSTEM NEURONAL CULTURE CELLS

4.1. Introduction

NMDA mediated excitotoxicity (Olney JW *et al*., 1972) has been implicated in several neurogenic pathophysiology (Lipton SA *et al*., 1994) such as stroke. NMDA receptors are composed of a tetramer of different subunits: NR1 (mandatory), NR2A-D, and, in some cases, NR3A or B subunits. The difference in the subunit composition as well as the alternative splicing of some subunits, contributes to the pharmacological properties of the receptor-ion channel complex. Also, it has been reported that the subunits are differentially expressed both regionally in the brain and temporally during development (Kemp JA *et al*., 2002). Studies have also shown that potential neuroprotective agents that block virtually all NMDA receptor activity will very likely have unacceptable clinical side effects indicating that physiological NMDA receptor activity is essential for normal neuronal function (Lees KR *et al*. 2000, Kemp JA *et al*., 1999, Sacco RL *et al*, 2000).

The mechanism of NMDA receptor mediated excitotoxicity is primarily via oxidative stress. Briefly, receptor overactivation causes an excessive amount of Ca^{2+} influx into the neurons leading to Ca^{2+} overload of mitochondria, resulting in the generation of ROS and activation of caspases, Ca^{2+}-dependent activation of neuronal NOS, leading to increased NO production which reacts with ROS to form toxic peroxynitrite ($ONOO^{-}$), and stimulation of MAPK p38 signaling pathway (Okamoto SI *et al*., 2005), thus activating transcription factors (c-Jun, c-fos) that trigger apoptosis (Dawson VL *et al*. 1991, Dawson VL *et al*. 1993, Budd SL *et al*., 2000, Yun HY *et al*., 1998, Tenneti L *et al*., 1998, Lipton SA *et al*., 1993). Recent studies

have shown that activation of specifically NMDA receptors but not AMPA receptors causes activation of sGC and formation of cGMP to produce physiological responses in the NTS (Chianca *et al*., 2004). Thus, NMDA receptor leads to formation of NO or NO moieties that activate sGC. Although the mechanism of neuronal injury in the brain is primarily due to receptor overstimulation, the NMDA receptors in the heart follow a different mechanism under sympathetic overactivation. Studies (Lu J *et al*., 2012) have shown that sympathetic hyperinnervation due to cardiac nerve sprouting with or without Myocardial Necrotic Injury upregulated the myocardial expression of NMDA receptor, thus remodeling the myocardial glutamate signaling and induced cardiomyocyte apoptosis. Intravenous infusion with NMDA (12 mg/kg) triggered ventricular tachycardia and ventricular fibrillation in rats with healed MNI plus sympathetic hyperinnervation; prevented by NMDAR antagonist. Hence the primary mechanism of NMDA receptor excitotoxicity is via enhanced NO production by nNOS.

NO and nNOS play an important role in the regulation of cardiac physiology which includes calcium turnover and adrenergic response (Barouch LA *et al*., 2002; Burkard N *et al*., 2007). NO produced by nNOS is important for NMDA receptor-dependent neurotransmitter release, neurotoxicity, and cyclic GMP elevations (Jaffrey SR *et al*., 1998). The coupling of NMDA receptor-mediated calcium influx and nNOS activation is suggested to be due to a physical coupling of the receptor and the enzyme by an intermediary adaptor protein, PSD95, through a PDZ-PDZ domain interaction between PSD95 and nNOS. NOS1AP competes with PSD95 for interaction with nNOS, and overexpression of NOS1AP results in a loss of PSD95/nNOS complexes in transfected cells (Chang KC *et al*., 2008; Jaffrey SR *et al*., 2002;

Jaffrey SR *et al*., 1998). These and other evidences indicate that NOS1AP might be a negative regulator of NO production.

Previous studies with NOS1AP have shown that NOS1AP could be responsible for directing the specificity of NO signal by regulating the nNOS activity at postsynaptic sites of neuron. NOS1AP also regulate nNOS stability, localization and possibly expression during synapse formation. These functions of the NOS1AP lead us to speculate the role of NOS1AP in NMDAR mediated excitotoxicity. Thus, the objective of the present study was to determine whether NOS1AP knockdown lead to excessive NO production and consequently neurotoxicity causing neuronal cell death. To test this hypothesis, we used the lentiviral system containing shRNA against NOS1AP for NOS1AP knockdown in primary neuronal cells cultured from the brainstem of SD rat pups. Firstly, we determined the sensitivity of NOS1AP knockdown neurons at different concentrations of NMDA. Next, we measured the relative NO/cGMP levels in control and NOS1AP knockdown cells and determined the relative level of nNOS bound to NMDAR under those conditions.

4.2. Materials and methods

4.2.1. Animals

Twelve-week-old Sprague-Dawley (SD) rats were obtained from Charles River Farms (Wilmington, MA). Rats were housed at 25 ± 2°C on a 12:12-h light-dark cycle and provided with food and water ad libitum. All animal protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee.

Preparation of primary neuronal cultures: Neuronal co-cultures were prepared from the brain stem of newborn SD rats exactly by following the established protocol of our

laboratory. Briefly, Trypsin (375 U/ml) and DNase I (496 U/ml) dissociated brain cells were suspended in DMEM containing 10% PDHS and were plated in poly-L-lysine-precoated 35-mm-diam tissue culture dishes at 3.0 x 10^6 cells/dish. Cultures were grown in a humidified incubator at 37°C for 7-12 days prior to use.

4.2.2. Excitotoxicity

Rapidly triggered excitotoxicity was induced by 30 min exposure to high concentrations of NMDA, carried out at room temperature in a HEPES buffer solution (HBSS) containing (in mM): NaCI, 120; KCI, 5.4; $MgCI_2$, 0.8; $CaCI_2$, 1.8; HEPES, 20; glucose,15; glycine, 0.01 (pH 7.4). The exposure solution was then washed away and replaced by conditioned media before returning cultures to the incubator for 24-48hrs.

4.2.3. WST-1 cell proliferation assay

WST-1 reagent (Roche, Indianapolis, IN) is a simple, colorimetric assay designed to measure the relative proliferation rates of cells in culture medium. The principle of WST-1 assay is based on the conversion of the tetrazolium salt, WST-1 (slightly red) into water soluble formazan dye (dark red) by mitochondrial dehydrogenase present only in viable cells. The activity of mitochondrial dehydrogenase is increases proportionally to the number of viable cells, leading to an increase in the conversion of WST-1 to formazan dye. The resulting increased color intensity is in turn quantified by measuring the absorbance at 440 nm wavelength. Procedure in brief, after treatment with different concentrations of NMDA, primary neuronal culture cells were incubated with WST-1 for 4 hours at 37°C in a humidified atmosphere maintained at 5 % CO2 as recommended by manufacturer. The absorbance of the formazan dye was measured colorimetrically.

4.2.4. Co-immunoprecipitation assay

The interaction of NMDAR with Nnos was assessed by co-immunoprecipitation studies in neuronal cultured cells. Cultured cells were washed with ice cold PBS and lysed in 500 µl of IP Lysis buffer (20 mM Tris, 150 mM NaCl, 0.4% CHAPS, 2 mM dithiothreitol, pH 7.4) by incubation for 1 h at 4 °C. A specific antibody for NMDA receptor was added at the appropriate dilution, and the sample was incubated for 2 h at 4 °C with continuous mixing, followed by addition of 50 µl of protein G Agarose beads (Santacruz Biotechnology, CA) and incubation for a further 2 h. Protein immunocomplexes were isolated with the beads after washing three times with IP buffer and eluting the protein immunocomplexes in SDS PAGE loading buffer by heating at 85°C for 5 mins. Immunoprecipitated proteins were resuspended in SDS-PAGE loading buffer, and analyzed by SDS-PAGE and autoradiography. The membrane containing the protein complexes was probed with an antibody against nNOS. This would give the relative amount of nNOS bound to the NMDA receptor on the membrane. Lysates collected at the first step were probed for the presence of NOS1AP to verify that the NOS1AP knockdown neuronal cells have reduced NOS1AP protein levels in the lysates.

4.2.5. Measurement of NO production

For fluorometric NO determination, the cell-permeable fluorophore, diaminorhodamine-4M (DAR-4M) was used (Alexis Biochemicals, Gruenberg, Germany). Washed cultured primary rat brain neuronal cultured cells were pre-incubated with 10 µM DAR-4M for 20 min at 37°C in darkness on a rotary shaker and then rinsed with fresh suspension buffer to remove excess fluorophore. DAR-4M fluorescence was measured using a spectrofluorometer (SPF-500™ Ratio, American Instrument Company, MD, USA) with 495 nm excitation and 515 nm emission

wavelength (2 nm band width). After the images were captured, they were then quantified for their respective fluorescence intensity using ImageJ software (NIH). Results were presented as Mean fluorescence intensity per cell.

4.2.6. Measurement of cGMP production

A sensitive and well characterized method of measuring NO is to use the guanylate-cyclase-coupled NO receptor as a biosensor and to follow the production of cGMP (57, 58). We used this method to assay the activity of nNOS, but note that it is an indirect measure of NO production. cGMP levels was assayed in the primary rat brain culture cell lysates using a cGMP Direct Biotrak Enzyme Immunoassay kit (GE Healthcare, Pittsburgh, PA) Measurements of Total Cellular cGMP from Cells in Culture. Cells were subjected to lysis following the instruction of BIOTRAK Kit. The amount of cGMP was calculated according to the standard curve that was generated from parallel reactions within the same experiment.

4.2.7. Detection of apoptosis by Flow Cytometry

Primary neuronal cells treated with Lv-SCR (control) and LV-NOS1AP-shRNA were harvested after desired treatment and washed in cold phosphate-buffered saline (PBS). Cell density was measured using hemocytometer and cells were resuspended in annexin-binding buffer to $1x10^6$ cells/mL. 5 µL Annexin V conjugate (Alexa Fluor 647 purchased from Invitrogen) was added to 100 µL of cell suspension and cells were incubated at room temperature (RT) for 15 minutes. After the incubation period, 400 µL of annexin-binding buffer was added to the cell suspension, gently mixed and cells were analyzed immediately by an Accuri C6 Flow Cytometer.

4.2.8. Data analysis

Results are expressed as means ± SE. Statistical significance was evaluated with the use of a one-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at $P < 0.05$.

4.3. Results and data analysis

4.3.1. Effect of NOS1AP knockdown on NMDA mediated excitotoxicity

To assess the sensitivity of NOS1AP knockdown neurons with respect to NMDAR mediated neurotoxicity, we performed a cell viability assay under different concentrations of NMDA. 7-10 day old primary neuronal cultures were transduced with Lv-SCR (control) and Lv-NOS1AP shRNA. The viral particles have been shown to transduce cells at a very high level. To determine the effects of NMDA on primary neuronal culture cells, we incubated cells with various concentrations of NMDA for 30 minutes of excitotoxic insult. The cells where then washed with PBS and returned to conditioned media for 24-48hrs. At the end of the incubation WST-1 reagent (Roche, Indianapolis, IN, USA) was added and placed in the incubator for 30 min. Finally absorbance was measured at 440 nm wavelength and the percentage growth inhibition was reported in Figure 18, by considering control (untreated primary neuronal culture cells) absorbance as 100 percent. It was observed from the results (Figure 18), that with increasing concentration of NMDA, the percentage cell growth is significantly decreases both in control and NOS1AP knockdown primary neuronal cells. This decrease in neuronal viability is significantly lower in NOS1AP knockdown neuronal cells than the decrease in control untreated cells. Thus the results indicate that NOS1AP dysfunction in the neurons renders them more sensitive towards NMDA induced excitotoxic cell death.

