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**HYPERHOMOCYSTEINEMIA ALTERS SINOATRIAL AND ATRIOVENTRICULAR
NODAL FUNCTION: ROLE OF THE CARDIAC NMDA RECEPTOR**

By

Chirag V. Soni

B.A., Northwestern University, 1999

M.S., University of Louisville, 2005

A Dissertation

Submitted to the Faculty of the
School of Medicine of the University of Louisville

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

Department of Physiology and Biophysics

University of Louisville

Louisville, KY

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A Dissertation Approved on

August 27th, 2012

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DEDICATION

This dissertation is dedicated to my late grandfather Shantilal Soni, my grandmother Shardaba Soni, my grandfather Pujalal Soni, my grandmother Susilaba Soni and my foi Kantaben Soni.

Foremost, this dissertation is dedicated to my loving parents Dr. Vinod and Geeta Soni, who worked very hard to be able to provide opportunities for their children which they did not have growing up in rural India, my sisters Hina and Amita who always keep me in line, and my baby niece Ava, the light of my life.

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Finally, I would like to thank the members of the Pathology Core, as well as Dr. Rong Wan for assistance with immunohistochemistry.

ABSTRACT

HYPERHOMOCYSTEINEMIA ALTERS SINOATRIAL AND ATRIOVENTRICULAR NODAL FUNCTION: ROLE OF THE CARDIAC NMDA RECEPTOR

Chirag V. Soni

August 27, 2012

Introduction: Hyperhomocysteinemia (HHcy) is a pathological condition characterized by excessive levels of plasma homocysteine (Hcy). Patients with HHcy are reported to be at risk for arrhythmias like atrial fibrillation and sudden cardiac death (SCD); however, the causative mechanisms remain unknown. The effects of HHcy on sinus node function, atrioventricular conduction and ventricular vulnerability were investigated by *in vivo* electrophysiological (EP) analysis, and the role of the cardiac *N*-methyl-D-aspartate receptor (NMDA-R) in promoting Hcy-induced conduction abnormalities was explored.

Materials and Methods: Anesthetized wild-type control mice (*WT*), mice receiving Hcy in the drinking water for 12 weeks (*DW*), and heterozygous cystathionine- β -synthase knockout mice (*CBS*^{+/-}) were subjected to electrocardiographic (ECG) analysis and programmed electrophysiological (EP) studies. To examine the role of the NMDA-R in eliciting conduction changes in HHcy, animals from the three groups were subjected to paired-design repeat EP studies before and after intraperitoneal injection of magnesium sulfate (MgSO₄, 20mg/kg), an endogenous blocker of NMDA-R. Another set of studies

utilized a specific NMDA-receptor antagonist, dizocilpine (MK-801), in similarly paired-design studies in an acute, intraperitoneal infusion model of HHcy. Expression levels of cardiac NMDA-R in all three groups of animals were quantified by immunoblotting, and immunohistochemical identification of cardiac NMDA-Rs at the mouse atrioventricular (AV) junction was undertaken.

Results: *DW* compared to *WT* had significantly shorter RR, PR, QT, and HV intervals, corrected sinus node recovery times (CSNRT), Wenckebach periodicity (WP), AV nodal effective refractory periods (AVNERP), and right ventricular effective refractory periods (RVERP). No ventricular arrhythmias were induced in either *WT* or *DW*. In the paired-design studies, blockade of cardiac NMDA-R with MgSO₄ had no effect on any ECG or EP variables in *WT* but significantly prolonged RR, QT, HV, CSNRT, WP, and AVNERP in *DW* and *CBS*^{+/-} animals. Immunopositive staining for cardiac NMDA-R at the mouse AV junction was detected.

Conclusions: Significant changes in conduction were observed between wild-type mice and mice with diet-induced or genetically-induced hyperhomocysteinemia (HHcy). Low-dose magnesium administration did not alter ECG or EP conduction variables in wild-type mice, but had a profound effect on mice with HHcy. These results suggest that shortened sinoatrial and atrioventricular conduction time in mild HHcy may involve cardiac NMDA-Rs.

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CHAPTER I

INTRODUCTION

Increased plasma homocysteine (Hcy) is an independent risk factor for a significant number of cardiovascular diseases, including hypertension, coronary artery disease, atherosclerosis, and heart failure. Recent reports demonstrate a link between hyperhomocysteinemia (HHcy) and arrhythmogenesis leading to atrial fibrillation (AF) as well as lethal arrhythmias leading to sudden cardiac death (SCD). The exact mechanism by which higher levels of Hcy may facilitate induction of arrhythmias has not been elucidated.

Hcy is known to activate the N-methyl-D-aspartate receptor (NMDA-R) in the brain, increasing intracellular calcium $[Ca^{2+}]_i$ and the excitability of cells¹. The NMDA-R is expressed in several peripheral tissues, including the heart, kidney, lungs, testes, GI tract² as well as in the vascular endothelium³ and smooth muscle⁴. In the heart it has been identified in both the myocardium as well as in the specialized conductive tissues^{5, 6}. Although subunits of NMDA-R are expressed in various cardiac cells, the role of cardiac NMDA-Rs in normal or pathological cardiac conduction is unknown.

Additionally, HHcy induces mitochondrial oxidative stress of cardiomyocytes, increasing levels of reactive oxygen species (ROS) which activate matrix metalloproteinases (MMPs) involved in extracellular matrix (ECM) remodelling^{7, 8}.

MMP-mediated remodelling is a ubiquitous phenomenon that results in the degradation and recycling of membrane-associated proteins, including ion channels like the N-methyl-D-aspartate receptor (NMDA-R), and connexins (Cxs) which mediate intercellular communication via formation of gap junction channels (GJCs). Changes in NMDA-R subunit composition and receptor density can alter cell-to-cell signal propagation in the myocardium and the specialized conduction system (SCS) of the heart. Communication between cells at the sinoatrial node (SAN), internodal pathways, atrioventricular node (AVN) and His-Purkinje fibers could be altered by MMP-mediated remodelling in patients with HHcy. Changes in NMDA receptor subunit expression can have a profound effect on normal impulse propagation, but more importantly on signal conduction between atria and ventricles across the AVN.

The overall hypothesis of this dissertation was arrhythmogenesis may arise in HHcy due to higher circulating levels of Hcy, which acts as an agonist at the cardiac NMDA receptor. This hypothesis was tested by 1) measuring electrocardiographic (ECG) and electrophysiological (EP) variables in order to compare cardiac conduction in wild-type and hyperhomocysteinemic mice, 2) characterizing the role of cardiac NMDA-R in the conduction changes observed in HHcy by utilizing pharmacological inhibition of the receptor, and 3) comparing cardiac NMDA receptor expression levels in wild-type and HHcy mice. In this dissertation, evidence that supports potential mechanisms by which increased plasma Hcy may increase nodal cell excitability, and thereby facilitate the initiation of arrhythmias, is presented.

BACKGROUND

Homocysteine

Homocysteine (Hcy) is a non-proteinogenic, sulfur-containing excitatory amino acid. It is similar to cysteine, another thiolated amino acid which is formed using Hcy as a precursor, except that Hcy contains one more methylene group (CH_2). It is generated as a consequence of methionine metabolism, and is involved in the transmethylation and trans-sulfuration pathways. In the transmethylation pathway, methionine, which is orally consumed as protein, becomes adenosylated via transfer of adenosine from ATP by methionine adenosyltransferase. This forms S-adenosylmethionine (SAM) which is a universal methyl group ($-\text{CH}_3$) donor. Methyl transferases, such as those which catalyze the methylation of DNA and RNA, transfer the methyl group bonded to the sulfur atom in SAM to its target. Phenylethanolamine-N-methyltransferase (PNMT) uses SAM to donate a methyl group to norepinephrine to produce epinephrine. Once SAM is demethylated, it becomes S-adenosylhomocysteine (SAH). SAH is hydrolyzed by SAH hydrolase to yield homocysteine.

Hcy at this point can continue in the transmethylation pathway and be remethylated to methionine, or it can be further metabolized by the trans-sulfuration pathway. In the transmethylation pathway, methionine can be regenerated from homocysteine by methionine synthase (MS), which is another methyltransferase. 5-methyltetrahydrofolate, which is formed following the vitamin B_{12} -dependent reduction of 5,10-methylenetetrahydrofolate by methylenetetrahydrofolate reductase (MTHFR), is used as a methyl donor by MS to re-methylate Hcy and regenerate methionine. In the

trans-sulfuration pathway, Hcy condenses with serine to form cystathionine via a vitamin B₆-dependent reaction catalyzed by cystathionine- β -synthase (CBS). Cystathionine is cleaved to create cysteine and α -ketobutyrate by cystathionine- γ -lyase (CGL), which is also vitamin B₆-dependent. Cysteine can be further metabolized by the trans-sulfuration pathway to form glutathione, an important cellular antioxidant.

Accumulation of Hcy

Elevated plasma and tissue accumulation of Hcy can arise in several ways. As previously described, Hcy is generated as methionine is converted to SAM/SAH. Increased dietary intake of methionine can therefore lead to higher levels of Hcy. Also, increased activity of methyl transferases will yield more SAH, and therefore more Hcy, following methylation reactions. In addition, genetic polymorphisms have been identified in several of the enzymes involved in the transmethylation and trans-sulfuration pathways. Genetic polymorphisms in MTHFR, CBS, MS, and MS reductase have been identified, and several of these are associated with varying degrees of hyperhomocysteinemia or homocystinuria, an inherited disorder affecting the metabolism of sulfur-containing amino acids⁹. One of the most extensively studied MTHFR polymorphisms, the substitution of C $\rightarrow$ T at nucleotide position 677 (C677T) which results in the substitution of valine for alanine, causes enzyme deficiency wherein homozygous individuals have MTHFR enzyme activity reduced to 30% of wild-type activity¹⁰. Over 100 different mutations have been identified in the CBS gene, and many have been linked to varying degrees of hyperhomocysteinemia¹¹. The classical genetic disease homocystinuria, caused by a CBS deficiency, results in extremely high levels of

plasma and urinary homocysteine and methionine. Homocystinurics present with mental retardation, musculoskeletal deformities, motor and gait dysfunction, and ectopia lentis⁹.

B vitamin deficiency can also cause elevated levels of Hcy, since several of the enzymes involved in Hcy metabolism require B vitamins as cofactors (MTHFR requires B₁₂, CBS and CGL both require B₆). Folate (B₉) is also required – the tetrahydrofolate cycle provides the methyl group required to regenerate methionine from Hcy. Without proper intake of B vitamins these processes will be hindered, resulting in increased plasma Hcy. Indeed, B vitamin supplementation is shown to decrease plasma Hcy.

Renal dysfunction can also lead to HHcy. Plasma Hcy levels are determined by intracellular metabolism of Hcy, import/export of Hcy between the plasma and the various tissues most largely associated with Hcy metabolism¹², and renal clearance of Hcy. Renal clearance of Hcy is hindered in patients with renal disease/dysfunction, resulting in the accumulation of Hcy in the plasma.

