mRNA VARIANTS OF A NEURONAL SODIUM CHANNEL IN RAT CARDIAC MYOCYTES

by

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Abstract

Death due to ischemic heart disease (IHD) is the result of cardiac arrhythmias and loss of cardiac pump function. One of the putative underlying mechanisms is impaired inactivation of voltage-gated sodium channels (VGSC), resulting in small persistent sodium currents. Nine VGSC (Na\textsubscript{v}1.1-Na\textsubscript{v}1.9) have been cloned and functionally expressed. Electrophysiological/pharmacological evidence suggests that “neuronal” isoform(s) exist in cardiomyocytes along with the cardiac-dominant Na\textsubscript{v}1.5. Given that persistent currents have been shown to be fundamental to the function of neuronal isoforms and that pharmacological evidence suggests that neuronal VGSC underlie increases in persistent currents during ischemic events, we hypothesized that neuronal VGSC are present in cardiomyocytes. Specifically, the purpose of this study was to demonstrate that the neuronal VGSC Na\textsubscript{v}1.1 exists in rat right ventricular myocytes.

The full-length Na\textsubscript{v}1.1 coding sequence was cloned in overlapping segments. Through sequencing, we identified one amino acid difference from a published brain sequence (c.2935A>G) and four deletion variants (c.[del266_473], c.[del2012_2044], c.[del4004_4258], and c.[del4003_4284]). The deletion variants were not present in all sequenced amplicons. Deletions at the first and the third deletion sites were of particular interest as these involved regions of conserved sequence, likely essential to channel function. To explore this further, PCR primers were designed to amplify a partial transcript spanning both of these regions. Three RT-PCR bands were produced: 1) an ~4500 bp band that is likely a composite of two amplicons, one with a deletion at the first deletion site (c.[del266_473]) and one with a deletion at the third deletion site (either c.[del4004_4258] or c.[del4003_4284]); 2) an ~4200 bp band confirmed to contain an amplicon with a deletion at both the first and the third deletion sites (either c.[del266_473] and c.[del4004_4258], or c.[del266_473] and c.[del4003_4284]); and 3) an ~4075 bp band suggesting further unidentified deletion variants. We were unable to identify any amplicons that did not contain at least one deletion involving conserved sequence.
Overall, these results demonstrate that multiple sequence variances of Na\textsubscript{v}1.1 exist in adult rat cardiomyocytes. Moreover it appears that under normal cellular conditions, at least one deletion that is likely to be devastating to channel function is always present.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>APD</td>
<td>action potential duration</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>bp</td>
<td>basepair</td>
</tr>
<tr>
<td>Br</td>
<td>brain</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy deoxyribonucleic acid</td>
</tr>
<tr>
<td>D-#</td>
<td>digestion cloning primer pair #1, #2, #3….</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DI, DII, DIII, DIV</td>
<td>VGSC domains</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
</tr>
<tr>
<td>Ht</td>
<td>heart</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>IHD</td>
<td>ischemic heart disease</td>
</tr>
<tr>
<td>IₙaP</td>
<td>persistent sodium currents</td>
</tr>
<tr>
<td>K₂ATP</td>
<td>dipotassium ATP</td>
</tr>
<tr>
<td>KCL</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>potassium phosphate</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
</tbody>
</table>
MgSO₄ magnesium sulfate
MI myocardial infarction
MOPS 3-(N-morpholino) propansulfonic acid
MTSEA methanethiosulfonate reagents
NaCl sodium chloride
Na₂HPO₄ sodium phosphate
NaOH sodium hydroxide
Naᵥ¹.x isoforms of voltage-gated sodium channels
ng nanogram
NHE sarcolemmal proton pump exchanger
NMD nonsense-mediated decay
NP-# nested primer pair #1, #2, #3…
O₂ oxygen
PCR polymerase chain reaction
PKC protein kinase C
RNA ribonucleic acid
ROS reactive oxygen species
RT reverse transcription
RT-PCR reverse transcription polymerase chain reaction
S₁, S₂, S₃, S₄, S₅, S₆ VGSC transmembrane segments
TA-# TA cloning primer pair #1, #2, #3…
TTX tetrodotoxin
µl microlitre
UTE untranslated exon
UTR untranslated region
VGSC voltage-gated sodium channels
18S & 28S 18 or 28 Svedberg units
Chapter 1