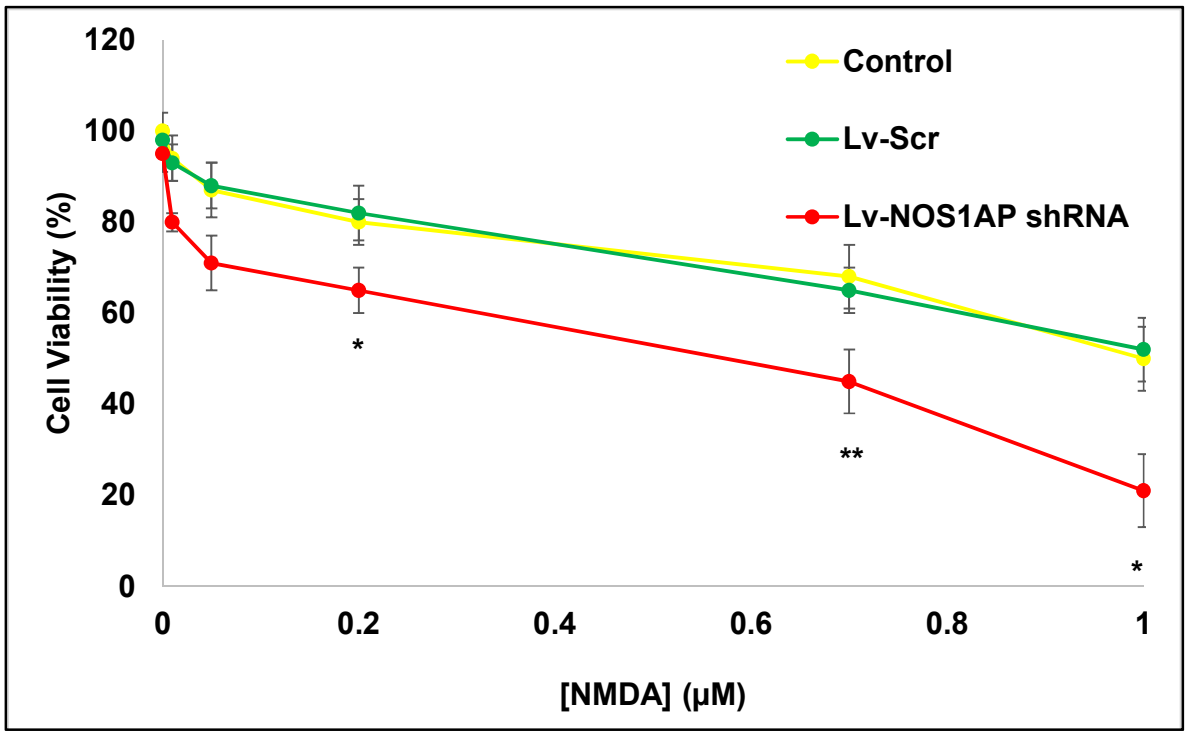

Figure 18: Effect of NOS1AP knockdown on NMDA induced neurotoxicity in primary cultured neurons. Quantitative assessment of NMDA induced neurotoxicity in primary cultured neurons by WST-1 Assay. Cell viability was markedly decreased by a 10-minute application of NMDA followed by an overnight incubation in glutamate-free medium. The NOS1AP knockdown cells showed increased sensitivity towards NMDA in a concentration-dependent manner from 10nM to 1μM. *p<0.05, n=6, Data are presented as mean±SEM.

4.3.2. Effect of NOS1AP knockdown on the level of Nitric Oxide in primary rat brain neuronal culture cells

Studies have suggested that NOS1AP is a negative regulator of nNOS. NMDAR /PSD95 /nNOS have been implicated in a variety of neurological disorders. In order to assess the direct effect of NOS1AP knockdown on NO production, we used DAR-4M that is a dye that emits fluorescence when bound to NO. This dye has a sensitivity in the nanomolar range for NO detection whereas other conventional NO detectors such as griess reagent have sensitivity in micromolar range. For detecting relative NO levels, we loaded the cells with the DAR-4M dye

and then incubated the cells for 30 minutes at 37°C. After that the cells were washed and were imaged under the microscope with laser of 495 nm excitation and 515 nm emission wavelength. After imaging the cells at basal conditions, the cells were then treated with 10µM NMDA and were imaged immediately upon drug addition. The results are shown in Figure 19.

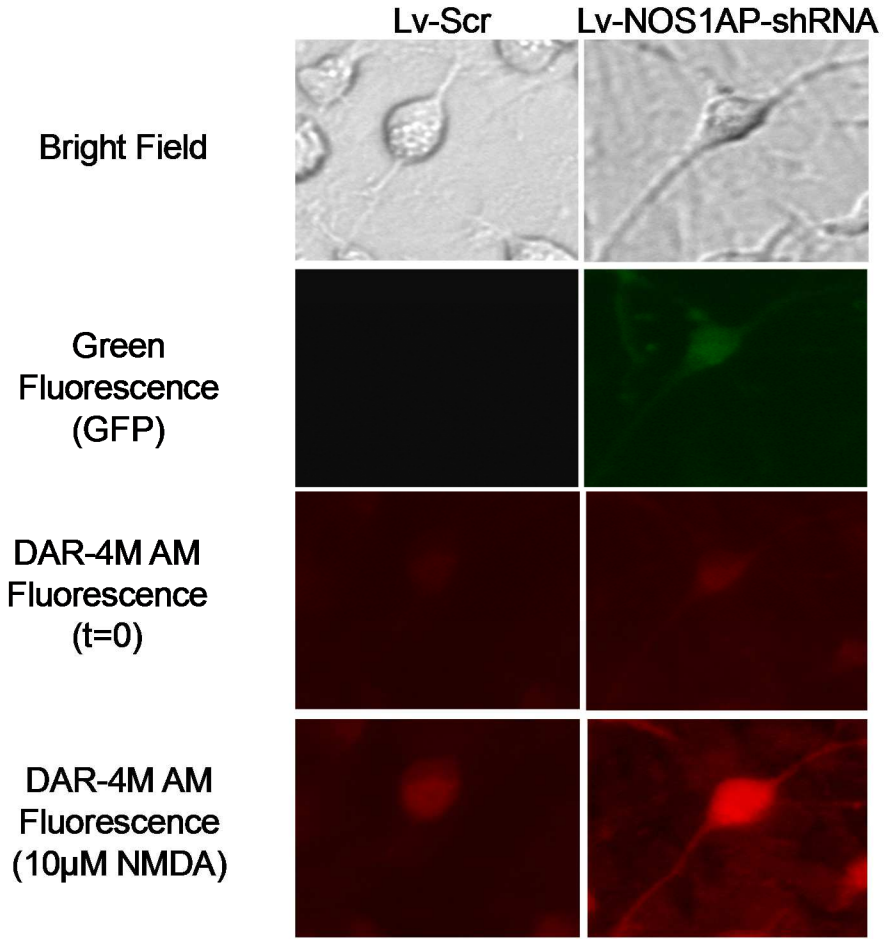

Figure 19: Effect of NOS1AP knockdown on Nitric Oxide levels in primary cultured neurons. DAR-4M AM Fluorescence images before and after 10uM NMDA addition. Primary neuronal culture cells were treated with 2uM DAR-4M AM for 20 mins and time lapse fluorescence imaging was performed before and after NMDA addition. A significant increase in NO induced fluorescence was seen after NMDA treatment in NOS1AP knockdown cells vs. control cells.

The images were also quantified for their mean fluorescence intensity per cell in response to NMDA treatment (Figure 20). The fluorescence intensity results corresponds to relative NO levels. The fluorescence intensity in the basal conditions remains the same in both control as well as NOS1AP knockdown neurons. However, as seen from the images as well as after quantification of fluorescence intensity, the cells with NOS1AP knockdown showed enhanced fluorescence as compared to control neurons upon NMDA addition. This indicated the enhanced NO production in NOS1AP knockdown neurons in response to NMDA.

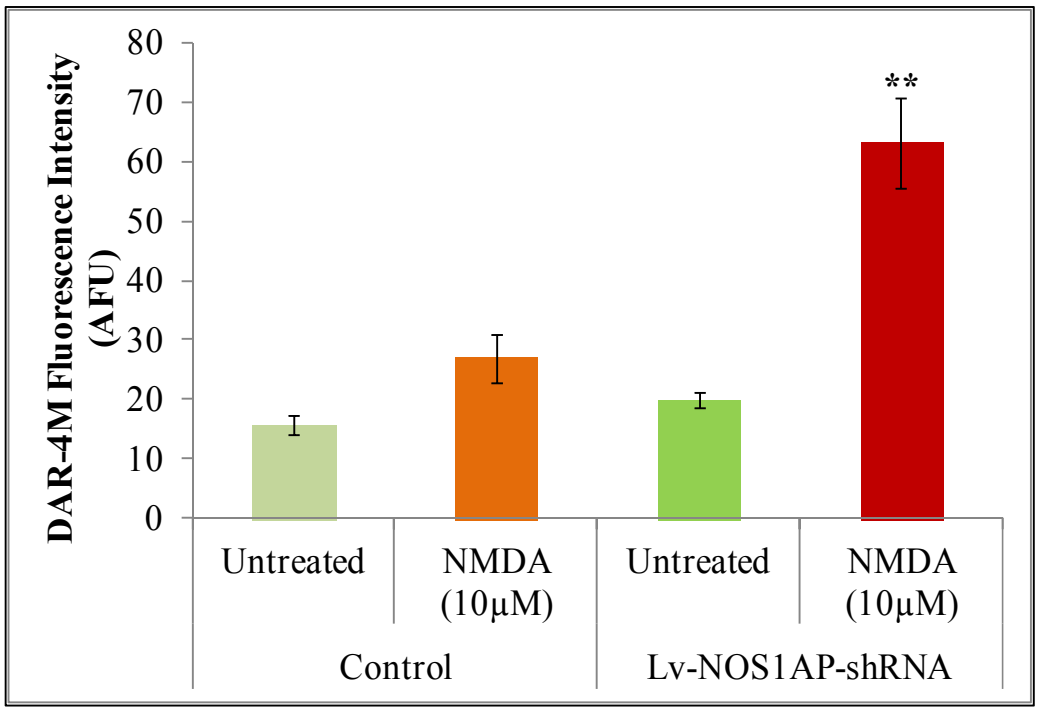

Figure 20: Quantification of Nitric Oxide levels in NOS1AP knockdown cells vs. control cells. Bar graph depiction of the relative fluorescence intensity of DAR-4M before and after 10uM NMDA addition. Cells were treated with 2uM DAR-4M AM for 20 mins and time lapse fluorescence imaging was performed before and after NMDA addition. The fluorescence images were quantified for their intensity using ImageJ software. A significant increase in NO level in NOS1AP knockdown cells vs. control cells is observed after NMDA addition. **$p<0.01$, $n=5$, Data presented as mean±SEM

4.3.3. Effect of NOS1AP knockdown on cGMP level in primary rat brain neuronal culture cells

An indirect measure of NO levels is the estimation of cGMP levels in the cells. cGMP levels were measured in control and NOS1AP downregulated primary rat brain neuronal culture cells in response to different concentrations of NMDA. Briefly, 48 hrs after the respective lentiviral vector transduction (Control= Lv-SCR and Experimental= Lv-NOS1AP-shRNA), the cells were given NMDA treatment as mentioned in the Material and method section. After NMDA treatment and overnight incubation, the cells were lysed for cGMP determination by the cGMP Elisa kit according to manufacturer protocol. Results are shown in Figure 21.