Normal fasting plasma Hcy values generally fall within 5-10 μ M. Values greater than 10 μ M (10-30 μ M) are considered mildly hyperhomocysteinemic. Moderate HHcy is characterized by plasma Hcy values between 30-100 μ M, and severe HHcy is characterized by plasma Hcy levels above 100 μ M.

Hcy and Disease

Kilmer S. McCully first postulated the homocysteine theory of arteriosclerosis in 1975¹³. It is now well-established that increased plasma Hcy is a risk factor for cardiovascular, cerebrovascular, and neurodegenerative disease. High plasma Hcy levels have been linked to coronary artery disease, hypertension, acute myocardial infarction,

stroke, atherosclerosis, heart failure, atrial fibrillation, and sudden cardiac death (SCD)^{9, 14-23}. Increased plasma Hcy is also a risk factor for neurologic disorders like Alzheimer's and Parkinson's diseases, as well as psychiatric disorders like schizophrenia.

Cardiac Arrhythmias in HHcy

The risk factors for arrhythmogenesis are multifactorial. HHcy is associated with hypertension, coronary artery disease, and cardiac interstitial fibrosis²⁴. The presence of one or a combination of these risk factors induces structural derangements to the ventricular myocardium, thereby increasing the chance of creating substrate for the induction of ventricular tachycardia (VT) and possibly ventricular fibrillation (VFib). In the absence of intervention, VT degenerates into VFib and sudden cardiac death (SCD). Several studies associate elevated Hcy with SCD^{9, 14, 16}.

Additionally, several studies assert an association between HHcy and atrial fibrillation²⁵, especially in the context of the thrombogenic potential of increased plasma Hcy and the consequent risk of stroke^{22, 23}. Atrial fibrillation arises due to extensive structural and electrical atrial remodelling. HHcy-induced MMP activation plays a role in the structural and electrical remodelling of the atria^{26, 27}. Following orthotopic heart transplantation, HHcy is associated with altered atrial electrical conduction manifesting as P wave dispersion²⁸. Hcy also modulates sodium and potassium currents in human atrial myocytes^{29, 30}, highlighting another plausible mechanism by which elevated Hcy may initiate atrial arrhythmias.

Cardiac Action Potentials

The basis for cardiac impulse generation and conduction is cardiac cell excitability and spatiotemporal ionic permeability changes. Ionic conductances through specific types of Na^+ , K^+ , and Ca^{2+} channels underlie the different phases of the cardiac action potential, as well as the different morphologies of action potentials elicited by the various cardiac tissues.

The cardiomyocyte action potential typically has four phases: in Phase 4 the cell is at rest, rapid depolarization occurs during Phase 0, early repolarization begins during Phase 1, delayed repolarization and a slow inward calcium current underlie the plateau of Phase 2, and repolarization is completed during Phase 3. Not all cardiac action potentials exhibit all four phases. Nodal cells of the specialized conduction system only exhibit three phases: an unstable Phase 4, Phase 0 depolarization, and Phase 3 repolarization. The unstable Phase 4 of nodal cells is responsible for cardiac automaticity.

In working myocytes, an inward sodium current (I_{Na}) activated by opening of $\text{Na}_v1.5$ (*SCN5A*) voltage-gated sodium channels causes the rapid phase 0 depolarization. The phase 1 repolarization is mediated by a transient outward K^+ current (I_{to}) mainly through $\text{K}_v4.3$ (*KCND3*) assembled voltage-gated K^+ channels. The plateau in phase 2 is due to opening of L-type voltage-gated calcium channels ($\text{Ca}_v1.2$), giving rise to inward conductance $I_{\text{Ca,L}}$. Repolarization is mediated by three outward delayed-rectifier K^+ channels: at the beginning of phase 2, the ultra-rapidly activating delayed-rectifier K^+ channel ($\text{K}_v1.5/3.1$) permits outward current I_{Kur} ; midway through phase 2 the slowly activating delayed-rectifier K^+ channel ($\text{K}_v\text{LQT1}$) opens to conduct I_{Ks} ; finally, late

repolarization in phase 3 is mediated by the rapidly activating delayed-rectifier K^+ channel (hERG), which conducts I_{Kr} .

An important difference between nodal cells and cells of the working myocardium is that nodal cells of the proximal conduction system lack the fast, voltage-gated Na^+ channel ($Na_v1.5$) responsible for rapid phase 0 depolarization in working myocytes. The Purkinje fibers of the distal conduction system, however, do express the fast Na^+ channels; this allows the Purkinje network to rapidly convey action potentials to the ventricular endocardium and epicardium. Phase 0 depolarization in nodal cells is mediated by activation of T-type voltage-gated calcium channels ($Ca_v3.1$, $I_{Ca,T}$) as well as the L-type channels found in non-pacemaker cells. Additionally, nodal cells express channels which give rise to the “funny” current I_f , also known as the pacemaker current. Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels at the sinoatrial node are responsible for the unstable phase 4 which confers automaticity. HCN channels are expressed at the AV node as well as in Purkinje fibers.

There are other currents which play a role in shaping the cardiac action potential, namely other K^+ currents such as the ATP-gated I_{KATP} , acetylcholine-gated I_{KAch} , and voltage-gated inward-rectifying I_{K1} currents.

Glutamate Receptors--The NMDA Receptor

Although they have been most extensively studied in the brain, glutamate receptors (GluRs) are present in many tissues outside the nervous system, including the heart^{6, 31}. GluRs are activated by the excitatory amino acid L-glutamate and are divided into 2 subtypes: metabotropic glutamate receptors (mGluRs) and ionotropic glutamate

receptors (iGluRs). Both types, when activated, result in an increase in intracellular Ca^{2+} by different mechanisms. Metabotropic GluRs are G-protein-coupled receptors; binding of glutamate to mGluRs initiates signal transduction events such as activation of phospholipase C, which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Subsequently, IP_3 activates the release of intracellular Ca^{2+} from stores within the sarcoplasmic reticulum. Acidic derivatives of Hcy, like homocysteic acid (HcyA), activate mGluRs in neuronal and non-neuronal tissues³², initiating G-protein mediated signalling and causing an increase in $[\text{Ca}^{2+}]_i$. mGluRs have also been identified in mammalian heart³³.

Ionotropic GluRs are present in various mammalian tissues as well^{31, 34}. There are 3 classes of iGluRs, each named after its highest affinity agonist: N-methyl-D-aspartate (NMDA) receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (also known as quisqualate receptors), and kainate (Ka) receptors. Activation of iGluRs predominantly induces Ca^{2+} influx, besides permitting Na^+ influx and K^+ efflux, causing depolarization and excitation of the cells. The NMDA receptor is unique in that its activation is both ligand- and voltage-dependent. Both glutamate and the co-agonist glycine are required to bind, and a voltage-dependent Mg^{2+} block occluding the central pore must be relieved by local membrane depolarization before the channel becomes permeable to Ca^{2+} , Na^{2+} , and K^+ .

Most NMDA receptors are assembled from NR1 subunits (of which there are eight alternative splice variants) and NR2 subunits (of which there are 4 isoforms, NR2A-D). NR3 subunits have also been identified, which confer an inhibitory effect on the receptor. NR1 and NR2 subunits contain binding sites for glycine and glutamate,

respectively. Hcy activates the NMDA-R in neurons¹, endothelial cells³, and vascular smooth muscle cells⁴. Hcy is capable of binding to both the glycine and glutamate binding sites of NMDA-R. Interestingly, Hcy acts as an agonist at the glutamate binding site on NR2, but acts as a partial antagonist at the glycine binding site on NR1¹. Glycine elevated above physiological levels therefore increases the agonistic effects of Hcy.

Supporting evidence that excitatory amino acids acting on the NMDA-R play a role in arrhythmogenesis is a study demonstrating that blockade of the receptor decreases ischemia/reperfusion-induced arrhythmias but not ischemia-induced arrhythmias³⁵. These findings suggest that circulating factors are involved in arrhythmia induction; also in the setting of myocardial ischemia/reperfusion, blocking the NMDA-R is important in reducing arrhythmogenic substrate. In this context, exposure to high levels of Hcy or other circulating excitatory amino acids may overactivate the cardiac NMDA-R, increasing $[Ca^{2+}]_i$ and altering the rate of cell-to-cell conduction, thereby creating arrhythmogenic substrate.

Hcy and Oxidative Stress

Increased plasma Hcy induces oxidative stress by activating inducible nitric oxide synthase (iNOS) and NADPH oxidase^{7, 36-38}. Both contribute to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) like peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical ($OH^\cdot$), and peroxynitrite⁷. Increased levels of myocardial iNOS are associated with arrhythmogenesis and SCD³⁹. HHcy increases mitochondrial oxidative stress of cardiomyocytes, increasing levels of ROS which activate matrix metalloproteinases (MMPs) involved in extracellular matrix (ECM)

remodelling⁸. Hcy also diminishes activity of superoxide dismutase (SOD) and many redoxins which reduce ROS, further exacerbating oxidative stress⁷.

It is established that Hcy activates MMPs by oxidizing inhibitors of MMPs, such as tissue and cardiac inhibitors of MMPs (TIMPs and CIMP, respectively). Latent MMPs associate with nitric oxide (NO) and TIMPs in a ternary complex; ROS and RNS generate nitrosylated tyrosine residues in TIMPs, thereby liberating active MMPs^{7, 40}. Overactivation of MMPs is responsible for abnormal myocardial remodelling, which results in increased interstitial collagen associated with fibrosis²⁴. In paradigms where cardiac remodelling associated with fibrosis occurs (e.g. heart failure, myocardial infarction or atrial fibrillation), an increased likelihood of re-entrant arrhythmias is a common feature. Conduction abnormalities observed in infarction and cardiac failure appear to be related to connexin dysfunction and increased action potential duration (APD), whereas in atrial fibrillation, reduction of APD appears to play a major role⁴¹.

Connexins

Intercellular communication between cardiomyocytes, nodal cells, and endothelial cells is important in the coordinated excitation and contraction of the working myocardium, as well as in impulse propagation through the SCS of the heart. Intercellular communication is mediated by gap junction channels (GJCs) composed of connexins (Cxs). Six connexins join to form a connexon (a hemichannel assembly comprising half of a GJC) which communicates with a hemichannel from an adjacent cell to form a functional GJC between the two cells. The selective permeability, unitary conductance of ions, and gating characteristics of an assembled GJC is determined by the Cx isotype

composition of the channels. Twenty-one human Cx isoforms have been identified, with five present in the heart: Cx31.9, Cx37, Cx40, Cx43, and Cx45⁴¹⁻⁴⁸. The latter three have been extensively studied in the heart. Cx43 is the most abundantly expressed Cx in the heart; it is present in the atrial and ventricular working myocardium, as well as the distal SCS^{41, 42}. GJCs composed entirely of Cx43 allow a unitary conductance of 115-120 picoSiemens (pS)^{49, 50}. Cx40 is present in and around the AVN, as well as in the atrial myocardium⁴¹. Channels composed of Cx40 allow faster conductance at 180-200 pS^{50, 51}. Cx45 is found in the SAN, AVN, and the internodal conduction system⁵². Cx45 channels exhibit slower unitary conductance, at 35 pS⁵³. A recently discovered connexin isoform Cx31.9 in humans (Cx30.2 in mouse) is thought to play a role in slowing the impulse conduction across the AVN^{54, 55}. Cx31.9 is predominantly localized to the SAN and AVN, and allows the slowest conductance, at 9 pS^{50, 54, 55}.