Introduction

Death due to ischemic heart disease (IHD) has been declining, partly as a result of improved implementation of thrombolysis and angioplasty to return blood flow in the setting of acute myocardial infarction (MI) (Fox et al, 2007). However, acute MI and the long-term effects of these events still account for 1 in 6 deaths in the United States (Go et al, 2013). Researchers have worked to understand the cellular events that account for these deaths, both during ischemia and during therapeutic reperfusion. This thesis contributes to these ongoing efforts, focusing on voltage-gated sodium channels (VGSC) that have been implicated in both the cardiac arrhythmias and loss of cardiac contractile function that collectively are responsible for all deaths due to MI.

1.1 The Structure and Function of Voltage-Gated Sodium Channels
1.1.1 The Central Role of Voltage-Gated Sodium Channels in Excitable Cells

Excitable membranes use action potentials (AP) to carry signals and coordinate muscle contraction (Hodgkin & Huxley, 1952; Ulbricht, 2005). These AP are shaped by currents flowing across the membrane through a variety of ion channels (Hodgkin & Huxley, 1952; Ulbricht, 2005). VGSC are the first to open, and are responsible for the rapid AP upstroke and large peak currents that are important to AP conduction (Hodgkin & Huxley, 1952; Ulbricht, 2005). A unique feature of sodium channels is a “fast off” gating mechanism, which shuts off this current through the majority of channels within 1 to 2 milliseconds (ms) of their initial opening (Hodgkin & Huxley, 1952; Ulbricht, 2005). Channels enter an intermediate refractory state of “inactivation”, only returning to the ready state of “closed” channels after AP repolarization (Hodgkin & Huxley, 1952; Ulbricht, 2005). This refractory period sets the maximal rate of depolarization and ensures that the AP travels in a forward direction (Hodgkin & Huxley, 1952; Ulbricht, 2005).
1.1.2 The Structure and Identified Functional Components of Voltage-Gated Sodium Channel Alpha Subunits

VGSC are made up of a large pore-forming alpha subunit (~260 kDa), associated with one or two smaller (33-36 kDa) beta subunits (Hartshorne et al, 1982; Barchi et al, 1983; Lombet & Lazdunski, 1984; Cohen & Levitt, 1993; Malhotra et al, 2001; Zimmer et al, 2002b). While beta subunits are capable of modifying channel gating and/or trafficking to the cell membrane (Isom et al, 1992; Isom et al, 1995a; Nuss et al, 1995; Zimmer et al, 2002b), the alpha subunit expressed alone in heterologous expression cells is capable of passing current across the cell membrane (Goldin et al, 1986; Noda et al, 1986b). Nine sodium channel alpha subunit isoforms have been cloned and functionally expressed. The nomenclature used to identify these isoforms (Nav1.1 through Nav1.9) was described by Goldin (2000). Na denotes the primary ion permeability and V signifies the control of these channels by membrane voltage. Systematic mutation of the parts of the sequence that are conserved across isoforms (e.g. Stuhmer et al, 1989; Hartmann et al, 1994) has identified the parts of the channels central to the basic role of voltage-dependent gating of sodium.

The primary structure of rat brain Nav1.2 and the putative relationship of sequence segments to the cell membrane is illustrated in Figure 1. Sodium channel alpha subunits contain 24 transmembrane segments, divided across four highly similar but not identical, channel domains. Both termini are located within the cell. Conserved functional components across all isoforms include the following:

a) the S4 transmembrane segments with evenly dispersed positively charged amino acids, that sense and translate membrane voltage changes into channel gating;

b) the fast inactivation “particle” (IFM) contained within the DIII/DIV linker that acts as a ball and chain, binding to and physically occluding the pore opening (Vassilev et al, 1988; Vassilev et al, 1989; Stuhmer et al, 1989; West et al, 1992);