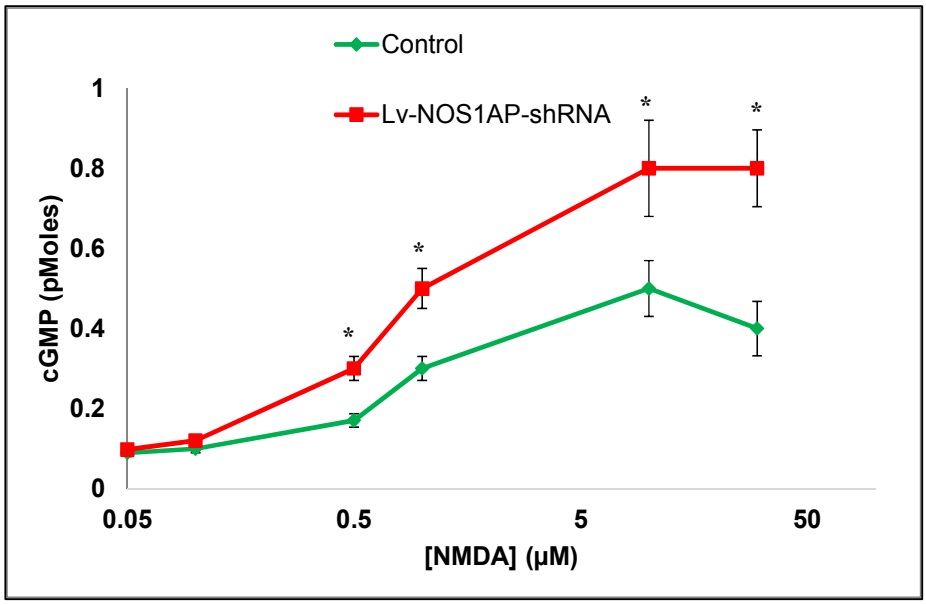

Figure 21: Effect of NOS1AP knockdown on cGMP levels. Primary neuronal culture cells treated with Lv-NOS1AP-shRNA show enhanced cGMP production with increasing concentrations of NMDA. This enhancement in cGMP production was significantly higher in NOS1AP knockdown cells than control cells. *$p<0.05$, n=4, Data presented as mean±SEM.

The results (Figure 21) indicate that NOS1AP downregulated neurons treated with higher conc. of NMDA caused an enhancement in the levels of cGMP as compared to control neurons. Also, at the highest NMDA concentration treatment the cGMP levels slightly decrease presumably due to excitotoxic neuronal death.

4.3.4. Effect of NOS1AP knockdown on the level of NMDAR/nNOS coupling in the primary rat brain neuronal culture cells

Previous studies have shown that NOS1AP sequesters nNOS by disrupting its ternary complex of NMDAR-PSD95-nNOS which is functionally active in the production of NO. We observed the impact of NOS1AP knockdown on the biochemistry of this ternary complex in primary neuronal cells to assess whether there is any change in the amount of the functional ternary complex after NOS1AP knockdown. This would help us to reason the overproduction of NO in the NOS1AP knockdown neuronal culture cells.

For this we performed Co-Immunoprecipitation studies where we collected the protein with the NMDAR antibody and detect any interacting nNOS protein (with their antibodies) which might have co-immunoprecipitated. Therefore, we treated the primary neuronal culture cells (DIV 12) with Lv-NOS1AP-shRNA (48 hrs.) and untreated cells were used as control. NMDAR were purified by overnight incubation with their respective antibodies. Cell lysates and purified proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. The densitometric analysis of the co-immunoprecipitation studies was performed by calculating the intensity ratio of NMDAR bound nNOS to the total nNOS present in the lysate fraction.

The results of the co-immunoprecipitation and consequent densitometric analysis (Figure 22) showed that for the same amount of protein pulled by NMDAR, there is significantly more nNOS bound to the protein complex isolated from NOS1AP downregulated cells. This indicated that NOS1AP downregulation led to release of nNOS from sequestration by NOS1AP and this nNOS then binds with NMDAR via PSD-95 to form functional NMDAR-PSD95/nNOS complex.

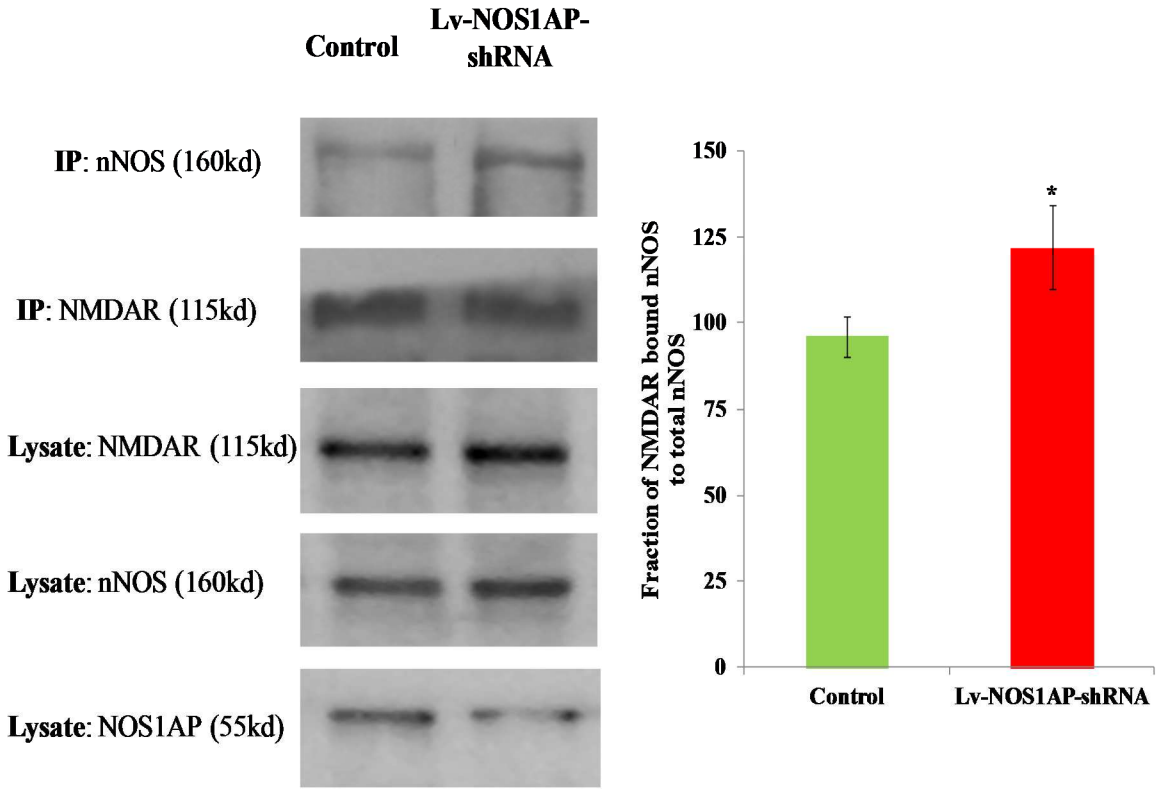

Figure 22: Effect of NOS1AP knockdown on NMDAR/nNOS coupling. Immunoblots depicting the presence of different proteins in lysate and IP fractions pulled by anti-NMDAR antibody. Bar graph indicates the quantification of NMDAR coupled nNOS with respect to total nNOS performed by protein densitometric analysis using imageJ. Results indicate significantly higher association of nNOS and NMDAR in NOS1AP knockdown neurons. Data are mean$\pm$SEM, n=3,*P<0.05, statistically significant from control.

4.3.5. Effect of NOS1AP knockdown on the type of cell death in the primary rat brain neuronal culture cells undergoing NMDA neurotoxicity: Apoptosis versus necrosis

In order to detect the type of cell death undergone by the neurons after NMDA excitotoxicity with downregulated NOS1AP, we co-stained the control, Lv-SCR treated and neurons with NOS1AP shRNA with Annexin V and Propidium iodide (PI). Annexin V antibody binds to the Annexin V receptors that are commonly expressed by the cells during apoptosis and later stages of cell death. Propidium iodide binds to the cells undergoing necrosis and not apoptosis. The co-stained cells were then analyzed under flow cytometry to determine the percentage of cells that are apoptotic verses cells that undergo necrosis.

The results (Figure 23) indicate that the control cells as well as the Lv-SCR transduced cells showed characteristics of apoptosis or necrosis but the NOS1AP knockdown neurons revealed a significant amount of cells that are apoptotic indicated by higher surface expression of Annexin V, detected by flow cytometry. Also, no significant necrotic cells were observed based on the PI staining of cells.

4.4. Data summary and conclusion

The purpose of this part of the study was to evaluate the role of NOS1AP in the NMDA mediated excitotoxicity in primary rat brain neuronal culture cells. This was achieved by observing and evaluating the effects of NOS1AP knockdown, mediated by lentivirus expressing NOS1AP shRNA, on neuronal cell viability, NO and consequently cGMP production, the binding of NMDA Receptor to nNOS and the type of cell death (apoptosis vs necrosis) undergone in the NOS1AP knockdown cells.

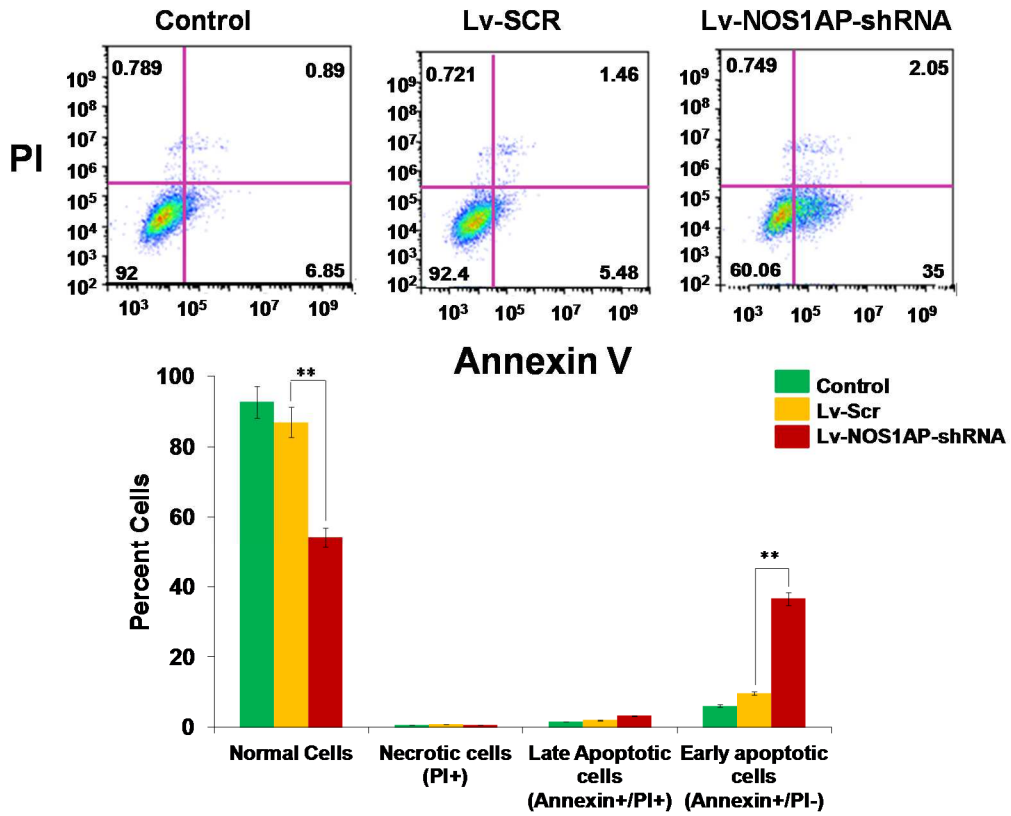

Figure 23: Effect of NOS1AP knockdown on the type of neuronal cell death. Apoptosis of neuronal cells treated with Lv-Scr and Lv-NOS1AP-shRNA was assessed using Annexin V/PI Staining and Flow cytometry after NMDA (100nM) treatment. The percentage of early apoptotic cells are significantly increased after NOS1AP knockdown. Data are mean±SEM, **P<0.01, statistically significant from control in each group, n=3.