Assembly of Cxs into hemichannels, and ultimately into GJCs, can result in 1 of 3 types of channels. Homotypic channels are composed of the same Cx isotypes in both hemichannels. Heterotypic channels are formed by hemichannels assembled from 1 isotype apposed to hemichannels composed entirely of a different Cx isotype. Heteromeric channels are the most variable; each hemichannel can have a variable Cx isotype configuration. If you consider only 2 of the 21 identified Cx isoforms, which can be assembled into any configuration of hemichannels and GJCs, there would be at least 196 different channel configurations⁵⁶. Due to the several possible configurations of heterotypic and heteromeric channels, the permeability, conductance, and gating properties of each channel varies as the summation of the properties of individual Cx subunits. In HHcy, activation of MMPs induces MMP-mediated cardiac remodelling²⁴,

and as observed in heart failure and myocardial infarction⁵⁷⁻⁶², remodelling alters the expression, distribution, and regulation of Cxs, ultimately modifying electrical continuity between cardiac cells.

Cardiac Remodelling and Cx Dysfunction

Cardiac remodelling in patients with heart failure is associated with various types of arrhythmias and conduction abnormalities⁶³. A common finding in these patients is down-regulation and dysfunction of Cx43⁵⁷⁻⁵⁹. Changes in Cx43 expression may be regionally determined and aggravated by dyssynchronous contraction^{57, 64}. Similarly, conduction abnormalities are frequently found in patients with healed myocardial infarctions. Surviving cells in the peri-infarct zone have abnormal action potentials due to abnormal cell coupling^{53, 65}. Significant changes in GJC organization and distribution of Cx43 occur in healed myocardial infarctions⁶⁰⁻⁶². In experimental studies, genetically engineered mice expressing <50% of normal Cx43 levels exhibit increased propensity towards arrhythmic activity in response to acute ischemia⁶⁶. Targeted inactivation of Cx43 in myocardium leads to increased arrhythmic activity and sudden death^{67, 68}. In heart failure and myocardial infarction, activation of c-Jun N-terminal kinase (JNK) appears to be the mechanism by which Cx43 is down-regulated⁶⁹. In addition to decreasing Cx43 expression, phosphorylation changes impairing Cx43 function effectively reduce cell-to-cell communication^{58, 59}.

Conduction is further impaired in heart failure and myocardial infarction by lateralization of GJCs and increased heterogeneity of Cx isotypes in hemichannels^{60, 70}. In normal hearts, GJCs are primarily localized at the end-to-end junctions of adjacent

myocytes. In healed myocardial infarction there are fewer and smaller GJCs, with a change in proportion of side-to-side versus end-to-end intercellular connections⁶⁰. In heart failure, the percentage of lateralized GJCs expressing Cx43 increases along the length of myocytes while end-to-end connections decrease⁷⁰. Furthermore, increased Cx43 heterogeneity in heart failure patients is associated with VT⁷¹. Recent studies demonstrate that homotypic Cx43 and Cx45 GJCs have higher conductance than heterotypic and heteromeric (Cx43 and Cx45 combined) GJCs^{72, 73}. Since Cx43 is down-regulated in heart failure and Cx45 levels are unchanged or increased, the Cx45:Cx43 ratio increases, potentially promoting the formation of more heterogeneous GJCs with lower conductance than in normal hearts^{56, 73}.

The similarity of events in HHcy-, heart failure- and myocardial infarction-induced remodelling supports the hypothesis that elevated levels of Hcy may create arrhythmogenic substrate. In fact, recent evidence demonstrates that long-term diet-induced HHcy down-regulates Cx43 expression⁷⁴. Down-regulation of Cx43 expression in heart failure produces a positive feedback system in which slow ventricular impulse conduction contributes to mechanical dysfunction, and further promotes ventricular remodelling⁶⁴. Impaired impulse conduction also creates substrate for re-entrant arrhythmias⁵⁷. These arrhythmias require two parallel conducting pathways connected proximally and distally by conducting tissue, thus forming a potential electric circuit. For re-entry to occur 3 prerequisites need to be present: 1) one of the two pathways conducts more slowly, 2) the faster conducting pathway has a substantially longer refractory period, and 3) an appropriately timed premature impulse is needed to initiate a continuous circular propagation of the impulse. In heart failure, subepicardial Cx43 expression is

reduced by approximately a third compared with deeper layers of the left ventricle, accentuating uncoupling between the epicardial-midmyocardial interface ⁵⁷. The development of steep gradients of repolarization occurring within the epicardial-midmyocardial interface underlies the mechanism of conduction block and re-entrant arrhythmogenesis in heart failure ⁷⁰. However, in HHcy-induced ventricular remodelling, the levels of Cx43 expression and function at the various myocardial layers have not been studied, therefore it is unknown whether uncoupling of myocardial layers due to HHcy is of sufficient magnitude to create re-entrant substrate.

Remodelling at the SAN and AVN

The sinoatrial node (SAN) and atrioventricular node (AVN) are part of the heart's SCS. The SAN is specialized in pacemaking, whereas the AVN is responsible for propagating cardiac impulses from the SAN to the ventricles. The AVN has two important functions: 1) reduce impulse conduction velocity to allow time between atrial and ventricular depolarization, thereby allowing time for ventricular filling prior to systole, and 2) protect ventricles from premature aberrant beats generated in the atria. To perform their functions, both the SAN and AVN have special requirements regarding the electrical coupling between cells, which is determined by Cx isoform expression in GJCs. The electrical coupling of cells at the center of the SAN needs to be weak to protect them from the inhibitory electrotonic influence of hyperpolarized non-pacemaking atrial myocytes surrounding the SAN. Conversely, for the SAN to be able to drive the atrial muscle, electrical coupling should be strong in the periphery of the SAN ⁷⁵. Indeed, at the center of the mouse SAN there is little or no expression of high conductance Cx40 and

Cx43 (the principal Cxs of cardiac muscle), but there is expression of lower conductance Cx45 and Cx30.2, whereas in the periphery of the SAN, Cx43 as well as Cx45 is expressed⁵⁵. In the AVN, a similar pattern of Cx expression as in the center of the SAN is observed; cell-to-cell coupling is mediated by abundant expression of Cx30.2 and Cx45, and to a lesser extent expression of Cx40⁵⁵. This Cx expression pattern is likely to be in large part responsible for the slow conduction of the action potential. In heart failure patients, atrial remodelling is characterized by abnormalities of conduction, sinus node dysfunction, and structural changes along the crista terminalis. These abnormalities may be responsible in part for the increased propensity for atrial fibrillation in heart failure^{76, 77}.

At present, it's unknown whether HHcy-induced cardiac remodelling has an effect on SAN function. However, recent evidence from electrophysiological studies indicates that in diet-induced HHcy, atrioventricular (AV) conduction is altered⁷⁸. Anatomically, the AVN is not as discrete a body as the SAN, and the modern view describes it as a dual atrioventricular transmission system wherein diffuse fibers arising in the right atrium coalesce into two pathways which approach the central, compact body of the AVN^{79, 80}. The anterior pathway, which runs along the Tendon of Todaro, has been determined to be the fast (or normal) pathway with a longer refractory period. The posterior pathway, running along the tricuspid annulus, comprises the slow pathway with a shorter refractory period. The existence of dual pathways with differing conduction velocities has been hypothesized to be the substrate for AV nodal re-entrant supraventricular arrhythmias^{79, 81}. The question however is whether MMP-mediated GJC remodelling, induced by pathological conditions such as HHcy, alters AV nodal Cx

expression (Cx30.2, Cx45, and Cx40) to sufficiently create re-entrant substrate. Recent studies have provided biochemical evidence for the ability of Cx30.2 to hetero-oligomerize with the other cardiac Cxs⁸². In cells expressing Cx43 and Cx45, the introduction of Cx30.2 alters the properties of GJC currents to an intermediate level between that of either Cx alone. These intermediate properties may be explained by the formation of heteromeric GJCs or they might correspond to the aggregate of a mixture of homomeric GJCs. In contrast, when Cx40 and Cx30.2 are co-expressed, the physiological properties of GJCs are similar to those in cells expressing Cx30.2 alone; these findings can be explained by the formation of heteromeric GJCs. In these studies, Cx45 expression showed the most extensive overlap with regions of Cx30.2 expression; thus Cx30.2-Cx45 interactions may influence conduction in those areas⁸². Studies using genetically altered animals have shown that Cx40 deficient mice exhibit slower AV nodal conduction⁸³. Conversely, mice deficient in Cx30.2 exhibit faster AV conduction⁵⁴. The latter study demonstrates the important role Cx30.2 plays in the slowdown of impulse propagation across the AVN, and in limiting the maximal number of beats conducted from atria to ventricles.

The evidence that diet-induced HHcy in mice shortens the PR interval in the ECG and the AH interval in the intracardiac electrogram suggests accelerated conduction across the AVN⁷⁸. However, the mechanisms for increased AVN conduction were not elucidated in the study, and it remains to be determined whether AV nodal conduction changes are due to HHcy-induced GJC remodelling and/or overactivation of the NMDA-R. Furthermore, an added level of complexity is presented by the potential effects of remodelling and cardiac NMDA-R activation on the slow and fast AVN pathways. To

explore these mechanisms a multidisciplinary approach that includes electrophysiological studies in combination with molecular techniques will be needed to understand the underlying changes in conduction properties of slow and fast pathways associated with HHcy. It remains to be determined whether the AVN pathways in HHcy will become slower due to fewer GJCs, lateralization of GJCs, and decreased Cx40 expression or whether they will have accelerated conduction due to increased GJC heterogeneity with greater ratios of Cx40 expression, and/or increased cardiac NMDA-R stimulation.

Cardiac Electrophysiological Pacing and Recording

The advent of clinical cardiac electrophysiology studies has provided a better understanding of AV junctional arrhythmias and the dual AV nodal pathway physiology⁸⁴. In the last 8 years, techniques to record intracardiac electrical activity, as well as the ability to deliver impulses to the right atrium and ventricle of mice using programmed stimulation, have been developed^{54, 83, 85}. To perform the electrophysiological studies, a 2Fr octopolar ring electrode catheter with 0.5mm electrode width and 0.5mm interelectrode distance is introduced into the right atrium and ventricle of the mouse via the right external jugular vein. Once the catheter tip is placed at the apex of the right ventricle, electrode pairs are selected to stimulate and record signals from the atria, AV node, and ventricles. Basic electrophysiologic variables are obtained using standard clinical electrophysiologic pacing protocols adjusted to the murine heart rate (~600 beats/min). Information gathered from these studies includes assessment of sinoatrial (SA)- and AV-nodal automaticity and conductivity as well as assessment of His-Purkinje system conductivity and refractoriness. It is also possible to study whether

mice have a propensity to develop atrial or ventricular tachyarrhythmias by using stimulation protocols that include up to three premature extra stimuli.