c) the S5 and S6 segments, that together with the large intervening “pore loops”, are believed to form the ion conducting pore (Ragsdale et al, 1994; Chen et al, 2002); and
VGSC are composed of four domains (I through IV) with similar but not identical sequence. Each contains six transmembrane segments (S1 (gold) through S6 (red)), with the four S5 to S6 connecting loops making up the channel’s outer pore. The amino (NH$_2^+$) and carboxy (COO$^-$) terminals are placed intracellularly. A 9 bp sequence in the intracellular loop connecting domains III and IV codes for isoleucine (I), phenylalanine (F) and methionine (M), and plays a critical role in VGSC fast inactivation.
d) a section of the pore loops that makes up the selectivity filter controlling conductance and ion-selective permeation (reviewed in Fozzard & Hanck, 1996).

### 1.1.3 The Na\(\text{V}1\) Subfamily

Of the nine functionally expressed VGSC, seven (Na\(\text{V}1.1, \text{Na} \text{V}1.2, \text{Na} \text{V}1.3, \text{Na} \text{V}1.6, \text{Na} \text{V}1.7, \text{Na} \text{V}1.8\) and Na\(\text{V}1.9\)) are primarily expressed in the nervous system, and are collectively referred to as neuronal VGSC. Na\(\text{V}1.1\) (Noda et al, 1986a; Smith & Goldin, 1998; Escayg et al, 2000), Na\(\text{V}1.2\) (Noda et al, 1986a), Na\(\text{V}1.3\) (Kayano et al, 1988) and Na\(\text{V}1.6\) (Smith et al, 1998; Plummer et al, 1998) are expressed in the brain (Westenbroek et al, 1989; Whitaker et al, 2001); whereas Na\(\text{V}1.3, \text{Na} \text{V}1.6, \text{Na} \text{V}1.7, \text{Na} \text{V}1.8\) and Na\(\text{V}1.9\) are expressed in the peripheral nervous system (Novakovic et al, 2001). Na\(\text{V}1.5\) (Rogart et al, 1989b) is the predominant isoform in the heart (Zimmer et al, 2002a); whereas Na\(\text{V}1.4\) (Trimmer et al, 1989; Schaller et al, 1992) is the predominant VGSC isoform in skeletal muscle (Trimmer et al, 1989).

Remarkable similarity exists across the VGSC isoforms, with amino acid sequence identities that place all nine functionally expressed isoforms spread over four chromosomes in the same subfamily. VGSC alpha subunit isoforms share 75% or greater amino acid identity across the transmembrane segments, pore loops, and the DIII/IV intracellular loop that makes up the fast inactivation gate (Catterall et al, 2003). When only the cardiac-dominant and the prevailing brain isoforms including Na\(\text{V}1.1\) are considered, this value increases to 88% or greater amino acid identity (Catterall et al, 2003). Gene similarity across chromosomes includes a high level of conservation of intron/exon structure (Plummer et al, 1998; Plummer & Meisler, 1999; Escayg et al, 2000). Furthermore, conservation extends across orthologous genes (Noda et al, 1984; Noda et al, 1986a; Salkoff et al, 1987; Plummer et al, 1998; Martin et al, 2007). Plummer & Meisler (1999) make the point that these similarities across VGSC are consistent with their essential functional role.

On the other hand, channel segments that are more variable may house important functional differences across isoforms (Plummer & Meisler, 1999). The first two intracellular loops are the least
well-conserved channel segments, with less than 50% amino acid identity across the nine VGSC isoforms (Catterall et al, 2003) and more variability across isoforms in the intron/exon structure (Plummer et al, 1998). In addition putative phosphorylation sites exist in the amino termini and the inactivation gate (DIII/DIV linker), and are concentrated within the first two cytoplasmic loops (Scheuer, 2011). Both phosphorylation sites and changes in channel function in response to phosphorylation events, have been shown to be isoform-specific (Murphy et al, 1996; Smith & Goldin, 1996; Frohnweiser et al, 1997; Scheuer, 2011). Despite this variability across isoforms, there is strong conservation of the first two intracellular loops when the same isoform (Na\textsubscript{v}1.6) is compared across human, rat and mouse (Dietrich et al, 1998; Plummer et al, 1998; Plummer & Meisler, 1999).