The major findings of the present study are listed below:

1. NOS1AP downregulation sensitizes the primary neurons to NMDA mediated excitotoxicty which was NMDA concentration dependent.
2. Enhanced NO/cGMP has been shown be present in NOS1AP downregulated cells treated with NMDA. cGMP production is enhanced in a dose dependent manner with very high NMDA concentration tends to decrease the cGMP levels presumably due to increased cell death.

3. There is an increase in the amount of ternary NMDAR/PSD95/nNOS complex in NOS1AP knockdown cells when compared with untreated control neuronal culture cells. The lysates probed with NOS1AP showed a decreased expression of NOS1AP confirming that NOS1AP downregulation was responsible for the enhanced coupling of nNOS with NMDAR.
4. The NOS1AP downregulated cells showed an enhanced expression of apoptotic proteins such as Annexin V as compared to the control cells when under NMDA induced neurotoxic insult. PI staining of dead/necrotic cells were not significantly observed in any cell type. This indicated that the NOS1AP downregulated cells are under pre-apoptotic condition during which any excitotoxic stress due to NMDA treatment might result in cell death.

Thus, the current study confirms that decreased NOS1AP in the rat brain primary neuronal cells leads to enhanced NO/cGMP production causing neuronal death under excitotoxic condition due to NMDA treatment. This enhancement of NO production might be due to increase in the functional NMDAR/PSD95/nNOS complex after NOS1AP downregulation leading to NMDAR overactivation and enhanced nNOS activity. The NOSIAP downregulation leads to release of more nNOS which was otherwise sequestered with the NOS1AP and was rendered inactive for NO production.

These observations are consistent with previous findings indicating that NOS1AP might be a negative regulator of NO production (Chang KC *et al*., 2008; Jaffrey SR *et al*., 2002; Jaffrey SR *et al*., 1998). NOS1 and NO play an important role in the regulation of cardiac physiology which includes calcium turnover and adrenergic response (Barouch LA *et al*., 2002; Burkard N

et al., 2007). NO produced by nNOS is important for NMDA receptor-dependent neurotransmitter release, neurotoxicity, and cyclic GMP elevations. The coupling of NMDA receptor-mediated calcium influx and nNOS activation is suggested to be due to a physical coupling of the receptor and the enzyme by an intermediary adaptor protein, PSD95, through a PDZ-PDZ domain interaction between PSD95 and nNOS. NOS1AP competes with PSD95 for interaction with nNOS, and overexpression of NOS1AP results in a loss of PSD95/nNOS complexes in transfected cells (Chang KC *et al*., 2008; Jaffrey SR *et al*., 2002; Jaffrey SR *et al*., 1998).

Some of the studies conducted in neuronal cells have shown contradictory results in that NOS1AP downlegulation is protective in NMDA mediated neurotoxicity. This can be explained by the variability of NMDA receptors in neurons cultured from different brain regions. Also, older neurons are more resistant to excitotoxicty than the younger neurons, thus, the level of NOS1AP downregulation might play an important role in deciphering its effects on the NMDA mediated neurotoxicity.

It is important to show the NMDA receptor activity and expression under altered NOS1AP conditions in the neurons. This might further add one more dimension to the regulation of NMDA mediated neurotoxicity by NOS1AP. Previous studies have shown that altered nNOS activity leads to Na^+-channel-mediated cLQTS in HEK293T cells. NOS1AP regulates nNOS subcellular location (Jaffrey SR *et al*., 2002; Jaffrey SR *et al*., 1998). Recent study showed that alteration in the HEK293T cells that increase the nNOS activity and released NO promotes the direct hyper-S-nitrosylation of $Na_v1.5$ and increases I_{NaL}, which is the characteristic biophysical dysfunction for Na^+-channel-mediated cLQTS (Ueda K *et al*., 2008; Tamargo J *et al*., 2010). In

addition to mediating several physiological functions, NO has been implicated in the cytotoxicity observed following excess stimulation of neurons by glutamate. This neurotoxicity can be a mode of neurogenic damage causing impaired transmission from the NTS leading to myocardial damage and cardiac arrhythmias.

CHAPTER V. EFFECT OF NOS1AP KNOCKDOWN IN THE NTS ON CARDIOVASCULAR FUNCTIONS AND ELECTROPHYSIOLOGY IN SD RATS

5.1. Introduction

Myocardial damage due to lesions in the central nervous system have been implicated in various neurological and cardiovascular pathophysiologies. Neural control of the heart is integrated at all levels of the neuraxis (Palma J *et al*., 2014). Briefly, sympathetic activation is triggered by neurons of the rostral ventrolateral medulla (RVLM), which send excitatory glutamatergic neurons to the sympathetic preganglionic neurons located in the intermediolateral nucleus of the spinal cord. The RVLM receives inhibitory GABAergic neurons from the CVLM that receives excitatory neurons from the NTS. PVN that receives afferent neural projections from the NTS, influences the regulation of sympathetic outflow through the interactions of excitatory and inhibitory neurotransmitters (Swanson LW *et al*., 1983). Thus, NTS lies at center of this sympathovagal balance of the autonomic control of the heart. Perturbation of this sympathovagal balance leads to activation of peripheral neurons innervating the myocardium and leads to alterations in neurotransmitter release from these cardiac neurons. 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 (Triposkiadis F *et al*., 2009). Nitric oxide (NO) is one such neuromodulator. Neuronal nitric oxide synthase (nNOS) is localized in both intrinsic cardiac vagal neurons and stellate sympathetic ganglia innervating the sino-atrial node (Herring *et al*. 2002; Paton *et al*. 2002). In cholinergic (vagal) neurons, NO acts to increase acetylcholine release via stimulation of soluble guanylate cyclase. The resultant generation of cGMP leads to inhibition of PDE3 and an increase

in cAMP–PKA-dependent phosphorylation of N-type calcium channels and calcium-induced exocytotic release of acetylcholine (Herring N & Paterson DJ, 2001). NO produced in sympathetic ganglia reduces the release of noradrenaline by a soluble guanylate cyclase–cGMP-dependent pathway that reduces calcium influx (Choate JK *et al*, 1999; Wang L *et al*. 2007), via stimulation of PDE2 and/or protein kinase G. Acetylcholine released from the vagal neurons binds to the Muscarinic receptors located in the Sinoatrial node and causes bradycardia. Conversely, Noradrenalin released from the sympathetic neurons binds to the β-adrenergic receptors located in the Sinoatrial node and causes tachycardia (Reviewed in Herring N *et al*., 2009). Thus, NO is an important modulator of myocardial function by relaying the perturbations in the sympathovagal balance from the central nervous system to the heart. Studies have shown that long term sympathetic overexcitation or increased plasma noradrenalin directly leads to myocardial damage and apoptosis (Singh K *et al*., 2000, Cohn JN *et al*., 1984). Thus, the lesions in the brain leading to sympathetic excitation cause myocardial damage and/or cardiac arrhythmia.

Studies conducted in the previous chapter have shown that NTS in the brain have the most abundant NOS1AP mRNA. These results are consistent with recent evidences that NTS lesions interrupting the baroreflex may induce cardiac arrhythmias and myocardial changes similar to those seen in humans with central lesions (Talman WT *et al*., 1993, Nayate A *et al*., 2008). The altered baroreflex transmission was associated with sudden death in approximately one third of the experimental animals (Nayate A *et al*., 2008). Previous studies have shown that NOS1AP is a negative regulator of NO, in that it sequesters nNOS preventing its binding to the scaffolding protein PSD-95 and hence altering the NO production (Jafferey SR *et al*., 1998).

Based on our findings and previous studies on NOS1AP, we hypothesized that NOS1AP knockdown in the NTS leads to more functional NMDA-PSD95-nNOS interaction causing overproduction of NO which causes neurotoxicity. This neurotoxicity of neurons in the NTS might lead to myocardial damage and/or cardiac arrhythmia manifested by QT interval prolongation. To test the hypothesis we knocked down the NOS1AP expression in the neurons in the NTS by using a lentiviral vector (Lv-NOS1P-shRNA) carrying shRNA against NOS1AP gene for stable and long term expression. This lentiviral vector had been successfully tested for its efficacy in neuronal cultures in the previous chapters.

The objective of this chapter was to assess the biological function of NOS1AP by genetic knockdown in the NTS. More specifically, we monitored changes in different cardiovascular variables (blood pressure, heart rate), and ECG parameters (RR interval, QT interval) and Heart Rate Variability. Also, we examined the myocardial damage in the NTS-NOS1AP knockdown animals and compared them with control rats.

5.2. Materials and methods

5.2.1. Animals

Twelve-week-old Sprague-Dawley (SD) rats were obtained from Charles River Farms (Wilmington, MA). Rats were housed at 25 ± 2°C on a 12:12-h light-dark cycle and provided with food and water ad libitum. All surgical procedures were performed under sterile conditions as approved by the North Dakota State University Institutional Animal Care and Use Committee.

5.2.2. Hematoxylin and eosin staining

To examine pathological changes in the heart, we removed the heart after the rat had been perfused and stored the heart in 4% paraformaldehyde for paraffin embedding. Transverse

sections were then cut, and hematoxylin and eosin-stained slides were prepared at 2-mm intervals throughout the entire heart.

5.2.3. Recording of arterial pressure

Chronic BP recording was carried out with a radiotelemetry system. Rats were anesthetized with a mixture of oxygen (1 l/min) and isoflurane (3%; Halocarbon, River Edge, NJ), which was delivered through a nose cone. A telemetry BP probe (model TA11PA-C40, Data Sciences International, St. Paul, MN) was positioned intra-abdominally and secured to the ventral abdominal muscle with the catheter inserted into the lower abdominal aorta. During the surgery, rats received an intraperitoneally delivered warm sterile 0.9% saline (~3% of body weight) to ensure proper fluid balance. The telemetry signals were processed and digitized as radio frequency data, which were recorded and stored in a computer using the Dataquest IV system (Data Sciences International). In the conscious state, mean values of BP and heart rate (HR) were recorded continuously for 1 h every day between 10:00 AM and 11:00 AM. Continuous recordings were started 4 days after probe implantation. The basal BP and HR were recorded for three days prior to NTS microinjection of Lv-NOS1AP-shRNA or Lv-SCR as described above.