CHAPTER II

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

Increased plasma homocysteine (Hcy) levels cause increased activation of cardiac NMDA receptors at the sinoatrial and atrioventricular nodes, increasing intracellular calcium and enhancing excitability of nodal cells, and thereby facilitating abnormalities in sinoatrial and atrioventricular conduction. This hypothesis was tested through three specific aims.

Specific Aim 1

To determine whether increased plasma Hcy, as observed in hyperhomocysteinemia (HHcy), affects cardiac conduction, surface electrocardiographic (ECG) data was obtained and programmed electrophysiological (EP) studies were performed *in vivo* to characterize conduction variables in wild-type C57BL/6J mice and diet-induced hyperhomocysteinemic C57BL/6J mice. Comparison of these data may illustrate any changes to cardiac conduction due to higher levels of plasma Hcy.

Specific Aim 2

To investigate whether cardiac NMDA-R activation is involved in normal and/or pathologic cardiac conduction, pharmacological inhibitors of the NMDA receptor were utilized and ECG and EP variables at baseline were compared with those obtained following NMDA-R blockade. ECG and EP data were collected from wild-type C57BL/6J mice, diet-induced hyperhomocysteinemic C57BL/6J mice, and genetically-induced hyperhomocysteinemic C57BL/6J mice. In a second set of experiments, HHcy was induced in wild-type C57BL/6J mice by intraperitoneal administration of Hcy solution in an acute infusion model.

Specific Aim 3

In order to compare expression levels of cardiac NMDA receptor in wild-type, diet- and genetically-induced hyperhomocysteinemic mice, immunoblotting was performed on hearts from animals from all three groups. Additionally, immunohistochemical identification of cardiac NMDA-R at the mouse atrioventricular node was undertaken.

CHAPTER III

HYPERHOMOCYSTEINEMIA ALTERS SINOATRIAL AND ATRIOVENTRICULAR CONDUCTION

Introduction

Recently, remote telemetric ECG recordings from wild-type mice and mice with diet-induced hyperhomocysteinemia (HHcy) have been shown to display irregular cardiac rhythms⁷⁴. In animals with diet-induced HHcy, long pauses in the ECG traces were recorded, indicating the presence of conduction abnormalities. HHcy is associated with hypertension, coronary artery disease, and cardiac interstitial fibrosis²⁴. These risk factors induce structural and functional changes to the specialized conductive tissues as well as to the working myocardium, altering normal conduction. Conduction abnormalities increase the chance of creating substrate for the induction of ventricular tachycardia (VT) and possibly ventricular fibrillation (VFib). In the absence of intervention, VT degenerates into VFib and if not treated, ultimately sudden cardiac death ensues. Several studies associate elevated Hcy with sudden cardiac death^{9, 14, 16}.

Additionally, several studies assert an association between HHcy and atrial fibrillation^{25, 28}. Atrial fibrillation arises from extensive structural and electrical atrial remodelling. HHcy-induced MMP activation plays a role in the structural and electrical remodelling of the atria^{26, 27}. Following orthotopic heart transplantation, HHcy is

associated with altered atrial electrical conduction manifesting as P wave dispersion²⁸. Hcy also modulates sodium and potassium currents in human atrial myocytes^{29, 30}, highlighting another plausible mechanism by which elevated Hcy may initiate atrial arrhythmias.

My aim in this chapter was to investigate for the first time the electrophysiological characteristics of the cardiac conduction system in wild-type and hyperhomocysteinemic mice. The goal of these studies was to gain a better understanding of the effects of HHcy on sinoatrial and atrioventricular function. The hypothesis tested was that elevated plasma Hcy alters excitability of sinoatrial and atrioventricular nodal cells, resulting in abnormal nodal conduction.

Methods

Specific Aim 1 consisted of experiments performed on groups of 5 month old male C57BL/6J mice weighing 28-32g. In this set of studies, wild-type control mice (*WT*, total n=17) and long-term diet-induced HHcy mice receiving 400 mg/L of homocystine, the dimerized form of Hcy, for a period of 12 weeks in their drinking water (*DW*, total n=16) were used to characterize electrocardiographic and electrophysiologic variables, including ventricular refractoriness and arrhythmia induction.

Mice were housed 5 per cage and allowed *ad libitum* access to food and water in a facility with 12-hour light and dark cycles. All animals were housed and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Publication No. [NIH] 86-23), the Association for the Assessment and Accreditation of Laboratory Animal

Care, and by the Institutional Animal Care and Use Committee of the University of Louisville.

Electrocardiographic and EP Studies

For each procedure, animals were anesthetized with 2,2,2-tribromoethanol (400 mg/kg IP). Supplemental doses were administered as needed to maintain an adequate plane of anesthesia throughout the experiment. Anesthesia was monitored by checking the pedal reflex. Body temperature was maintained using a feedback system consisting of a rectal probe and a heating pad (Fine Science Tools, Foster City, CA). A lead II surface ECG was recorded during all procedures using subcutaneous needle electrodes, and RR, PR, QRS, and QT intervals were measured. Corrected QT (QTc) intervals were calculated using Bazett's formula⁸⁶. A right external jugular vein cannulation was performed to advance a 2-Fr octapolar catheter (CIBer mouse-EP; NuMED Inc., Hopkinton, NY) into the right atrium and ventricle. Intracardiac bipolar recordings were performed to verify catheter placement. Intracardiac electrogram recordings were obtained using a customized Biopac MP150 recording/stimulation system controlled by Acknowledge software (Biopac Systems Inc., Goleta, CA). The catheter was manipulated to allow bipolar atrial stimulation, and recording of signals with clear visualization of the His bundle spike. The AH and HV intervals of intracardiac electrograms were measured. EP studies were performed using standard atrial and ventricular programmed stimulation protocols that were modified to accommodate mouse heart rates (~600 bpm). Mice were first paced from the right atrium and then paced from the right ventricle. Stimulation was performed using square waves with a pulse width of 2ms and with DC current set at twice

the diastolic threshold. The recording sampling rate of signals was 2kHz. EP variables measured included: sinus node recovery time (SNRT), corrected SNRT (CSNRT), Wenckebach periodicity (WP), 2:1 AV block, AV nodal effective refractory period (AVNERP), right ventricular effective refractory period (RVERP) and arrhythmia induction using one and two premature ventricular stimuli. WP and 2:1 AV block were assessed using programmed stimulation protocols in which basic cycle trains were reduced by 2.5ms decrements starting at an interval rate just below the intrinsic heart rate. The basic cycle length of trains in effective refractory period protocols was 100ms (S1) and premature beats (S2) were serially reduced by 5ms decrements.

Blood Analysis

At the end of the EP studies, 300 to 500 μ L of blood were collected and centrifuged for 10 min at 10,000 rpm. The plasma was separated and frozen until analysis. Plasma Hcy was measured independently using a recombinant enzymatic cycling assay (Carolina Liquid Chemistries Corp., Winston-Salem, NC). The assay determines total plasma Hcy, which represents protein-bound Hcy plus free Hcy. Free Hcy encompasses the sulfhydryl form (HcyH), the dimerized form homocystine (Hcy-Hcy), and Hcy-cysteine mixed disulfides. Values of plasma levels are reported as mean $\pm$ SEM.

Statistical Analysis

Unpaired *t*-tests were performed to compare the means between groups for ECG and EP variables, as well as plasma Hcy values. Values are given as mean $\pm$ SEM for each group. A *P* value less than 0.05 was considered significant.

Results

In the first experiment, we evaluated the effects of HHcy on surface ECG intervals, intracardiac electrograms, sinoatrial function, AV nodal conduction and ventricular conduction. Tables 1 and 2 summarize findings from this experiment. Surface ECG analysis revealed that the RR (122.2 ± 3.6 ms vs. 141.0 ± 7.7 ms, $P < 0.05$), PR (46.6 ± 2.0 ms vs. 53.8 ± 2.6 ms, $P < 0.05$), and QT (56.7 ± 2.3 ms vs. 64.5 ± 1.9 ms, $P < 0.05$) intervals were significantly shortened in mice with HHcy compared to controls (Table 1). The intracardiac HV interval was significantly shortened compared to controls (8.1 ± 0.5 ms vs. 10.3 ± 0.5 ms, $P < 0.01$) while the AH interval was not significant (39.0 ± 1.8 ms vs. 44.0 ± 2.3 ms, $P = \text{NS}$) in HHcy mice compared to controls (Table 1). Figure 1 shows representative surface and intracardiac electrograms obtained from *WT* and *DW* mice, and the shorter PR, and HV intervals observed in *DW* mice. Programmed atrial stimulation revealed that in HHcy there was a significant shortening of CSNRT (30.9 ± 4.3 ms vs. 51.2 ± 3.0 ms, $P < 0.001$), WP (84.1 ± 2.3 ms vs. 95.9 ± 2.5 ms, $P < 0.01$), 2:1 AV block (73.8 ± 1.8 ms vs. 81.2 ± 2.4 ms, $P < 0.05$), AVNERP (55.9 ± 3.0 ms vs. 70.0 ± 3.2 ms, $P < 0.01$), and RVERP (38.1 ± 1.9 ms vs. 52.8 ± 3.3 ms, $P < 0.01$) as compared to controls (Table 2). Arrhythmia induction using one and two premature ventricular beats was

unsuccessful. Mean plasma levels of Hcy were $7.9 \pm 0.67 \mu\text{mol/L}$ in *WT* vs. $11.2 \pm 1.03 \mu\text{mol/L}$ in *DW* ($P < 0.05$); the latter is considered to be mildly elevated.

Table 1. Comparison of Surface ECG and Intracardiac Electrograms.

	WT Control (n=17)	DW (n=16)	P
RR interval (ms)	141.0±7.7	122.2±3.6	<0.05
HR (bpm)	444.4±21.9	497.6±15.4	NS
PR interval (ms)	53.8±2.6	46.6±2.0	<0.05
QRS duration(ms)	18.4±0.4	17.4±0.7	NS
QT interval (ms)	64.5±1.9	56.7±2.3	<0.05
QTc interval (ms)	54.6±0.8	51.4±2.1	NS
AH interval (ms)	44.0±2.3	39.0±1.8	NS
HV interval (ms)	10.3±0.5	8.1±0.5	<0.01
His duration (ms)	3.3±0.1	3.3±0.1	NS

WT, wild-type control; DW, mice receiving homocysteine (400mg/L) in drinking water;

HR, heart rate; NS, not significant. Values are mean ± SEM.

A. WT

B. DW

Figure 1. Representative surface ECGs and intracardiac His bundle electrograms (HBE) from: A) a wild type control (WT) mouse, and B) a mouse receiving homocysteine (400mg/L) in the drinking water for 12 weeks (DW). Notice shorter PR and HV intervals in mice receiving homocysteine compared to controls.