1.2 Voltage-gated Sodium Channel Function in the Heart

1.2.1 Normal Function

The path and the timing of the spread of electrical activity in the heart must be tightly controlled in order to achieve coordinated muscle contraction. A critical component of this is provided by the fast on, large sodium currents of the action potential (AP) upstroke that promote uniform spread of depolarization through the cardiac conduction system and muscle (Katz, 2006). In addition, the duration of the AP and therefore of the refractory period that it controls, is between 200 and 400 ms in the heart as opposed to 1 to 2 ms in nerve. These periods are required for cardiac relaxation and filling, but play an additional important role: differences in AP duration (APD) across the myocardium are designed to corral the spreading depolarization through cardiac muscle in a prescribed unidirectional path (Ashamalla et al, 2001).

1.2.2 Genetic Mutations Provide Insight into the Critical Role of Action Potential Duration

A growing list of genetic mutations involving ion channels, or cellular components that interact with ion channels, provides insight into the dramatic impact of changes to ion channel function in the heart. These mutations are all associated with changes in the duration of the cardiac action potential.
Central to proposed arrhythmia mechanisms, is the fact that the level of impact on the APD varies across the heart wall (Antzelevitch, 2007). Evidence supporting an increase in the variability of APD, and, therefore, of the refractory period, across the heart wall has been reported for two clinical syndromes associated with high death rates, Long QT Syndrome (LQTS) (Yan & Antzelevitch, 1998) and Brugada Syndrome (Yan & Antzelevitch, 1996; Rosso et al, 2008; Obeyesekere et al, 2013).

The QT in Long QT Syndrome refers to the phase of cardiac repolarization on a surface electrocardiogram that approximates the APD. LQTS mutations are frequently associated with a particularly lethal form of tachycardia (Lankipalli et al, 2005). Roughly 15 to 23% of patients diagnosed with LQTS, will have a life-threatening cardiac event or experience sudden cardiac death (reviewed in Goldenberg & Moss, 2008). Mutations in the Na\textsubscript{v}1.5 gene or in genes for cell components associated with VGSC function, account for 10 to 15% of LQTS cases (Wilde & Brugada, 2011), and these are collectively referred to as LQT3. It is the LQT3 mutations that carry the highest risk for sudden cardiac death (Zareba et al, 1998). While a wide range of mutations have been associated with LQT3, the majority of Na\textsubscript{v}1.5 LQT3 mutations result in a defect in channel inactivation (Bennett et al, 1995; Wang et al, 1995).

Brugada Syndrome patients share a distinct electrocardiographic pattern, but are thought to be a mixed clinical population. The predominant explanation for the electrocardiographic pattern is more pronounced early repolarization (Meregalli et al, 2005; Yan & Antzelevitch, 1996; Obeyeskere et al, 2013). Early repolarization would be expected to result in marked decreases in APD in select areas of the heart (Krishnan & Antzelevitch, 1993; Obeyesekere et al, 2013). The majority of mutations identified are in the Na\textsubscript{v}1.5 gene or in genes for the cellular components that interact with VGSC (Hedley et al, 2009), with 20 to 25% of patients having a mutation in Na\textsubscript{v}1.5 (Kapplinger et al, 2010). Functional studies of these Na\textsubscript{v}1.5 mutations show faster channel inactivation or reduced peak sodium currents (Wang et al, 2000; reviewed in Hedley et al, 2009), and both of these are consistent with early repolarization (Obeyeskere et al, 2013). Obeyeskere et al (2013) make the point that the electrocardiographic pattern is
relatively common. However, Tikannen et al (2009) identified a group with more marked changes in the electrocardiogram and an adjusted relative risk of death due to arrhythmia of 3.94 (CI =1.96 to 7.9, p<0.001).