5.2.4. Chronic ECG recording

ECG recording was carried out with a radiotelemetry system. Male SD rats will be anesthetized. An ECG transmitter will be implanted into the peritoneal cavity of adult SD rats (Model TA10EA-F20, Data Sciences, Minneapolis, USA), with a position of the electrodes generating a lead II on ECG. After a recovery period from surgery of 1 week, a 24 h ECG was recorded with a sampling rate of 1 kHz. Measurements included heart rate, RR interval, QRS

interval, (rate corrected) QT interval ($QTc = QT_0/ (RR_0/100)^{1/2}$, whereas the end of the T-wave was defined as the point at which the slow component returned to the isoelectric line.

5.2.5. Microinjection experiment

Lv-NOS1AP-shRNA or Lv-SCR was microinjected bilaterally into the NTS, according to procedures described previously (Ito K et al., 2003, Tsukamoto K et al., 1993). In brief, the anesthetized animals were placed in a stereotaxic frame. A multiple-barrel glass injection pipette (tip size 20–40 μm) was positioned in the NTS. The coordinates for the NTS were determined from the Swanson rat atlas (Swanson LW., 2004), which were 0.5 mm rostral to the caudal tip of the area postrema, 0.5 mm lateral to the midline, and 0.5 mm below the dorsal surface of the brain stem. Proper placement was confirmed by checking for an l-glutamate-induced (200 pmol, in 50 nl) depressor response. This would induce a characteristically abrupt decrease in BP (ΔBP > 35 mmHg) and HR (ΔHR > 50 beats/min), if the needle tip was located precisely in the NTS. After a responsive site was identified by l-glutamate, the probe remained in this site throughout the remainder of the experiment. Lv-NOS1AP-shRNA (1×10^9 genome copy in 50 nL) will be microinjected bilaterally into the NTS over a 25-minute period with a microinjection device. Throughout the experiment, rat body temperature was maintained in the range of 36.5–37.5°C with a heating pad (Gaymar Industries, Orchard Park, NY).

After the protocol, the injection position was also confirmed by microinjection of methylene blue dye (50 nl). To avoid the spread into non targeted area of the brain other than NTS, the volume of each injection was not more than 100nl and was microinjected bilaterally into the NTS over a period of 25 minutes.

5.2.6. Heart Rate Variability analysis

HRV analysis is the ability to assess overall cardiac health and the state of the autonomic nervous system (ANS) responsible for regulating cardiac activity (Acharya RU *et al*., 2006; European Heart Journal,1996; Fuller BF., 1992; Bernsten GG *et al*., 1997; Hatch JP *et al*., 1992). Power spectral density analysis of the ECG, recorded during a previous experiment in Lv-NOS1AP-shRNA microinjected rats and control rats was performed. For time domain analysis, the parameters calculated were: mean normal-to-normal (NN) intervals in milliseconds, standard deviation of all NN intervals (SDNN, in ms) and square root of the mean square of successive differences between adjacent NN intervals (RMSSD, in ms). For Frequency domain analysis the parameters calculated were: high frequency (HF 1.5–5 Hz), low frequency (LF 0.15–1.5 Hz) and very low frequency (VLF 0–0.15 Hz) bands as defined for rats and the power (ms2) in these bands were calculated. LF and HF were expressed in normalized units (nu), which represents the relative value of each power component in proportion to the total power minus the VLF component, and the LF/HF ratio will be determined.

5.2.7. Data analysis

Results are expressed as means ± SE. Statistical significance was evaluated with the use of a one-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at $P < 0.05$.

5.3. Results and data analysis

5.3.1. Effect of NOS1AP knockdown on the Mean arterial pressure and Heart rate

Mean arterial pressure (MAP) and heart rate (HR) were recorded using telemetry in 6 rats per group treated with control Lv-SCR (NTS microinjection) and Lv-NOS1AP-shRNA (NTS

microinjection 50nL, $3X10^8$ TU/ml). The recordings were performed for 15 days post microinjection (Figure 24 for protocol).

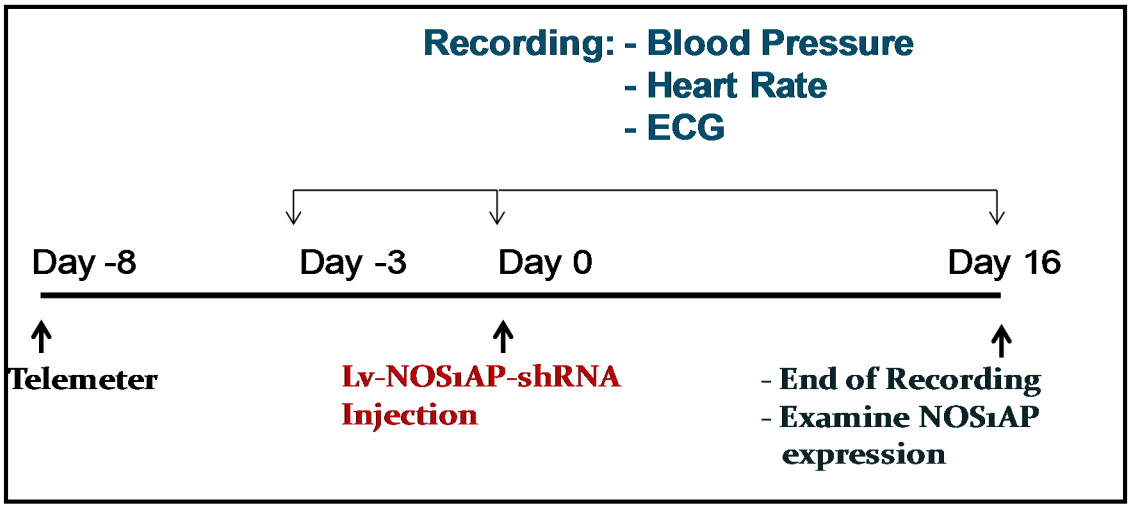

Figure 24: Schematic design of the protocol for lentiviral microinjection and recording of cardiovascular variables and ECG.

The results (Figure 25) demonstrated that no significant difference was observed in the HR and BP between Lv-NOS1AP-shRNA injected and control rats. The results indicated that NOS1AP dysfunction in the NTS might not have a significant cardiovascular effect on the control of blood pressure or heart rate.

5.3.2. Electrocardiographic changes due to NOS11AP knockdown

Previous studies have shown that variants of NOS1AP are linked to prolonged QT interval (Arking DE *et al*., 2006, Aarnoudse AJ *et al*., 2007, Eijgelsheim M *et al*., 2008, Lehtinen AB *et al*., 2008, Post W *et al*., 2007, Raitakari OT *et al*., 2008, Tobin MD*et al*., 2008, Circulation 2009) and QT interval is a risk factor for SCD (Newton CC *et al*., 2005, Hong Y *et al*., 2001, Straus SM *et al*., 2006). Therefore in order to provide a link between NOS1AP and SCD, it is important to study the effect of NOS1AP dysfunction in the NTS on the QT interval

that is a measure of ventricular repolarization. For this we followed the protocol shown in Fig. 24 and recorded the chronic ECG in the Lv-NOS1AP-shRNA microinjected and Lv-SCR microinjected SD rats in the NTS as described in methods section. Measurements included heart rate, RR interval, QRS interval, (heart rate corrected) QT interval ($QTc = QT_0/ (RR_0/100)^{1/2}$, whereas the end of the T-wave will be defined as the point at which the slow component returned to the isoelectric line.

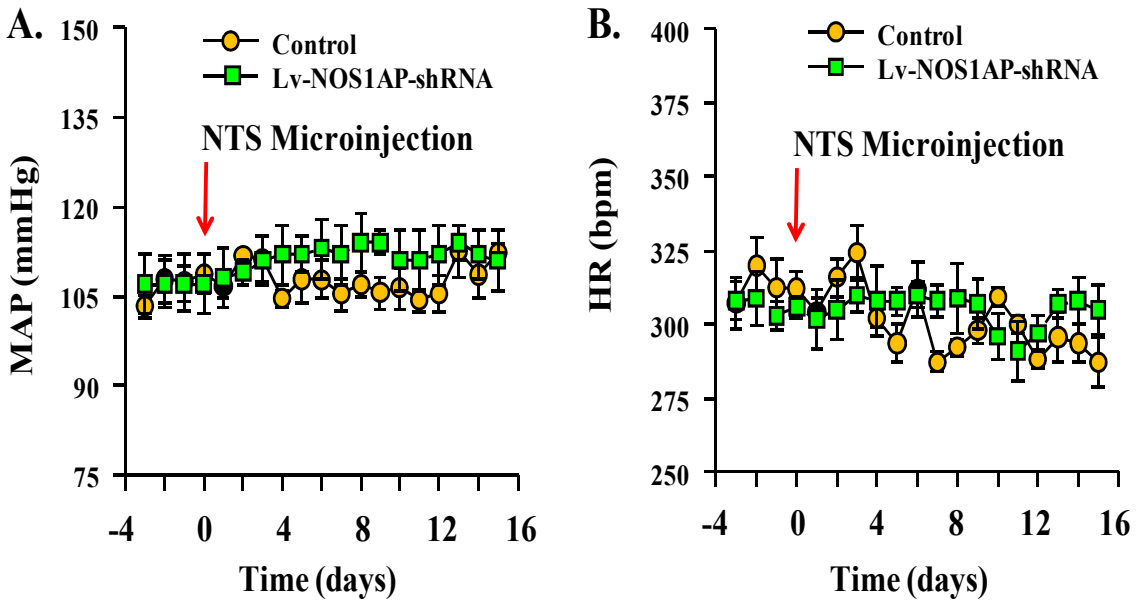

Figure 25: Effect of NOS1AP knockdown in the NTS on Mean arterial pressure and Heart rate. MAP and HR were recorded using radiotelemetry in rats treated with control (Lv-SCR microinjection) and Lv-NOS1AP-shRNA (NTS microinjection 50nL, $3X10^8$ TU/ml). No significant difference was observed in the HR and BP between Lv-NOS1AP-shRNA injected and control rats. Data are presented as mean±SD from all six rats in each group.

Results of the ECG analysis (Figure 26) showed that although the R-R interval was not significantly altered in the NOS1AP knockdown rats as compared to the control rats (193.9±10.3 ms vs 184.2±3.8 ms, n=6, P> 0.05), there was a significant prolongation of QT interval observed

after NOS1AP knockdown in the NTS as compared to control rats (72.4±4.3 ms vs 102.2±12.8 ms, n=6, P< 0.01). The results suggest that NOS1AP dysfunction in the NTS is responsible for prolongation of QT interval.