Table 2. Comparison of Atrial and Ventricular Programmed Stimulation Studies.

<i>Atrial Stimulation</i>			
	WT Control (n=17)	DW (n=16)	P
CSNRT (ms)	51.2±3.0	30.9±4.3	<0.001
WP (ms)	95.9±2.5	84.1±2.3	<0.01
2:1 AV Block (ms)	81.2±2.4	73.8±1.8	<0.05
AVNERP (ms)	70.0±3.2	55.9±3.0	<0.01
<i>Ventricular Stimulation</i>			
	WT Control (n=9)	DW (n=8)	P
RVERP (ms)	52.8±3.3	38.1±1.9	<0.01
Arrhythmia induction %	0	0	

WT, wild-type control; DW, mice receiving homocysteine (400mg/L) in drinking water; CSNRT, corrected sinus node recovery time; WP, Wenckebach periodicity; AVNERP, AV-nodal effective refractory period; RVERP, right ventricular effective refractory period. Arrhythmia induction was assessed using a train of 8 beats followed by two premature beats. Values are mean ± SEM.

Discussion

The major finding of this study was that mild, diet-induced HHcy in mice shortens the PR and HV intervals, suggesting accelerated conduction across the AVN. These findings were supported by EP data demonstrating that CSNRT, WP, 2:1 AV block, AVNERP, and RVERP were all significantly shorter in *DW* mice than in *WT* mice. These results indicate SA and AV nodal conduction abnormalities in mice with mild HHcy. There are several plausible mechanisms by which elevated Hcy may account for these changes. As discussed earlier, Hcy activates NMDA receptors, as well as modulates sodium and potassium channels. Activation of cardiac NMDA receptors, especially those at the SAN and AVN, allows more calcium influx, resulting in bathmotropic and dromotropic effects. High levels of Hcy increase sodium currents while inhibiting potassium currents in human atrial myocytes^{29, 30}. Increased sodium currents enhance cell excitability, while inhibition of potassium currents prolongs action potential duration (APD). HHcy-induced modulation of cardiac sodium and potassium channels, as well as cardiac NMDA receptor activity, may produce the changes observed in this study.

Additionally, HHcy induces oxidative stress by activating inducible nitric oxide synthase (iNOS) and NADPH oxidase^{7, 37, 38}. As discussed previously, increased levels of myocardial iNOS are associated with arrhythmogenesis and SCD⁸⁷. HHcy increases mitochondrial oxidative stress of cardiomyocytes, increasing levels of reactive oxygen species (ROS) which activate matrix metalloproteinases (MMPs) involved in extracellular matrix (ECM) remodelling⁸. Increased activation of MMPs is responsible for abnormal myocardial remodelling, which results in increased interstitial collagen associated with fibrosis⁷. Homocysteine-activated MMP-mediated cardiac remodelling

likely alters the expression, distribution, and regulation of cardiac channels and receptors, ultimately modifying electrical continuity between cardiac cells. Cardiac remodelling in patients with heart failure^{57, 58, 70}, myocardial infarction⁶⁰⁻⁶² and likely, HHcy, is associated with various types of arrhythmias and conduction abnormalities.

Impaired impulse conduction, as observed in this study, creates substrate for re-entrant arrhythmias⁵⁷. These arrhythmias require 2 parallel conducting pathways connected proximally and distally by conducting tissue, thus forming a potential re-entry or circular electrical circuit. For re-entry to occur 3 prerequisites need to be present: 1) one of the 2 pathways conducts more slowly, 2) the faster conducting pathway has a substantially longer refractory period, and, 3) an appropriately timed premature impulse is needed to initiate a continuous circular propagation of the impulse. Anatomically in the mouse and most mammals, the AVN is not a discrete entity, and the modern view describes it as a dual atrioventricular transmission system wherein diffuse fibers arising in the right atrium coalesce into two pathways which approach the central, compact body of the AVN^{79, 80}. The anterior pathway, which runs along the Tendon of Todaro, has been determined to be the fast (or normal) pathway with a longer refractory period. The posterior pathway, running along the tricuspid annulus, comprises the slow pathway with a shorter refractory period. The existence of dual pathways with differing conduction velocities has been hypothesized to be the substrate for AV nodal re-entrant supraventricular arrhythmias^{79, 81}

The mechanisms for accelerated nodal conduction were not elucidated in the study, and it remains to be determined whether SA and AV nodal conduction changes are due to HHcy-induced modulation of ion channel activity and/or MMP-induced cardiac

remodelling. To explore these mechanisms a multidisciplinary approach that includes electrophysiological studies in combination with molecular techniques will be needed to understand the underlying changes in conduction properties associated with HHcy.

Conclusions

The results of this study show acceleration of SA nodal and AV nodal conduction in HHcy. These findings suggest that acceleration of conduction has the potential to create substrate that ultimately may lead to arrhythmogenesis.

CHAPTER IV

HYPERHOMOCYSTEINEMIA-INDUCED CONDUCTION ABNORMALITIES ARE ASSOCIATED WITH CARDIAC NMDA RECEPTOR

Introduction

The NMDA receptor has been extensively studied in the CNS – its role in excitatory neurotransmission, synaptic plasticity, and memory formation has been well documented. It is known that homocysteine can activate the NMDA-R in the brain¹. Extraneuronal expression of NMDA receptors has been established in the heart, lungs, kidneys, gastrointestinal tract, ovaries, and testes². In the heart, the receptor has been identified in cardiomyocytes of the working myocardium, as well as in the specialized conductive tissues of the heart^{5, 6, 34}, however its function at these sites is poorly understood.

Recent studies characterizing the decrease in cardiomyocyte contractility that is observed in HHcy implicate a role for cardiac NMDA receptors. Use of MK-801, a specific non-competitive NMDA-R antagonist, attenuates the contractile dysfunction observed in HHcy⁸⁸. Further studies using cardiomyocyte-specific deletion of NMDA-Rs in the murine heart also show improvements in contractile function of isolated cardiomyocytes⁸⁹. Additionally, in remote telemetric ECG studies in hyperhomocysteinemic mice treated with MK-801, significant prolongation of RR, PR,

QRS, QT, and QTc is observed⁷⁴. Animals treated with Hcy alone or MK-801 alone have prolonged QT/QTc intervals over control animals, and animals treated with both Hcy and MK-801 together have even longer QT/QTc intervals. This may implicate homocysteine as well as the NMDA-R in the etiology of long QT syndrome. Taken together, it can be suggested that Hcy is acting on cardiac NMDA-Rs to effect the changes observed in HHcy.

My aim in this chapter was to investigate whether activation of cardiac NMDA receptors by Hcy underlies changes in conduction observed in diet-induced HHcy. I tested the hypothesis that elevated levels of Hcy increase activation of cardiac NMDA-Rs at the SA and AV nodes, altering sinoatrial and atrioventricular impulse conduction. The approach was to delineate a role for cardiac NMDA-Rs by comparing ECG and EP variables before and after NMDA-R blockade. A specific blocker (MK-801) and a non-specific blocker (MgSO₄) were selected to test the hypothesis, but ultimately Mg²⁺ blockade proved to have fewer confounding effects and was used in the study.

Materials and Methods

Study Design

Specific Aim 2 consisted of two sets of experiments performed on male C57BL/6J mice weighing 28-32g. In the first set of experiments, the feasibility of using an acute intraperitoneal infusion model of HHcy with specific NMDA-R blockade using MK-801 was explored. Hcy and MK-801 dose response studies were performed in wild-type C57BL/6J following baseline measurements of ECG and/or EP variables as described previously. ECG and EP variables were measured at 30-minute intervals for up

to 60 minutes. Minimal doses of Hcy eliciting conduction abnormalities and minimal doses of MK-801 which did not affect conduction were sought in these experiments. Once the minimal doses were established three experimental groups were studied: Hcy only group, MK-801 only group, and Hcy and MK-801 group where MK-801 was administered 30 minutes after Hcy administration. Blood was collected at the end of each experiment for plasma analysis as described previously.

In the second set of experiments, a long-term HHcy mouse model with non-specific NMDA-R blockade using MgSO_4 was utilized. Mg^{2+} is an endogenous blocker of the NMDA-R, a fact which gives rise to the receptor's voltage-dependence. In a pilot study, it was determined that a dose of 20mg/kg of MgSO_4 was ideal as it did not appear to have a confounding effect on mouse ECG variables. These experiments were performed on 20 week-old male C57BL/6J and heterozygous cystathionine β -synthase knockout mice (C57BL/6J-CBS^{tm1-Unc} The Jackson Laboratory, Bar Harbor, ME) mice weighing 28-32g. A paired-design study was performed on the following groups: wild-type controls (WT, n=8), diet-induced HHcy receiving 400 mg/L of homocystine in the drinking water for 12 weeks (DW, n=8), and C57BL/6J-CBS^{tm1-Unc} mice (CBS^{+/-}, n=7) were subjected to a baseline EP study followed by a repeat EP study 30 minutes after intraperitoneal (IP) administration of MgSO_4 (20mg/kg) in phosphate buffer saline (PBS).

Mice were housed 5 per cage and allowed *ad libitum* access to food and water in a facility with 12-hour light and dark cycles. All animals were housed and cared for in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services, NIH Publication No.

86-23), the Association for the Assessment and Accreditation of Laboratory Animal Care, and by the Institutional Animal Care and Use Committee of the University of Louisville.

Electrocardiographic and EP Studies

For each procedure, animals were anesthetized with 2,2,2-tribromoethanol (400 mg/kg IP). Supplemental doses were administered as needed to maintain adequate anesthesia throughout the experiment. Anesthesia was monitored by checking the pedal reflex. Body temperature was maintained using a feedback system consisting of a rectal probe and a heating pad (Fine Science Tools, Foster City, CA). A lead II surface ECG was recorded during all procedures using subcutaneous needle electrodes, and RR, PR, QRS, and QT intervals were measured. Corrected QT (QTc) intervals were calculated using Bazett's formula⁸⁶. A right external jugular vein cannulation was performed to advance a 2-Fr octapolar catheter (CIBer mouse-EP; NuMED Inc., Hopkinton, NY) into the right atrium and ventricle. Intracardiac bipolar recordings were performed to verify catheter placement. Intracardiac electrogram recordings were obtained using a customized Biopac MP150 recording/stimulation system running Acknowledge software (Biopac Systems Inc., Goleta, CA). The catheter was manipulated to allow bipolar atrial stimulation, and clear visualization of His bundle spike. The AH and HV intervals of intracardiac electrograms were measured. EP studies were performed using standard programmed stimulation protocols that were modified to accommodate mouse heart rates (~600 bpm). The heart was paced from the right atrium. Stimulation was performed using square waves with a pulse width of 2ms and with DC current set at twice the diastolic

threshold. The recording sampling rate of signals was 2kHz. EP variables measured include: sinus node recovery time (SNRT), corrected SNRT (CSNRT), Wenckebach periodicity (WP), 2:1 AV block, AV nodal effective refractory period (AVNERP), and arrhythmia induction using one and two premature ventricular stimuli. WP and 2:1 AV block were assessed using programmed stimulation protocols in which basic cycle trains were reduced by 2.5ms decrements. The basic cycle lengths of trains in AVNERP protocols in mice receiving Mg^{2+} were 100, 110, and 120ms, and were performed when heart rates allowed it. AVNERPs from the baseline and repeat EP studies were compared at the equivalent S1 cycle length. Premature beats (S2) in all studies were reduced by 5ms decrements.