1.3 Voltage-Gated Sodium Channels in Myocardial Ischemia and Reperfusion

1.3.1 Reactive Oxygen Species as Mediators of the Clinical Outcomes of Ischemia and Reperfusion

Myocardial ischemia is associated with lethal cardiac arrhythmias, increasing levels of reversible contractile dysfunction (“stunning”), and ultimately cell (cardiomyocyte) death (Stephenson et al, 1960; Imahashi et al, 1999; Becker, 2004). While improved prognosis with reperfusion has been clearly demonstrated (The I.S.A.M. Study Group, 1986; Guerci et al, 1987; The ISIS-2 Collaborative Study Group, 1988; Wilcox et al, 1988), return of blood flow may be more arrhythmogenic than ischemia (reviewed in Wit & Janse, 2001), is associated with increases in the degree of stunning (Imahashi et al, 1999; Padilla et al, 2000), and markedly increases the rate of cell death (Becker, 2004). A number of interacting cellular changes are implicated in the cellular response to ischemia and reperfusion, including ionic imbalance culminating in calcium overload, increased production of reactive oxygen species (ROS), depletion of high energy phosphate compounds, activation of proteases, and mitochondrial damage (reviewed in Powers et al, 2007).

Three key ROS are produced as a byproduct of cellular metabolism. Superoxide anion (O$_2^-$) in the presence of superoxide dismutase is converted to produce hydrogen peroxide (H$_2$O$_2$), which in turn may be broken down to form H$_2$O in the presence of catalase or glutathione peroxidases, or may be converted to produce hydroxyl anion (•HO) (Becker, 2004). ROS production increases during ischemia and shows a further marked increase described as a burst, during the initial minute post reperfusion (Grill et al, 1992; Ferrari, 1994).

ROS generating systems, such as substrate plus xanthine oxidase, have been shown to produce contractile dysfunction and myocardial cell injury in perfused hearts (Miki et al, 1988) and in a
myocardial tissue preparation (Burton et al, 1984). Potentially dangerous reperfusion arrhythmias can be increased or decreased using an ROS generating system or ROS degradation enzymes, respectively, in perfused rat hearts (Bernier et al, 1986). Also, in vivo animal studies have shown that treatment with ROS degradation enzymes just before reperfusion of ischemic myocardium, improves the rate of functional recovery (Myers et al, 1985), reduces lethal cardiac arrhythmias (Riva et al, 1987), and reduces infarct size (Jolly et al, 1984). While the least reactive of the three, the effectiveness of degradation enzymes targeting different ROS species suggests an important role of H$_2$O$_2$ in the lethal arrhythmias, myocardial stunning and cell damage produced by ischemia and reperfusion (Bernier et al, 1986; Miki et al, 1988; Kraemer et al, 1990), and it is used experimentally to simulate ischemia/reperfusion as it is more stable than the other two.

1.3.2 Reactive Oxygen Species Prolong Action Potential Duration by Impairing Channel Inactivation

The ability to examine the opening and closing events of a small number of ion channels under a pipette tip, referred to as “single channel recordings”, in the 1980’s added to our understanding of sodium channel inactivation. Yue et al (1989) summarize this literature as demonstrating a repetitive closing and reopening of individual channels before settling into a stable state of inactivation.

Under normal cellular conditions the net result of the repetitive reopening of many individual channels during VGSC inactivation, is small inward sodium currents (Ju et al, 1996; Ward & Giles, 1997; Maltsev et al, 1998; Sakmann et al, 2000). These have been recorded from isolated ventricular myocytes at different time points after the upstroke of the AP, including time points that cover the full duration of the AP (Ju et al, 1996; Ward & Giles, 1997; Maltsev et al, 1998; Sakmann et al, 2000). These “persistent currents” (I$_{Na,P}$) are not recorded in all experiments or in all cells within an experiment (Ju et al, 1996; Ward & Giles, 1997; Maltsev et al, 1998; Sakmann et al, 2000). I$_{Na,P}$ are within the range of 35 to 50 pA (Ju et al, 1996; Ward & Giles, 1997; Maltsev et al, 1998; Sakmann et al, 2000). For comparison, the
sodium currents that make up the AP upstroke are estimated to be around 2 to 3 nA (Ju et al, 1996; Ward & Giles, 1997; Maltsev et al, 1998; Sakmann et al, 2000).