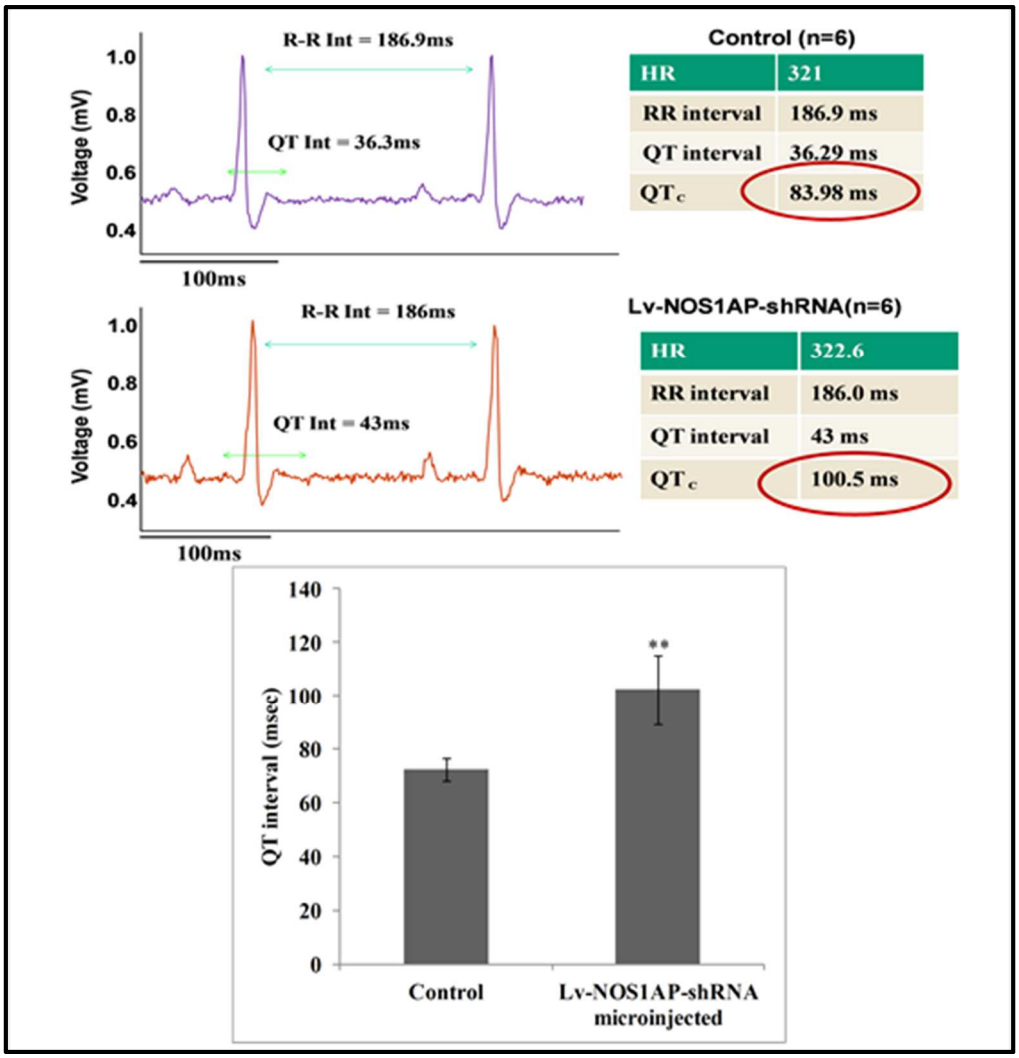

Figure 26: Effect of NOS1AP knockdown in the NTS on QT interval. ECG was recorded in rats treated with control (Lv-SCR microinjection) and Lv-NOS1AP-shRNA (NTS microinjection 50nL, $3X10^8$ TU/ml). Upper panel illustrates a sample recording of ECG and analysis of QT interval. The heart rate corrected QT (QTc) interval was markedly increased (72.4±4 vs 105.0±11 ms, n=6, P< .01) in rats with NTS microinjection of Lv-NOS1AP-shRNA. Data are presented as mean±SD from all six rats in each group.

5.3.3. Effect of NOS1AP knockdown on the Heart Rate Variability: Time and Frequency domain analysis

In order to provide a mechanistic link between SCD and NOS1AP and investigating the effect of NOS1AP downregulation in the NTS on the sympathovagal balance of the heart, it is important to assess the effect of this knockdown on the Heart rate variability (HRV) in rats. HRV is a reliable reflection of the many physiological factors modulating the normal rhythm of the heart. They provide a powerful means of observing the interplay between the sympathetic and parasympathetic nervous systems. HRV analysis is the ability to assess overall cardiac health and the state of the autonomic nervous system (ANS) responsible for regulating cardiac activity (Acharya RU *et al*., 2006; European Heart Journal,1996; Fuller BF, 1992; Bernsten GG *et al*., 1997; Hatch JP *et al*., 1992).

Therefore, to assess whether NOS1AP dysfunction in the NTS causes any modulations in the HRV, we studied the HRV for time and frequency analysis of the previously recorded ECG measurements from the SD rats, with control Lv-SCR microinjection and the Lv-NOS1AP-shRNA microinjection in the NTS. Results (Figure 27) from the time domain analysis reveal that HRV was significantly reduced (SDNN, 51.2±5.6 vs 5.0±1.3 ms, n=6, P< .001 and RMMSD,) in Lv-NOS1AP-shRNA injected rats vs control rats. SDNN is the Standard deviation of the mean NN interval derived from the ECG, This variable is directly related to HRV. Thus reduced SDNN implies a reduction in HRV.

Results from the power spectral density analysis are discussed as follows. There were two major spectral components in the power spectrum at low frequency (LF) (0.6 Hz) and high frequency (HF) (approximately 1.4 Hz). Results from the power spectral density analysis

revealed that the power of LF was higher in the NOS1AP knockdown rats (75.1±5 nu vs 54.7±3.5 nu, n=6, p<0.005) as compared to the control rats. Also, the power of HF was lower in the NOS1AP knockdown rats (24.9±5 nu vs 45.2±3.5 nu, n=6, p<0.005) as compared to the control rats. Next we analyzed the LF/HF ratio which is an index of parasympathetic and sympathetic interactions in the rat. Results indicated a decrease in the parasympathetic mechanism that was reflected by a slight increase in the LF/HF ratio in NOS1AP knockdown rats (2.9±0.9 vs 1.3±0.2, n=6, p<0.01) as compared to control rats.

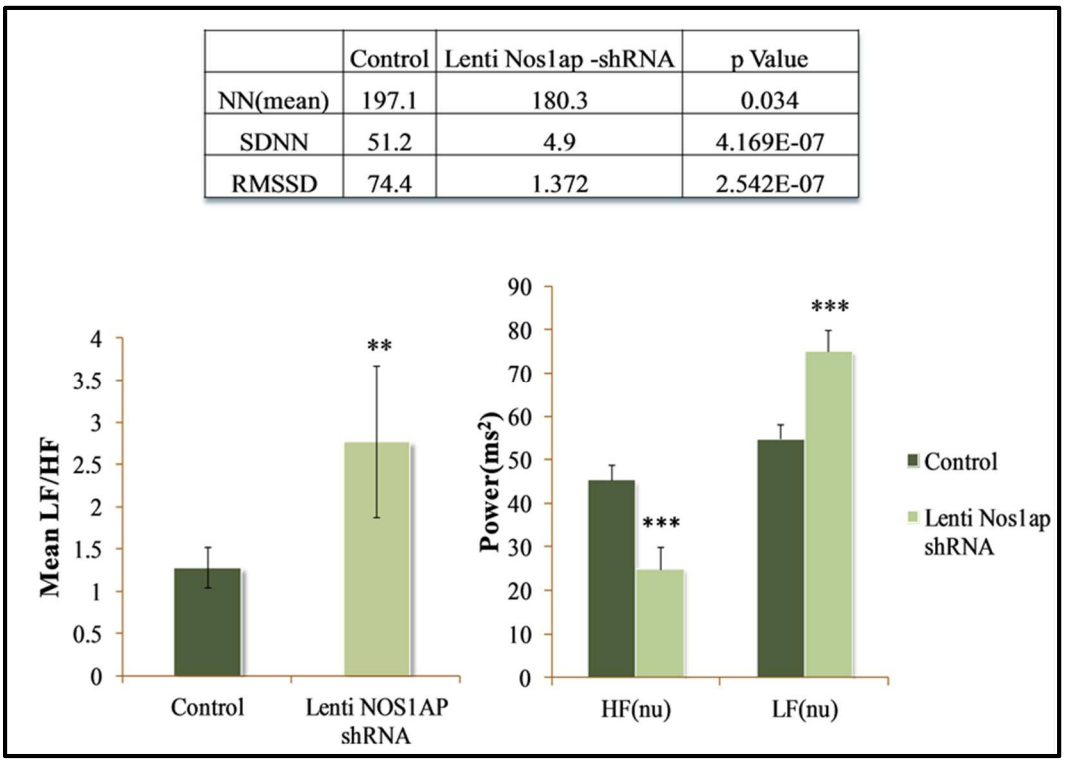

Figure 27: Effect of NOS1AP knockdown in the NTS on Heart Rate Variability (HRV). HRV in the form of Time (upper panel) and Frequency domain was analyzed using the ECG signals derived from radiotelemetric probes in rats treated with control (Lv-SCR microinjection) and, Lv-NOS1AP-shRNA (NTS microinjection 50nL, 3 X 10^8 TU/ml). HRV was significantly reduced (SDNN, 51.2±5.6 vs 5.0±1.3 ms, n=6, P< .001) in Lv-NOS1AP-shRNA injected rats vs control rats. Data are presented as means ± SD from all six rats in each group. **p <0.01, ***p <0.005, statistically significantly different from control group.

5.3.4. Pathological changes in the heart due to reduced NOS1AP expression in the NTS

Examination of hematoxylin and eosin-stained sections of hearts from the 6 rats that had received bilateral Lv-NOS1AP-shRNA in the NTS revealed that some had a presence of contraction band necrosis (Figure 28), in which the cell dies in a hypercontracted state with early myofibrillar damage and anomalous irregular cross-band formations. The contraction band necrosis was their only pathological change. None of the rats from control group displayed any pathologic abnormalities in the heart. The sections of the heart were also analyzed for cardiomyopathy as demonstrated by Sirius red staining (data not shown). None of the rats in either control or NOS1AP knockdown rats demonstrated cardiomyopathy.

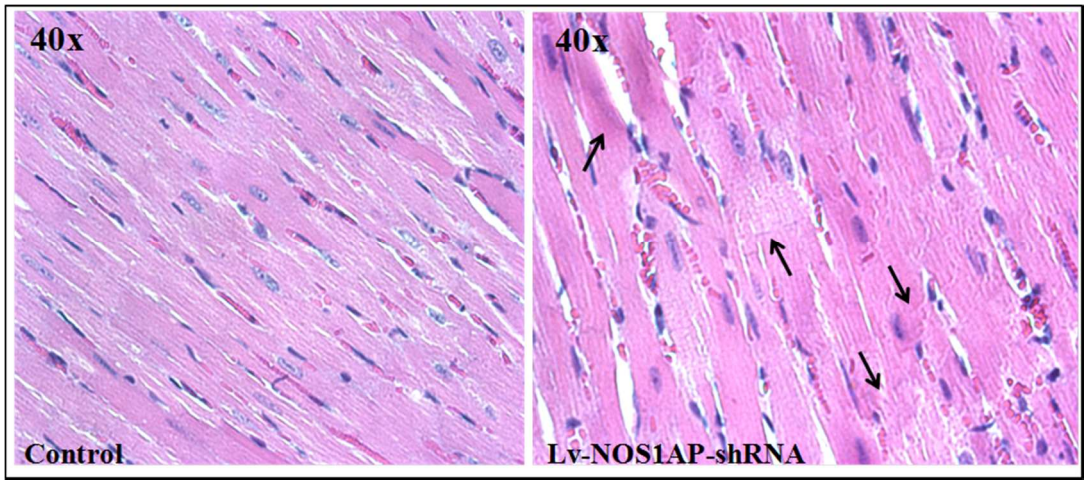

Figure 28: Effect of NOS1AP knockdown in the NTS on cardiac damage. Examination of hematoxylin and eosin-stained sections of hearts from the rats (n=6) that had received bilateral Lv-NOS1AP-shRNA in the NTS revealed a presence of contraction band necrosis (depicted by arrowheads), indicating cardiac damage in some cases.