Blood Analysis

At the end of the EP studies, 300 to 500 μ L of blood were collected and centrifuged for 10 min at 10,000 rpm. The plasma was separated and frozen until analysis. Plasma Mg^{2+} levels were measured using a clinical chemistry Beckman-Coulter DxC 800 instrument (Beckman-Coulter, Brea, CA). It should be pointed out that total Mg^{2+} levels (free + bound Mg^{2+}) were measured in the present study, however, free Mg^{2+} constitutes the active form. Values of plasma levels are reported as mean $\pm$ SEM. Hcy was measured independently using a recombinant enzymatic cycling assay (Carolina Liquid Chemistries Corp., Winston-Salem, NC). The assay determines total plasma Hcy, which represents protein-bound Hcy plus free Hcy. Free Hcy encompasses the sulfhydryl form (HcyH), the dimerized form homocystine (Hcy-Hcy), and Hcy-cysteine mixed disulfides. Values of plasma levels are reported as mean $\pm$ SEM.

Statistical Analysis

Single-factor ANOVA and unpaired *t*-tests were performed to compare the means between groups for ECG and EP variables, as well as plasma Hcy and Mg²⁺ values. Paired *t*-tests were performed to compare means within a group before and after Mg²⁺ administration. Values are given as mean $\pm$ SEM for each group. A *P* value less than 0.05 was considered significant.

Results

In the first set of experiments to determine minimal doses of Hcy eliciting conduction changes, acute intraperitoneal infusion of very high doses consisting of 100 and 200 mg/kg did not elicit any significant changes in PR interval, WP, 2:1 AV block, and AVNERP at 30 and 60 minutes. These findings are illustrated in Figures 2 and 3. Conversely, in experiments using the NMDA-R blocker MK-801, extremely pronounced changes in the PR interval, WP, 2:1 AV block, and AVNERP were observed following administration of blocker at very low doses (0.05, 0.16, or 0.25 mg/kg). These changes are illustrated in Figures 4 and 5.

Figure 2. PR interval (top panel) and Wenckebach periodicity (bottom panel) in wild-type C57BL6 mice at 30 and 60 minutes following acute intraperitoneal administration of 100 or 200 mg/kg homocysteine

Figure 3. 2:1 AV block (top panel) and AV nodal effective refractory period (AVNERP, bottom panel) in wild-type C57BL6 mice at 30 and 60 minutes following acute intraperitoneal administration of 100 or 200 mg/kg homocysteine

Figure 4. PR interval (top panel) and Wenckebach periodicity (bottom panel) in wild-type C57BL6 mice 30 minutes following acute intraperitoneal administration of 0.05, 0.16, or 0.25 mg/kg MK801, a non-competitive NMDA receptor antagonist.

Figure 5. 2:1 AV block (top panel) and AV nodal effective refractory period (AVNERP, bottom panel) in wild-type C57BL6 mice 30 minutes following acute intraperitoneal administration of 0.05, 0.16, or 0.25 mg/kg MK801, a non-competitive NMDA receptor antagonist.

In the second set of experiments, surface ECG and intracardiac electrogram changes before and after intraperitoneal administration of MgSO_4 (20mg/kg) in *WT*, *DW* and *CBS*^{+/-} groups are shown in Figures 6-8. Administration of Mg^{2+} increased duration of RR, PR, QRS and QTc intervals, as well as AH and HV intervals in *DW*, but had little to no effect on *CBS*^{+/-} and *WT* controls. Figure 9 shows EP variables before and after intraperitoneal Mg^{2+} in all three groups. Mg^{2+} administration had no effect on *WT* animals, but had a marked effect on both the *DW* and *CBS*^{+/-} animals. WP, 2:1 AV block, and AVNERP were all significantly prolonged following administration of MgSO_4 in *DW* and *CBS*^{+/-} animals, and CSNRT was significantly prolonged in *DW* mice.

There was no significant difference between Mg^{2+} plasma levels in all groups from blood samples collected at the end of EP studies. Normal Mg^{2+} plasma levels in wild-type animals before receiving intraperitoneal Mg^{2+} were $1.50 \pm 0.20 \text{ mmol/L}$. In animals receiving intraperitoneal Mg^{2+} the levels were $1.65 \pm 0.24 \text{ mmol/L}$ in *WT*, $1.71 \pm 0.22 \text{ mmol/L}$ in *DW*, and $1.40 \pm 0.17 \text{ mmol/L}$ in *CBS*^{+/-}. There were significant differences in plasma Hcy levels between *WT* mice ($8.43 \pm 2.75 \mu\text{mol/L}$), *DW* mice ($11.16 \pm 2.91 \mu\text{mol/L}$, $P < 0.02$ compared to *WT*) and *CBS*^{+/-} mice ($13.04 \pm 4.49 \mu\text{mol/L}$, $P < 0.02$ compared to *WT*). There was no difference in Hcy levels between *DW* and *CBS*^{+/-} mice.

A.

B.

Figure 6. A. Surface ECG variables in wild-type (WT) animals at baseline (Pre-Mg²⁺) and following MgSO₄ administration (Post-Mg²⁺). B. Intracardiac ECG variables in WT animals at baseline (Pre-Mg²⁺) and following MgSO₄ administration (Post-Mg²⁺). AH= Intracardiac interval measuring time between atrial depolarization and depolarization of the His bundle. HV= Intracardiac interval measuring infra-Hisian conduction.

A.

B.

Figure 7. A. Surface ECG variables in diet-induced hyperhomocysteinemia (DW) animals at baseline (Pre-Mg²⁺) and following MgSO₄ administration (Post-Mg²⁺). **B.** Intracardiac ECG variables in DW animals at baseline (Pre-Mg²⁺) and following MgSO₄ administration (Post-Mg²⁺). AH= Intracardiac interval measuring time between atrial depolarization and depolarization of the His bundle. HV= Intracardiac interval measuring infra-Hisian conduction.

A.

B.

Figure 8. A. Surface ECG variables in genetically-induced hyperhomocysteinemia ($CBS^{+/-}$) animals at baseline (Pre-Mg²⁺) and after 30 minutes following MgSO₄ administration (Post-Mg²⁺). **B.** Intracardiac ECG variables in $CBS^{+/-}$ animals at baseline (Pre-Mg²⁺) and after 30 minutes following MgSO₄ administration (Post-Mg²⁺). AH= Intracardiac interval measuring time between atrial depolarization and depolarization of the His bundle. HV= Intracardiac interval measuring infra-Hisian conduction.

A.

B.

C.

Figure 9. EP variables in wild-type (WT), diet-induced HHcy (DW), and genetically-induced HHcy (CBS^{+/-}) animals at baseline (Pre-Mg²⁺) and following MgSO₄ administration (Post-Mg²⁺)

Discussion

The major finding of this study was that administration of the non-specific, endogenous NMDA-R antagonist MgSO_4 prolonged AV nodal conduction in both diet-induced and genetically-induced HHcy, but not in wild-type animals. Additionally, an attempt to establish an acute model of HHcy via intraperitoneal infusion of Hcy was not successful in replicating results obtained from using chronic models of HHcy (such as dietary intake of Hcy for 12 weeks, or by genetic CBS knockout). No changes in ECG or EP variables were observed following intraperitoneal administration of high levels of Hcy, even after 120 minutes post-administration. These findings suggest that acute infusion of Hcy does not elicit the effects observed in our long-term models of chronic HHcy. Indeed, a recent report demonstrates that Hcy accumulation in tissues does not necessarily reflect plasma levels, and therefore tissue levels must be considered when analyzing the effects of plasma Hcy on target organs¹².

To delineate a role for cardiac NMDA-Rs in HHcy-induced conduction abnormalities, MK-801, a specific and noncompetitive NMDA-R antagonist, was utilized. MK-801 produces sustained elevations in mean arterial pressure and heart rate when administered systemically at therapeutic doses⁹⁰. Pilot studies were undertaken to determine a low dose of MK-801 which would not alter ECG and EP variables too drastically; however, at doses considerably lower than published therapeutic or experimental doses, the heart rate slowed considerably, suggesting that the blocker was inducing central effects. The use of alternative specific NMDA-R blockers (like 2-amino-5-phosphonovaleric acid and memantine) was avoided because they would induce similar effects as MK-801, affecting cardiovascular reflexes and neural tone⁹⁰⁻⁹², which would

confound results from EP studies.

The evidence presented regarding the role of the NMDA-R in shortening AV conduction time is based on the effects of Mg^{2+} , a known NMDA-R modulator. Electrophysiological effects of Mg^{2+} have been known clinically for over 70 years. Mg^{2+} has been used in the management of patients with torsades de pointes, atrial flutter, paroxysmal AV re-entrant supraventricular tachycardia and Wolff-Parkinson-White (WPW) syndrome^{93, 94}. In the present study, low-dose Mg^{2+} blockade was selected based on results from pilot studies. In these studies, the lowest Mg^{2+} dose (90mg/kg) reported to block central NMDA-Rs in rodents⁹⁵ produced severe bradycardia in the C57BL/6J mice used in the present study; thus, the Mg^{2+} dose was further lowered to 20mg/kg, to the point where no discernible effects on heart rate or blood pressure were observed. Mg^{2+} blood levels at the end of the second EP study were in the normal range indicating that peak levels following IP administration were not excessively high. In the present study, this dose had no effect on ECG and EP variables in *WT* mice. Pilot studies determined that plasma Mg^{2+} levels peaked between 40 and 50 minutes following IP administration. EP studies were started 30 minutes post-administration to coincide with peak Mg^{2+} blood levels. Elevation of extracellular Mg^{2+} interferes with the release of the Mg^{2+} channel block present in NMDA receptors, and thus reduces inward Ca^{2+} flow via this channel⁹⁵. In most human studies, Mg^{2+} administration increases cardiac conduction time to some degree; however, there is disagreement as to which conduction variables are affected. Rassmussen et al. and DiCarlo et al. demonstrate that Mg^{2+} treatment prolongs PR interval, QRS duration, AV nodal (AH) conduction time and AV nodal functional refractory period, but has no effect on sinus node function^{96, 97}. Alternatively, Kulick et

al. demonstrate that Mg^{2+} administration prolongs PR interval, AH interval, and AV nodal effective refractory period but has no significant effect on QRS duration, QT interval, HV interval, WP, and AVNERP⁹⁸. Differences reported in these studies can be attributed to the lower plasma Mg^{2+} levels achieved by Kulick's group. Indeed, in canine studies, low-dose Mg^{2+} administration elicited few conduction changes, but as dosing and levels of serum Mg^{2+} increase (up to 6.5 ± 2.1 mmol/L), a progressive prolongation in AH interval, HV interval, QRS duration, WP, AVNERP and RR cycle length is observed, while arterial blood pressure falls⁹⁹. Thus, low-dose administration of Mg^{2+} appears to have little effect on cardiac conduction and EP variables. The Mg^{2+} dose used in the present study appears to unmask Hcy-induced effects via cardiac NMDA-Rs because changes in conduction were only observed in mice with HHcy but not in *WT* mice receiving the same therapy.