$I_{Na,p}$ are more consistently recorded in isolated ventricular myocytes under experimental conditions simulating myocardial ischemia and reperfusion. Ju et al (1996) show loss of sodium currents over the initial 20 ms after a depolarization under normal conditions, as compared with remaining sodium currents at the 200 ms mark post depolarization under hypoxic conditions. Consistent with this, Ward & Giles (1997) show the loss of sodium currents around the 15 to 20 ms timepoint of a sustained depolarization under normal cellular conditions. These currents are still present at the end of their 20 ms recording after H$_2$O$_2$ application.

While $I_{Na,p}$ are small, ROS application to isolated cardiomyocytes is also associated with dramatic increases in the APD (Barrington et al, 1988; Beresewicz & Horackova, 1991; Ward & Giles, 1997). The VGSC-specific toxin tetrodotoxin (TTX) returns AP to control levels (Beresewicz & Horackova, 1991; Ward & Giles, 1997), demonstrating that the small $I_{Na,p}$ produce or provide an essential contribution to, the AP prolongation. Ward & Giles (1997) provide data that demonstrates that the small $I_{Na,p}$ are capable of the former. Injection of a hyperpolarizing current as small as -20 pA, returned the AP of the H$_2$O$_2$-treated cells to the resting membrane potential.

The increase in persistent sodium gating with ROS, appears to be largely the result of impaired channel inactivation. Bhatnager et al (1990) were the first to demonstrate that tert butylhydroperoxide, an oxidant that increases ROS levels (Kanupriya et al, 2007), slows VGSC inactivation. They also showed changes to the voltage-dependence of VGSC gating, with the potential to contribute to the inward sodium currents during the AP. Two groups present single channel tracings consistent with impaired channel inactivation with ROS. Ju et al (1996) present data showing an abrupt increase in channel openings with ROS application. Furthermore, data from Ward & Giles (1997) shows patterns of increased post-depolarization sodium channel openings and AP prolongation in response to H$_2$O$_2$, that are indistinguishable from the response to the sea anemone toxin anthopleurin A. This is part of a group of
toxins that act through changes in VGSC gating (reviewed in Moran et al, 2009), with anthopleurin A both slowing inactivation and leaving channels unable to completely inactivate (reviewed in Ulbricht, 2005).

1.3.3 The Role of Persistent Sodium Currents in the Clinical Outcomes of Ischemia and Reperfusion

Experiments using perfused hearts support an expected role of $I_{NaP}$ in producing the arrhythmias that appear during ischemia and reperfusion, and also provide evidence suggesting a central role of these currents in the development of myocardial stunning and cell death. Perfused hearts show increases in intracellular sodium concentration in response to ischemia (Imahashi et al, 1999), consistent with impaired VGSC inactivation in hypoxic conditions (Ju et al, 1996) and in response to ROS (Ward & Giles, 1997). Continuous recording of intracellular sodium after reperfusion shows a transient (~5 min) further increase (Williams et al, 2007), before a rapid decrease (Pike et al, 1995; Imahashi et al, 1999; Williams et al, 2007). Pike et al (1995) report that intracellular sodium levels are the strongest predictor of lethal ventricular arrhythmia when compared with pH and depletion of high energy phosphates, in ischemic hearts.

It is argued that the increases in intracellular sodium act through increases in intracellular calcium, to initiate the ischemia/reperfusion arrhythmia and myocardial stunning (Haigney et al, 1994; Imahashi et al, 1999; Song et al, 2006). Furthermore, the role of calcium overload in myocardial cell death with MI is well-established (reviewed in Bers, 2008). Continuous recordings of intracellular calcium in perfused hearts also show increases through ischemia (Haigney et al, 1994; Song et al, 2006), followed by an additional increase with reperfusion (Haigney et al, 1994). While other players have been suggested and may play some contributing role (reviewed in Haigney et al, 1994), evidence suggests that the primary mechanism behind increased intracellular calcium both during ischemia and in the first few minutes of reperfusion, is the exchange of intracellular sodium for calcium by the sarcolemmal sodium calcium exchanger (Pike et al, 1990; Haigney et al, 1992; Haigney et al, 1994; Imahashi et al, 2005). This
exchanger normally acts to remove elevated intracellular calcium, however, it is thought to act in a reverse mode under conditions of elevated intracellular sodium, exchanging intracellular sodium for extracellular calcium (reviewed in Blaustein & Lederer, 1999). Imahashi et al (1999) used manipulation of extracellular calcium levels and inhibition of the sodium calcium exchanger, to separate the roles of intracellular sodium and intracellular calcium in myocardial stunning in perfused hearts. Conditions expected to interfere with the ability of the heart to exchange intracellular sodium for extracellular calcium, slowed the recovery of intracellular sodium levels while improving the rate of contractile recovery. On the other hand, high extracellular calcium, expected to promote this reverse mode sodium/calcium exchange, improved intracellular sodium recovery and slowed contractile recovery.