5.4. Data summary and conclusion

The present study was aimed to understand the biological function of NOS1AP in cardiovascular and electrophysiological regulation. This was achieved by knocking down

NOS1AP expression in the NTS. The choice of NTS was made based on our previous studies that showed enhanced NOS1AP expression in the normal as well as heart failure conditions. This was consistent with other reports indicating that lesions in the NTS lead to myocardial damage, asystole and death in one third animals (Nayate A *et al*., 2008). Thus, we hypothesized that NOS1AP knockdown in the NTS causes neurotoxicity via NMDA-PSD95-nNOS interaction leading to overproduction of NO. This neurotoxicity in the NTS might induce arrhythmogenesis accompanied by cardiac damage. The role of NOS1AP in NMDA mediated neurotoxicity is the subject of next chapter, with the current chapter focused on the physiological role of NOS1AP.

Immediate findings from the present study are listed below.

1. The knockdown of NOS1AP expression in the NTS did not alter the BP or HR significantly.
2. The NOS1AP expression knockdown in the NTS led to prolongation of the QT interval without any change in the R-R interval.
3. The heart rate variability analysis demonstrated a reduced HRV in rats with reduced NOS1AP in the NTS. Also the frequency domain analysis revealed an enhanced LF/HF signifying a sympathetic preponderance in NOS1AP knockdown rats.
4. The analysis of ventricular myocardium revealed the presence of contraction band necrosis indicating cardiac damage in the rats with reduced NOS1AP expression in the NTS.

Results demonstrated that NOS1AP downregulation has no effect on the BP and HR. This is an interesting result since we initially thought that NOS1AP downregulation would lead to enhanced NO which could act to modulate the baroreceptors. Increased NO could lead to the

neurological damage of the NTS leading to a loss of baroreceptor reflex function. Thus, the NTS will not be able to regulate blood pressure or provide cardioprotection by maintaining the sympathovagal balance. Recent study has shown that NO in the NTS is integral to excitation of baroreflex pathways involved in reflex tachycardia, a largely sympathetically mediated response, but not reflex bradycardia, a largely parasympathetically mediated response, suggesting that, at the basal state, nNOS is maximally engaged (Lin LH *et al*., 2012) . Thus, its upregulation does not augment the baroreflex. Thus, since NOS1AP has been suggested to be a negative regulator of NO production by sequestering nNOS - we hypothesized that NOS1AP downregulation might cause an enhanced nNOS activity leading to enhanced NO production. In line with the above report, this enhancement of nNOS activity might not have any effect on the baroreflex activity since at the basal state all the nNOS has been reported to be maximally engaged. This could explain our result that showed NOS1AP downregulation had no effect on effect on BP and HR.

Another argument might be that due to different pharmacological interventions within the brain itself it is difficult to attain the exact effects. Since the level of downregulation might be insufficient to counteract those interventions.

NOS1AP dysfunction in the NTS leads to a prolongation of the QT interval. QT interval is a measure of ventricular repolarization and prolonged QT interval is a risk factor for SCD (Newton CC *et al*., 2005, Hong Y *et al*., 2001, Straus SM *et al*., 2006, Schouten EG *et al*, 1991). A large body of clinical and experimental observations demonstrate direct influences of cortical and brainstem activity upon the myocardium and cardiac electrophysiology (Surges R *et al*., 2005). Subarachnoid hemorrhage is associated with higher rates of ECG abnormalities and arrhythmias (Oppenheimer SM *et al.,* 1991, Rogers MC *et al*., 1973, Billman GE *et al*., 1982, La

Rovere MT *et al*., 1988). Evidence shows that focal stimulation of a discrete set of brain regions may produce changes in heart rate, blood pressure, ECG and arrhythmias (Greenshot JH *et al*., 1969; Kono T *et al*., 1994). Thus NOS1AP dysfunction in the NTS might have lead to the neurological damage leading to arrhythmia. This effect is not only restricted to neurons but other cell types as well. Recent Studies have shown that glial cell damage, with no discernible neuronal damage, interfered with cardiovascular reflex transmission through the NTS (Lin LH *et al*., 2013).

Reduced heart rate variability is an indicator of cardiac mortality after myocardial infarction. In order to investigate the effect of NOS1AP downregulation on the occurrence of cardiac arrhythmias, it is important to assess the effect of this downregulation on the HRV in rats. HRV is a reliable reflection of the many physiological factors modulating the normal rhythm of the heart. They provide a powerful means of observing the interplay between the sympathetic and parasympathetic nervous systems. HRV analysis is the ability to assess overall cardiac health and the state of the ANS responsible for regulating cardiac activity (Oppenheimer SM *et al.,* 1991, Rogers MC *et al*., 1973, Billman GE *et al*., 1982, La Rovere MT *et al*., 1988).

Results showed that NOS1AP dysfunction leads to reduced HRV and the power spectral density analysis showed that the heart is more under the sympathetic control. This observation is in line with our result demonstrating pathological damage in the ventricular myocardium similar to the ones observed during sympathetic overactivation.

The HE staining revealed the presence of contraction band necrosis in the ventricle myocardium of NOS1AP dysfunctional rats. Studies have shown that, enhanced sympathoexcitation could cause local cardiac release of neuromodulators such as NO that affect

the cardiac function by modulating the acetylcholine release and binding to muscarinic receptors as well as noradrenalin release and binding to β-adrenergic receptors (Herring N *et al*., 2009).

CHAPTER VI. OVERALL DISCUSSION

Despite recent advances in preventing SCD due to cardiac arrhythmia, it remains a leading cause of death in the United States, claiming an estimated 325,000 lives each year. Neurogenic cardiac damage has been found to be the most important contributing factor to sudden death. This neurogenic cardiac damage might be caused due to SAH, epilepsy, stroke or MI. The cardiac damage associated with central lesions might occur in the form of cardiac arrhythmia and/or damage to the myocardium. Two major factors determine an individual's risk for fatal cardiac arrhythmias– heritable genetic disorders such as Long QT interval Syndrome and genetic variations along with cardiac pathology. Recent studies have identified NOS1AP gene variants and provide evidence for the association between these variants and SCD through prolonged QTc interval. The specific mechanism(s) by which this effect is exerted remains to be elucidated. NOS1AP acts as a negative regulator of NO production and its downstream signaling events by competing with PSD95 for interaction with nNOS. Thus, overexpression of NOS1AP prevents NMDA–nNOS interaction. Results from our study demonstrated Nucleus tractus solitaries (NTS) in the brain of Sprague Dawley (SD) rats have the most abundant NOS1AP mRNA (Figure 7). This finding is consistent with a recent study showing damaging neurons in NTS causes increased rate of SCD in rats, making it an excellent additional target to study neurogenic cardiac damage due to cardiac arrhythmia or myocardial damage. However, the nature and fundamental neuronal mechanisms linking NOS1AP in the NTS to arrhythmogenesis and/or myocardial damage are largely unknown. Based on recent evidences and our study, we hypothesized that NOS1AP knockdown in the NTS leads to more NMDA-PSD95-nNOS

interaction causing overproduction of NO which causes neurotoxicity. This neurotoxicity in the NTS might induce arrhythmogenesis and/or cardiac damage (Figure 6).

We tested our hypothesis through the following specific aims:

1) To identify the intracellular mechanisms in cultured neurons from SD rats and the role of NOS1AP in NMDAR mediated neuronal excitotoxicity.

The purpose of this specific aim is to provide evidence to proposed hypothesis regarding the induction of arrhythmogenesis due to altered NOS1AP levels in the rat brain neuronal culture cells. In this specific aim we investigated whether NOS1AP knockdown in primary rat brain neuronal cultures lead to neurotoxicity due to overproduction of NO (Fig. 6) due to change in NMDAR/nNOS coupling.

2) To detect the effect of NOS1AP knockdown in the NTS on the cardiovascular functions and electrophysiology of SD rats.

The purpose of this specific aim was to assess the biological function of NOS1AP by genetic knockdown in the NTS.

Thus, our major objective was to provide evidences to support/or refute the above hypothesis. The combinations of *in vitro* and *in vivo* techniques with cellular, molecular and physiological approaches were used to accomplish the objective.

6.1. NOS1AP expression profiling in cardiovascular regulatory regions in SD rats

The results from the above study helped to successfully achieve NOS1AP expression profiling. The results showing that NOS1AP is abundantly expresses in the neurons of the NTS (Figure 7, Figure 8) are consistent with recent evidences that NTS lesions interrupting the baroreflex may induce cardiac arrhythmias and myocardial changes similar to those seen in

humans with central lesions. The altered baroreflex transmission was associated with sudden death in 33% of the experimental animals (Talman WT., 1993; Nayate A *et al*., 2008). Based on our results from this chapter and the previous data, we used NTS as the site of NOS1AP knockdown in our proposed experiments to test the effect of NOS1AP dysfunction on cardiovascular and electrophysiological functions. Results showed that cardiovascular pathophysiologies involving sympathoexcitation leads to alteration of NOS1AP mRNA expression in major cardiovascular regulatory areas- NTS, PVN and heart (figure 10). These results are in line to previous studies that have shown decrease in gene expression of neuronal nitric oxide synthase in hypothalamus and brainstem of rats in heart failure (Patel KP *et al*., 1996). This can be explained by our previous observation and corresponding hypothesis that NOS1AP acts as a negative regulator of NO production and its downstream signaling events by competing with PSD95 for interaction with nNOS. Thus, overexpression of NOS1AP prevents NMDA–nNOS interaction. Therefore, in conditions manifested with sympathetic overactivation, such as myocardial infarction, NOS1AP expression might be altered as a protective mechanism so that augmented NOS1AP will sequester more nNOS rendering them inactive and hence reduced NO production. Reduction in NO might serve as a protective mechanism considering it primarily plays an excitatory role. Also, reduced NO might also lead to a reduction in neuronal damage in the brain and myocardial damage in response to prolonged sympathoexcitation.

Taken together, the results from the present study point towards an important role of NOS1AP in the various cardiovascular centers across the brain and heart under normal physiological conditions, which gets augmented with the development of pathophysiologies underlying sympathetic activation.

6.2. Role of NOS1AP in NMDA induced neurotoxicity in primary rat brainstem neuronal culture cells

The results from the current study confirms that decreased NOS1AP in the rat brain primary neuronal cells leads to enhanced NO/cGMP production causing neuronal death under excitotoxic condition due to NMDA treatment. This enhancement of NO production might be due to increase in the functional NMDAR/PSD95/nNOS complex after NOS1AP downregulation leading to NMDAR overactivation and enhanced nNOS activity. The NOSIAP downregulation leads to release of more nNOS which was otherwise sequestered with the NOS1AP and was rendered inactive for NO production.

These observations are consistent with previous findings indicating that NOS1AP might be a negative regulator of NO production (Chang KC *et al*., 2008; Jaffrey SR *et al*., 2002; Jaffrey SR *et al*., 1998). NOS1 and NO play an important role in the regulation of cardiac physiology which includes calcium turnover and adrenergic response (Barouch L.A. *et al*., 2002; Burkard N *et al*., 2007). NO produced by nNOS is important for NMDA receptor-dependent neurotransmitter release, neurotoxicity, and cyclic GMP elevations. The coupling of NMDA receptor-mediated calcium influx and nNOS activation is suggested to be due to a physical coupling of the receptor and the enzyme by an intermediary adaptor protein, PSD95, through a PDZ-PDZ domain interaction between PSD95 and nNOS. NOS1AP competes with PSD95 for interaction with nNOS, and overexpression of NOS1AP results in a loss of PSD95/nNOS complexes in transfected cells (Chang KC *et al*., 2008; Jaffrey SR *et al*., 2002; Jaffrey SR *et al*., 1998).