The findings from the present study provide modest support to the hypothesis that increased levels of Hcy overstimulate cardiac NMDA-Rs, causing an increase in intracellular calcium $[Ca^{2+}]_i$ during depolarization, leading to increased nodal cell excitation and AV nodal conduction. This hypothesis was based on literature indicating that activation of NMDA-Rs by excitatory amino acids increases intracellular calcium $[Ca^{2+}]_i$, which in some cases leads to Ca^{2+} overload^{6, 100, 101}. Hcy increases NMDA-R activity by interacting with both glycine and glutamate binding sites, and the activation is enhanced when glycine levels are above normal¹. Hcy-induced NMDA-R activation increases cell excitability in various cell types^{3, 4, 88, 89, 102, 103}, and the results from the present study suggest that cardiac NMDA-Rs behave similarly, particularly those expressed in AV nodal cells. HHcy is known to increase $[Ca^{2+}]_i$ in various cells

expressing NMDA-Rs, including neural cells^{1, 103}, smooth muscle cells¹⁰², endothelial cells^{3, 4}, and cardiomyocytes^{88, 89}. Although the NMDA-R is expressed in cells of the AV node, bundle of His and Purkinje fiber system, this receptor's role in normal or pathologic cardiac conduction is largely unknown. Future studies using cardiac conduction system-restricted NMDA-R knockout mice will be necessary to elucidate more specifically the mechanisms by which HHcy alters atrioventricular conduction.

Conclusions

The mechanisms associated with conduction changes observed in mild HHcy appear to involve stimulation of cardiac NMDA-Rs by Hcy. Blockade of NMDA-R with low-dose Mg^{2+} prolonged conduction time in animals with HHcy but had no effect on normal controls. The results of this study provide evidence that atrioventricular conduction abnormalities associated with HHcy may involve increased stimulation of AV nodal cardiac NMDA-Rs, which may provide substrate for arrhythmogenesis.

CHAPTER V

CARDIAC NMDA RECEPTOR EXPRESSION IN MILD HYPERHOMOCYSTEINEMIA

Introduction

Modulation of NMDA receptors in different tissues is associated with various pathologic conditions including HHcy. Ethanol is a potent, selective inhibitor of NMDA-R function, and chronic ethanol consumption causes an adaptive upregulation of central NMDA-R mRNA transcription as well as protein expression^{104, 105}. In gentamicin-induced nephrotoxicity, both mRNA and protein levels of the obligatory NR1 subunit of kidney NMDA-R are increased in renal proximal tubules, and treatment with the NMDA-R antagonist MK-801 ameliorates tubular injury¹⁰⁶. Several lines of evidence demonstrate modulation of NMDA-R expression in HHcy. Elevated homocysteine upregulates expression of NMDA receptor NR1 subunit mRNA and protein in rat aortic endothelial cells³ and rat aortic smooth muscle cells⁴. In uni-nephrectomized rats fed a folate-free diet for 6 weeks to induce HHcy, glomerular NR1 and NR2C subunits are upregulated, an effect that is attenuated by MK-801¹⁰⁷. Chronic HHcy also enhances long-term potentiation (LTP) in rat hippocampus, suggesting an upregulation of NMDA-R expression¹⁰⁸. In the brain, LTP underlies the processes of learning and memory formation, and is mediated by NMDA-R activity.

In this chapter, I examined whether the electrophysiological changes observed in mild HHcy are in part explained by differences in cardiac NMDA-R expression. My aim was to investigate cardiac NMDA-R expression levels by Western blot analysis of total NMDA receptor NR1 subunit expression in whole heart, as well as to characterize NR1 expression at the murine AV node by immunohistochemical analysis. I hypothesized that NMDA-R expression levels in the heart, and in particular at the AV node, differ based on duration of exposure to elevated Hcy. To test this hypothesis, levels of cardiac NMDA-R expression were quantified, and NR1 subunit expression at the AV node was characterized.

Methods

Study Design

Specific Aim 3 consisted of two sets of experiments performed on male C57BL/6J mice weighing 28-32g. In the first set of experiments, total cardiac expression of NR1 was studied in three experimental groups of animals: wild-type control (*WT*, n=3), diet-induced HHcy receiving 400 mg/L of homocystine in the drinking water for 12 weeks (*DW*, n=3), and heterozygous CBS knockouts (*CBS*^{+/-}, n=3). In the second set of experiments, wild-type (*WT*, n=4) C57BL/6J mice were utilized for immunohistochemical identification of NMDA receptor NR1 subunit at the murine AV node.

Western Blotting

Hearts from *WT*, *DW*, and *CBS^{+/-}* mice were harvested at the end of the experiment and frozen in liquid nitrogen. Cardiac tissue homogenates were prepared from whole hearts using protein extraction buffer (RIPA buffer, Sigma Aldrich, St. Louis, MO). The extracted protein was collected by centrifugation at 1500 g for 10 minutes. The supernatant was collected and centrifuged again until the aqueous portion was clear. Protein content was estimated using a BCA protein assay kit (Thermo Scientific, Rockford, IL). Equal amounts of protein were separated by SDS-PAGE on 12% Tris-glycine gels (Bio-Rad), transferred onto PVDF membrane overnight, and probed with a polyclonal anti-NR1 antibody (Abcam Inc., Cambridge, MA) conjugated with horseradish peroxidase (HRP). Enhanced chemiluminescence reagents were applied and band intensity was recorded and analyzed with a Kodak image analyzer (Perkin-Elmer, Waltham, MA)

Immunohistochemistry

Hearts from *WT* mice were harvested and fixed by coronary perfusion with 8% paraformaldehyde. The free wall of the right ventricle was removed, as was most of the right atrium, in order to gain access to the AV junction. Tissue samples were stored in 8% paraformaldehyde until they were embedded in paraffin (a suture was tied to the apex of the heart for orientation), and sectioned transversely beginning at a level just superior of the tricuspid valve. HRP-conjugated polyclonal anti-NR1 antibody (Abcam Inc., Cambridge, MA) was used to probe for NMDA-R expression. Diaminobenzidine (DAB) was used as a chromogen.

Statistics

Single-factor ANOVA and unpaired *t*-tests were performed to test for significance between groups. Values are given as mean $\pm$ SEM for each group. A *P* value less than 0.05 was considered significant.

Results

To characterize and compare cardiac NMDA-R expression in hearts from the three experimental groups, Western blotting for NMDA receptor NR1 subunit in whole heart was performed. Western blotting analysis showed a significant decrease of NR1 expression in *CBS*^{+/-} hearts compared to *WT* hearts (*P*<0.05) and *DW* hearts (*P*<0.05). Figure 10 shows the densitometric means from the three groups.

In order to identify NMDA-R NR1 subunit expression at the murine AV node, immunohistochemical analysis of dissected AV junction tissue was undertaken. Figures 11-12 show representative images of sections of AV tissue stained with hematoxylin and eosin, and probed for NR1 expression. Positive immunoreactivity for NR1 is indicated by brown punctate staining as well as diffuse brown staining of tissue. These results were difficult to interpret since the AV node is not a discrete structure that is easily identified due to the small size of mouse heart.

Figure 10. Relative abundance of NR1 subunit of NMDA receptor in wild-type (WT, n=3), genetically-induced hyperhomocysteinemic (CBS^{+/-}, n=3), and diet-induced hyperhomocysteinemic (DW, n=3) mouse hearts. NR1 expression in CBS^{+/-} is significantly decreased compared to WT as well as DW.

Figure 11. Tissue at the murine atrioventricular junction stained with hematoxylin and eosin and probed with anti-NR1 polyclonal antibody conjugated with horseradish peroxidase. Diaminobenzidine was used as a chromogen. 20X magnification shown. Positive immunoreactivity for NR1 is indicated by brown punctate staining as well as diffuse brown staining of cardiomyocytes (arrows).

Figure 12. Tissue at the murine atrioventricular junction stained with hematoxylin and eosin and probed with anti-NR1 polyclonal antibody conjugated with horseradish peroxidase. Diaminobenzidine was used as a chromogen. 40X magnification shown. Positive immunoreactivity for NR1 is indicated by brown punctate staining (arrows).

Discussion

The major finding from this study was that in life-long, genetically-induced HHcy, the cardiac NMDA-R appears to be down-regulated compared to *WT* animals as well as animals with diet-induced HHcy. The difference in NMDA-R NR1 subunit expression between the *CBS*^{+/-} and *DW* groups may be associated with the duration of exposure to elevated Hcy, life-long versus 12 weeks respectively. The results suggest that life-long HHcy in *CBS*^{+/-} mice induces a down-regulation of cardiac NMDA-R expression. Interestingly, plasma Hcy levels were similar in *CBS*^{+/-} and *DW* groups; however down-regulation of cardiac NMDA-R NR1 subunit expression was only observed in *CBS*^{+/-}. Moreover, NR1 expression was significantly higher in *DW* compared to *CBS*^{+/-}. In the literature, conflicting evidence exists regarding the effect of increased agonism of NMDA-R in different cells/tissues on receptor expression. A rapid down-regulation (within 3 hours) of NMDA-R NR1 subunit occurs when cerebellar granule cells are treated with NMDA, the most potent agonist of the receptor¹⁰⁹. Other studies demonstrate upregulation of NR1 in HHcy. In cultured rat aortic endothelial cells, simulating moderate levels of HHcy (50μM of Hcy) causes increasing NR1 subunit expression that peaks at 6 hours³. In rat vascular smooth muscle cells, moderate levels of HHcy produce an increase in expression of NR1 which peaks at 12 hours post-treatment⁴. *In vivo* studies using uni-nephrectomized rats fed a folate-free diet for 6 weeks to induce mild HHcy demonstrate upregulated glomerular NR1 expression¹⁰⁷. Based on these studies, the expectation was that an upregulation of cardiac NMDA-R would occur in *CBS*^{+/-} and *DW* animals. However, a down-regulation was observed in *CBS*^{+/-} animals compared to *WT*, and no change was observed in *DW* animals compared to *WT* animals.