Pharmacological studies provide evidence for involvement of sodium influx through VGSC, and in particular of the persistent currents through these channels, in the high intracellular sodium levels believed to be the initiating event for all three clinical outcomes associated with ischemia/reperfusion. Hypercontracture, with the return of blood flow and energy substrate to cardiomyocytes with elevated intracellular calcium, can be prevented in isolated ventricular myocytes with the use of TTX (Haigney et al, 1994), and Abraham et al (1989) demonstrated antiarrhythmic effects of TTX during myocardial ischemia in anesthetized rats. TTX doses used eliminated or significantly reduced the fast-on transient sodium currents of the action potential upstroke that control AP conduction.

In contrast with TTX, blockers that have been used clinically all take advantage of VGSC gating mechanisms to target currents from the ischemic areas of the heart. The binding site for these drugs is within the inner channel pore, and VGSC blockade by these agents requires access provided by channels in the open and/or inactivated state (Hanck et al, 2009). Channels with defective inactivation are open longer; ischemic tissue is depolarized resulting in a high proportion of channels in the inactivated state; and rapid firing rates, i.e. frequent openings, are the early component of the majority of cases of sudden arrhythmic death.

Lidocaine is the prototypical antiarrhythmic used in the setting of ischemia/reperfusion, and
application to isolated ventricular myocytes has been shown to attenuate both the increase in persistent sodium current (Ju et al, 1996) and the increase in intracellular sodium (Haigney et al, 1994) in response to hypoxic conditions. As well, reperfusion hypercontracture is eliminated with lidocaine (Haigney et al, 1994). However, while an earlier study demonstrated lidocaine’s selectivity for persistent versus the transient sodium currents (Ju et al, 1992), it should be noted that doses used in these experiments also produced a decrease in the peak sodium current.

Ranolazine is the result of efforts to further target the persistent sodium currents in clinical settings (Song et al, 2004; Fredj et al, 2006; Undovinas et al, 2006; Wang et al, 2008b). Song et al (2006) demonstrated the ability of ranolazine to decrease sodium influx after the AP upstroke, to reduce the rise in intracellular calcium, and to attenuate AP prolongation, in ventricular myocytes treated with H2O2. Of note, the drug concentration used by Song et al (2006) did not change the appearance of the AP upstroke.

It has also been proposed that the increase in intracellular sodium during ischemia/reperfusion could be a result of activity of the sarcolemmal sodium proton exchanger (NHE). A number of papers using NHE inhibitors and perfused hearts have presented data in support of this. NHE inhibition has been associated with an attenuation or elimination of the intracellular increases in sodium (Pike et al, 1993; Pike et al, 1995) and calcium (Murphy et al, 1991) during ischemia. In addition, these agents have been shown to improve the rate of functional recovery, decrease cell injury, and decrease arrhythmias associated with ischemia/reperfusion (Murphy et al, 1991; Mochizuki et al, 1993; Pike et al, 1993; Yasutake et al, 1994; Pike et al, 1995).

However, alternate explanations have been proposed for the beneficial effects of NHE inhibition. Ward & Moffat (1995a) suggest that the decreases in pH during ischemia/reperfusion, may be protective by decreasing the calcium sensitivity of the contractile apparatus under conditions of high intracellular calcium. They report that NHE inhibition during exposure of cardiomyocytes to lactate acidosis, further reduces the pH and is associated with a reduced sensitivity of the contractile apparatus to calcium. Hypercontractilility during reperfusion has been associated with increased cell damage (Siegmund et al,
Hartmann & Decking (1999) agree, demonstrating reduced contracture on reperfusion of ischemic hearts, improved functional recovery in the 30 minutes following an ischemic period, and improved availability of high energy phosphate compounds, with NHE inhibition.