Some of the studies conducted in neuronal cells have shown contradictory results in that NOS1AP downlegulation is protective in NMDA mediated neurotoxicity. This can be explained by the variability of NMDA receptors in neurons cultured from different brain regions. Also, older neurons are more resistant to excitotoxicty than the younger neurons, thus, the level of NOS1AP downregulation might play an important role in deciphering its effects on the NMDA mediated neurotoxicity.

It is important to show the NMDA receptor activity and expression under altered NOS1AP conditions in the neurons. This might further add one more dimension to the regulation of NMDA mediated neurotoxicity by NOS1AP. Previous studies have shown that altered nNOS activity leads to Na^+-channel-mediated cLQTS in HEK293T cells. NOS1AP regulates nNOS subcellular location (Jaffrey SR *et al*., 2002; Jaffrey SR *et al*., 1998). Recent study showed that alteration in the HEK293T cells that increase the nNOS activity and released NO promotes the direct hyper-S-nitrosylation of $Na_v1.5$ and increases I_{NaL}, which is the characteristic biophysical dysfunction for Na^+-channel-mediated cLQTS (Ueda K *et al*., 2008; Tamargo J *et al*., 2010). In addition to mediating several physiological functions, NO has been implicated in the cytotoxicity observed following excess stimulation of neurons by glutamate. This neurotoxicity can be a mode of neurogenic damage causing impaired transmission from the NTS leading to myocardial damage and cardiac arrhythmias.

6.3. Effect of NOS1AP knockdown in the NTS on cardiovascular functions and electrophysiology in SD rats

Results demonstrated that NOS1AP downregulation has no effect on the BP and HR (Figure 25). This is an interesting result since we initially thought that NOS1AP downregulation

would lead to enhanced NO which could act to modulate the baroreceptors. Increased NO could lead to the neurological damage of the NTS leading to a loss of baroreceptor reflex function. Thus, the NTS will not be able to regulate blood pressure or provide cardioprotection by maintaining the sympathovagal balance. Recent study has shown that NO in the NTS is integral to excitation of baroreflex pathways involved in reflex tachycardia, a largely sympathetically mediated response, but not reflex bradycardia, a largely parasympathetically mediated response, suggesting that, at the basal state, nNOS is maximally engaged (Lin LH *et al*., 2012) . Its upregulation does not augment the baroreflex. Thus, since NOS1AP has been suggested to be a negative regulator of NO production by sequestering nNOS - we hypothesized that NOS1AP downregulation might cause an enhanced nNOS activity leading to enhanced NO production. In line with the above report, this enhancement of nNOS activity might not have any effect on the baroreflex activity. This could explain our result that showed NOS1AP downregulation had no effect on effect on BP and HR. This can also be explained in response to different pharmacological interventions within the brain itself it is difficult to attain the exact effects. Since the level of downregulation might be insufficient to counteract those interventions.

NOS1AP dysfunction in the NTS leads to a prolongation of the QT interval (Figure 26). QT interval is a measure of ventricular repolarization and prolonged QT interval is a risk factor for SCD (Newton CC *et al*., 2005, Hong Y *et al*., 2001, Straus SM *et al*., 2006). A large body of clinical and experimental observations demonstrate direct influences of cortical and brainstem activity upon the myocardium and cardiac electrophysiology (Surges R *et al*., 2005). Subarachnoid hemorrhage is associated with higher rates of ECG abnormalities and arrhythmias (Oppenheimer SM *et al.,* 1991, Rogers MC *et al*., 1973, Billman GE *et al*., 1982, La Rovere MT

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The presence of contraction band necrosis in the ventricle myocardium of NOS1AP dysfunctional rats was shown by HE staining (Figure 28). Studies have shown that, enhanced sympathoexcitation could cause local cardiac release of neuromodulators such as NO that affect

the cardiac function by modulating the acetylcholine release and binding to muscarinic receptors as well as noradrenalin release and binding to β-adrenergic receptors (Herring N *et al*., 2009).

Taken together, all results indicate that NOS1AP plays an important role in the protection of neurons from glutamate-induced neurotoxicity. NOS1AP dysfunction in the NTS might increase the risk of neurogenic cardiac damage, leading to QT interval prolongation, even sudden cardiac death.

CHAPTER VII. CONCLUSION AND FUTURE DIRECTIONS

The major finding of the current study is that NOS1AP knockdown in primary rat brain neuronal cultures lead to neurotoxicity due to overproduction of NO and NOS1AP knockdown in the NTS lead to prolonged QT interval and cardiac damage.

7.1. Conclusion

The overall conclusions from the in vivo and in vitro studies are summarized as following:

7.1.1. In Vitro study on neuronal cultures from brainstem of neonatal SD rats

1. NOS1AP downregulation sensitizes the primary neurons to NMDA mediated excitotoxicty which increases with increase concentration of NMDA.
2. Enhanced NO/cGMP has been shown be present in NOS1AP downregulated cells treated with NMDA. cGMP production is enhanced in a dose dependent manner with very high NMDA concentration tends to decrease the cGMP levels presumably due to increased cell death.
3. There is an increase in the amount of ternary NMDAR/PSD95/nNOS complex in NOS1AP knockdown cells when compared with untreated control neuronal culture cells. The lysates probed with NOS1AP showed a decreased expression of NOS1AP confirming that NOS1AP downregulation was responsible for the enhanced coupling of nNOS with NMDAR.
4. The NOS1AP downregulated cells showed an enhanced expression of apoptotic proteins such as Annexin V as compared to the control cells in response to NMDA. PI staining of dead/necrotic cells were not significantly observed in any cell type. This indicated that

the NOS1AP downregulated cells are under pre-apoptotic condition during which any excitotoxic stress due to NMDA treatment might result in cell death.

7.1.2. In Vivo study in SD rats

1. The NOS1AP mRNA expression is enhanced in the brain cardiovascular centers, the RVLM, PVN and NTS. The NOS1AP mRNA expression was comparable among the heart, aorta and the kidney.
2. The NOS1AP mRNA expression was the highest in the NTS of the SD rats as compared to the kidney.
3. The NOS1AP is localized in the neurons of the NTS and the PVN in SD rats, although the expression is augmented in the NTS as compared to other regions such as the PVN or RVLM.
4. Under pathophysiological myocardial infarction condition, the NTS, PVN and heart showed an increase in NOS1AP mRNA expression as compared to the corresponding tissues in the normal control SD rats. The NOS1AP mRNA expression in the RVLM was similar in both normal and HF rat models.
5. The knockdown of NOS1AP expression in the NTS did not alter the BP or HR significantly.
6. The NOS1AP expression knockdown in the NTS led to prolongation of the QT interval without any change in the R-R interval.
7. The heart rate variability analysis demonstrated a reduced HRV in rats with reduced NOS1AP in the NTS. Also the frequency domain analysis revealed an enhanced LF/HF signifying a sympathetic preponderance in NOS1AP knockdown rats.

8. The analysis of ventricular myocardium revealed the presence of contraction band necrosis indicating cardiac damage in the rats with reduced NOS1AP expression in the NTS.

To summarize, all results indicate that NOS1AP plays an important role in the protection of neurons from glutamate-induced neurotoxicity. NOS1AP dysfunction in NTS might increase the risk of neurogenic cardiac damage, leading to QT interval prolongation, even cardiac sudden death.

7.2. Future directions

It has been reported earlier that the genetic variants of NOS1AP have been linked to prolonged QT interval in various populations and also SCD. But, the cellular and molecular mechanism behind such association is yet to be elucidated and needs be looked into in the future. Our present study has attempted to dissect out a functional role of NOS1AP in the neurons of the NTS in causing arrhythmogenesis and myocardial damage. Although we were able to successfully look into some intracellular mechanisms such as the role of NOS1AP in NMDA mediated neurotoxicity, the present study leaves a few unanswered questions which would be interesting to look into in the future. One such question is the effect of alterations in NOS1AP on perturbation of electrophysiological characteristics of NMDA receptor or NMDA-induced calcium response or NMDA receptor activity leading to calcium influx into the cell. However, the actual changes in cytoplasmic free calcium also depend on the calcium-handling machinery of the cell. Measurement of the calcium response of neuronal cells with alterations in NOS1AP cotransfecting a fluorescence resonance energy transfer (FRET)–based calcium reporter precocious chameleon or YC2.12 would yield interesting results. Also NMDAR activity itself

measured by patch clamp method under altered NOS1AP conditions would provide important insights into its regulatory mechanisms.

Another role of NOS1AP is that it acts along with other signaling proteins such as dexras and Synapsin I and some of this interaction have been shown to lead to an increase in the number of dendrites of neuronal cells. Future studies could target this type of NOS1AP interaction in order to gain a better understanding of how NOS1AP could cause neuronal remodeling in various pathophysiological conditions including neurodegenerative diseases.

In the present study, we have investigated the role of central NOS1AP dysfunction in the pathogenesis of cardiac arrhythmia and myocardial damage. It would be interesting to see a role for NOS1AP in the peripheral nervous system such as on the glutamate induced neurotoxicity by stellate ganglia or other peripheral ganglia innervating the heart, thus modulating cardiac function. It is also a possibility that NOS1AP through its interaction with other signaling proteins, might lead to neuronal remodeling in such peripheral neurons leading to various cardiovascular manifestations.

The current study utilizes the lentiviral mediated knockdown approach for studying the underlying mechanisms of NOS1AP. It would be of interest to see how the overexpression of NOS1AP in cellular systems or in vivo affects the cardiovascular functions. Knockdown of NOS1AP essentially mimics the loss or mutation causing a loss of the protein function as found in different pathophysiological conditions. On the other hand overexpression studies would lead to further understanding of protein function and more importantly its role in signal transduction pathways by enhanced interactions through its binding partners. Also, in vivo this could lead to cardiovascular outcomes associated with the protein, if any. Different approaches could be

utilized to perform the overexpression of NOS1AP. One such method could be to induce NOS1AP expression in different cellular systems such as myocardial cells or neuronal cells and observe the signal transduction pathways with respect to a neurogenic cardiovascular function. Also, NOS1AP expression could be induced in vivo using microinjection techniques or through an inducible expression system such as Cre-Lox to cause overexpression of NOS1AP protein. This could lead to pathophysiological outcomes measured by echocardiography. Combining the above in vitro and in vivo approaches further understanding of the NOS1AP role in cardiovascular system could be deciphered.

An important issue to address is the effect of exacerbated physiological stress such as exercise on normal individuals or survivors of various cardiovascular pathologies such as MI and whether NOS1AP has any role to play in it. Since, the present study demonstrated that NOS1AP dysfunction neurons are highly sensitive to glutamate induced neurotoxicity, one might speculate that high level of physical activity in the form of stress causing increased mortality in MI survivors could underlie signaling mechanisms involving NOS1AP in the center.

Thus, extensive studies in future, to fill the gap in knowledge regarding the intracellular mechanisms involved in NOS1AP-induced neurotoxicity might be critical for defining NOS1AP as a SCD risk marker. Further research will help establish NOS1AP as a potential target for the prediction, prevention, and therapy of neurogenic cardiac damage underlying various cardiovascular diseases.

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