A plausible explanation for the findings in *CBS*^{+/-} mice is that in life-long HHcy, a compensatory down-regulation of NMDA-R expression in the heart may occur during early embryonic development, and expression remains down-regulated throughout adulthood. An explanation as to why *DW* animals did not demonstrate upregulation of NR1 may be related to the lower plasma levels of Hcy achieved in *DW* mice (11.36±1.03 µM) compared to those reached (16µM) in uni-nephrectomized rats fed a folate-free diet, which did exhibit upregulation of glomerular NR1 expression. Thus it appears that receptor agonism can induce both upregulation and/or down-regulation of NMDA-R expression in different tissues, and is dependent on the agonist used as well as the duration of exposure to the agonist. Elevated Hcy primarily upregulates short-term receptor expression, but modulation may be dependent on plasma Hcy levels. Furthermore, modulation of receptor expression may be dependent on tissue type; in the present study, expression of NMDA-R in heart may exhibit decreased propensity towards upregulation as compared to the kidney, as observed in animals fed a folate-free diet for 6 weeks¹⁰⁷.

NR1 expression in the cardiac conduction system has been successfully characterized in larger mammals, such as rat, primate, and human. The larger size of the hearts in these animals precludes the difficulties associated with micro-dissection of a very small region of tissue. Prior immunohistochemical characterization of NR1 expression at the murine AV node had not been accomplished, possibly due to the small size of the mouse heart and the technical challenges inherent in micro-dissection of murine AV nodal tissue. Notwithstanding this challenge, to this author's knowledge this is the first attempt to identify NMDA-R expression at the AV junction in mouse heart.

Conclusions

Expression levels of cardiac NMDA-R were significantly decreased in genetically-induced HHcy, compared to diet-induced HHcy and controls. It appears that the electrophysiological changes observed in animals with HHcy are not due to receptor modulation, since the electrophysiologically-similar *CBS*^{+/-} and *DW* animals exhibited significantly dissimilar cardiac NMDA-R expression levels.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Hyperhomocysteinemia (HHcy) is a risk factor for cardiac arrhythmogenesis. Increased plasma homocysteine (Hcy) accelerates sinoatrial and atrioventricular conduction, as measured by electrophysiological studies. These findings suggest that acceleration of conduction has the potential to create substrate which ultimately may lead to arrhythmogenesis.

The mechanisms associated with conduction changes observed in mild HHcy appear to involve stimulation of cardiac N-methyl-D-aspartate receptors (NMDA-Rs) by Hcy. Blockade of NMDA-R with low-dose Mg^{2+} prolonged conduction time in animals with HHcy but had no effect on normal controls. Sinoatrial and atrioventricular conduction abnormalities associated with HHcy may involve increased stimulation of nodal cardiac NMDA-Rs, which may provide substrate for arrhythmogenesis.

Expression levels of cardiac NMDA-R were significantly decreased in genetically-induced HHcy, compared to diet-induced HHcy and controls. It appears that the electrophysiological changes observed in animals with HHcy are not due to receptor modulation, since the electrophysiologically-similar *CBS*^{+/-} and *DW* animals exhibited significantly dissimilar cardiac NMDA-R expression levels.

Future investigation is necessary to unequivocally determine the role of cardiac NMDA-R in Hcy-induced arrhythmias. Conditional knock-out technology, wherein genetic deletion of the protein of interest can be limited to one organ or one tissue, may be used to further delineate the role of cardiac NMDA-R in normal or pathological cardiac conduction. Additionally, it is necessary to investigate all known pathways in which Hcy exerts an influence. It may be through a direct interaction with a receptor like the cardiac NMDA-R, or through indirect means involving activation of MMPs and subsequent cardiac remodelling, that Hcy is able to evoke conduction abnormalities which underlie arrhythmogenesis.

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CURRICULUM VITAE

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A. Education

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B. Employment

Research Technician, Children's Memorial Hospital, Northwestern University
10/1999-8/2003

Research Technologist, Department of Physiology and Biophysics, University of
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C. Awards

1st Prize Winner, Research!Louisville 2004, Medical Student 2nd -4th Year Division
Khan M, Grossi FV, **Soni CV**, Delamere N, Okafor M, Maldonado C. Delivery of exogenous ATP preserves Na⁺, K⁺ pump function during chemical hypoxia. 8th Annual Research!Louisville, Louisville, KY. November 3-7, 2004.

1st Prize Winner, Research!Louisville 2008, Research Staff Division
Soni C, Todnem ND, Pushpakumar S, Tyagi S, Maldonado C. High levels of dietary homocysteine accelerate impulse propagation across the murine atrioventricular node. 12th Annual Research!Louisville, Louisville, KY, October 20-24, 2008.

1st Prize Winner, Research!Louisville 2010, Post-Doctoral Fellows Division
Pushpakumar SB, Perez-Abadia G, Todnem ND, **Soni CV**, Fensterer TF, Patibandla P, Barker JH, Maldonado C. Enhancing Complement Control on The Endothelial Barrier Reduces Renal Dysfunction Following Ischemia Reperfusion Injury. 14th Annual Research!Louisville, October 11- 5, 2010, Louisville, KY.

D. Presentations

1. Khan M, Grossi FV, **Soni CV**, Delamere N, Okafor M, Maldonado C. Delivery of exogenous ATP preserves Na⁺, K⁺ pump function during chemical hypoxia. 8th Annual Research!Louisville, Louisville, KY. November 3-7, 2004.
2. Maldonado C, Grossi FV, Khan M, **Soni C**, Okafor MC, Vasilic D, Ehringer WC, Perez-Abadia G, Delamere NA, Barker JH. Delivery of exogenous ATP maintains sodium-potassium pump activity in endothelial cells during chemical hypoxia. American College of Surgeons, 91st Annual Clinical Congress, San Francisco, CA. October 16-20, 2005.
3. Maldonado C, Moshal K, Todnem N, **Soni C**, Tyagi SC. High levels of dietary homocysteine (Hcy) accelerate impulse propagation across the murine atrioventricular node (AVN). Presented at Federation of American Societies for Experimental Biology, San Diego, CA, April 4-9, 2008.
4. **Soni C**, Todnem ND, Pushpakumar S, Tyagi S, Maldonado C. High levels of dietary homocysteine accelerate impulse propagation across the murine atrioventricular node. 12th Annual Research!Louisville, Louisville, KY, October 20-24, 2008.
5. Pushpakumar SB, Perez-Abadia G, Todnem ND, **Soni CV**, Fensterer TF, Patibandla PK, Barker JH, Maldonado C. Endothelium Targeted Anti-Complement Therapy Protects Renal Function Following Ischemia and Reperfusion Injury. Presented at 13th Annual Research!Louisville, Louisville, KY November 2009
6. Fensterer TF, Maldonado C, Perez-Abadia G, Pushpakumar S, **Soni CV**, Patibandla P, Todnem ND. Department of Physiology. A Novel Cuff Design to Facilitate Vessel Anastomosis for Cervical Heart Transplantation in Rodents. 13th Annual Research!Louisville, Louisville, KY. November 2009
7. Todnem ND, Maldonado C, **Soni CV**, Givvimani S, Pushpakumar SB, Fernandes TF, Villafane J, Tyagi SC. Elevated Homocysteine in the Blood Increases AV Nodal Conduction: Role of the Cardiac NMDA Receptor. 13th Annual Research!Louisville, Louisville, KY. November 2009.
8. Perez-Abadia G, Goga L, Pushpakumar S, Olson P, Anderson G, **Soni CV**, Barker JH, Maldonado C. Characterization of a novel liposome based therapy to reduce complement deposition on cell membranes. Presented at Federation of American Societies for Experimental Biology, Anaheim, CA, April 24-28, 2010.
9. Claudio Maldonado, Nathan D. Todnem, **Chirag V. Soni**, Srikanth Givvimani, Sathnur Pushpakumar, Phani K. Patibandla, Suresh C. Tyagi. Mild hyperhomocysteinemia increases atrioventricular nodal conduction: Role of the

cardiac NMDA receptor. Presented at Federation of American Societies for Experimental Biology, Anaheim, CA, April 24-28, 2010.

10. **Soni CV**, Maldonado C, Todnem ND, Givvimani S, Pushpakumar SB, Patibandla P, Villafane J, Tyagi SC. Hyperhomocysteinemia Alters Sinoatrial and Atrioventricular Nodal Function: Role of the Cardiac NMDA Receptor. 14th Annual Research!Louisville, October 11-15, 2010, Louisville, KY.
11. Pushpakumar SB, Perez-Abadia G, Todnem ND, **Soni CV**, Fensterer TF, Patibandla P, Barker JH, Maldonado C. Enhancing Complement Control on The Endothelial Barrier Reduces Renal Dysfunction Following Ischemia Reperfusion Injury. 14th Annual Research!Louisville, October 11- 5, 2010, Louisville, KY.
12. Fensterer TF, Patibandla P, Perez-Abadia G, David Song, BS, Pushpakumar SB, **Soni CV**, Claudio Maldonado, PhD. Complement Inactivation in Heart Transplantation. 14th Annual Research!Louisville, October 11-15, 2010, Louisville, KY.
13. Patibandla P, Fensterer TF, Perez-Abadia G, Pushpakumar SB, **Soni CV**, Harrison A, Maldonado C. Enhancing Complement Control to Protect the Endothelial Cell Injury. 14th Annual Research!Louisville, October 11-15, 2010, Louisville, KY.

E. Publications

1. Maldonado C, **Soni CV**, Todnem ND, Pushpakumar S, Rosenberger D, Givvimani S, Villafane J, Tyagi SC. [Hyperhomocysteinemia and sudden cardiac death: potential arrhythmogenic mechanisms](#). Curr Vasc Pharmacol. 2010 Jan;8(1):64-74.
2. **Soni CV**, Barker JH, Pushpakumar SB, Furr LA, Cunningham M, Banis JC Jr, Frank J. [Psychosocial considerations in facial transplantation](#). Burns. 2010 Nov;36(7):959-64. Epub 2010 Apr 7.
3. Pushpakumar SB, Barker JH, **Soni CV**, Joseph H, van Aalst VC, Banis JC, Frank J. [Clinical considerations in face transplantation](#). Burns. 2010 Nov;36(7):951-8. Epub 2010 Apr 21.
4. Goga L, Perez-Abadia G, Pushpakumar SB, Cramer D, Yan J, Todnem N, Anderson G, **Soni C**, Barker J, Maldonado C. [Cell membrane modification for rapid display of bi-functional peptides: a novel approach to reduce complement activation](#). Open Cardiovasc Med J. 2010 Jul 20;4:157-65.
5. Goga L, Pushpakumar SB, Perez-Abadia G, Olson P, Anderson G, **Soni CV**, Barker JH, Maldonado C. [A novel liposome-based therapy to reduce complement-](#)

[mediated injury in revascularized tissues.](#) J Surg Res. 2011 Jan;165(1):e51-7. Epub 2010 Oct 16.

6. Pushpakumar SB, Perez-Abadia G, **Soni C**, Wan R, Todnem N, Patibandla PK, Fensterer T, Zhang Q, Barker JH, Maldonado C. [Enhancing complement control on endothelial barrier reduces renal post-ischemia dysfunction.](#) J Surg Res. 2011 Oct;170(2):e263-70. Epub 2011 Jul 7.