As well, Williams et al (2007) point out that the NHE inhibitors used in earlier studies might also interfere with VGSC, providing an alternate explanation for their effects on the intracellular sodium and calcium responses to ischemia. They demonstrate that TTX prevents the increase in intracellular sodium during ischemia but not the increase in response to reperfusion. In contrast, one NHE inhibitor used in this study prevents all sodium increases through both ischemia and reperfusion while another prevents only the increase in intracellular sodium in the minutes post reperfusion.

1.3.4 Clinical Evidence of Cardiac Persistent Sodium Currents

To date, there is not strong clinical evidence for the ability of lidocaine or ranolazine to decrease mortality in the setting of MI. The long-standing use of lidocaine in the treatment of ischemia/reperfusion arrhythmias can be traced to small scale studies showing impressive results in an era that preceded the, now standard, use of techniques to restore blood flow. For example, Gianelly et al (1967) published a report of the interruption of ventricular tachycardia in each of 20 patients during acute MI. However its early acceptance has left it poorly investigated, and White et al (2001) conclude from their review of literature that the efficacy of lidocaine in the treatment of pre-hospital ventricular tachycardia or ventricular fibrillation has not been demonstrated. One large study was completed to examine the effects of prophylactic use of lidocaine in the setting of acute MI in the post-reperfusion era, with results suggesting no benefit for all cause death during acute MI or over the first 30 days post-MI (Alexander et al, 1999). In addition, it is well-documented that lidocaine has a not uncommon proarrhythmic effect in mixed populations of arrhythmia patients (e.g. Rae et al, 1988), that is believed to be related to its effect on peak sodium currents and AP conduction.

In contrast, large scale studies have examined the effects of ranolazine treatment in heart disease. The improved selectivity for $I_{Na,p}$ demonstrated for ranolazine, is associated with removal of the
proarrhythmic effect reported for other VGSC blockers (Scirica et al, 2007). However, prophylactic use of ranolazine in the setting of acute MI does not change the incidence of symptomatic arrhythmia, death in the acute setting, or death over the first year post-MI (Morrow et al, 2007). The author would argue that the literature suggests the potential for $I_{Na\,P}$ blockade in the treatment of ischemic/reperfusion injury. Next steps will require developing an understanding of intended and unintended effects in narrowly defined patient populations.

1.4 Persistent Currents in Neuronal Voltage-Gated Sodium Channels

With growing evidence for the existence of neuronal VGSC in the heart (reviewed below), the author would argue that these are the most likely candidates for mediating the increased $I_{Na\,P}$ and increased APD with $H_2O_2$ application to cardiomyocytes. Research (Llinas & Sugimori, 1980; Aracri et al, 2006) has demonstrated that, in contrast with the all-or-none depolarizations in cardiac muscle, persistent sodium currents are important contributors to subthreshold currents in neural tissue. This work shows that persistent currents are essential to summative processes such as signal integration or amplification, and act to promote neuronal firing patterns described as rhythmic, repetitive or bursting.

The strongest evidence implicating specific VGSC isoforms in functionally important persistent gating in neuronal tissue, comes from the Purkinje neurons of the cerebellar cortex. $NaV_{1.1}$ and $NaV_{1.6}$ are the only VGSC isoforms detected with single cell PCR (polymerase chain reaction) of these cells (Vega-Saenz de Miera et al, 1997). Persistent sodium currents are an important contributor to the electrophysiological patterns of these Purkinje cells (Llinas & Sugimori, 1980), and the literature suggests that both isoforms make important contributions to these currents (Burgess et al, 1995; Kohrman et al, 1995; Kohrman et al, 1996; Meisler et al, 1997; Raman et al, 1997; Vega-Saenz de Miera et al, 1997; Raman et al, 1997; Mulley et al, 2005; Kalume et al, 2007).

Consistent with an important role for persistent VGSC gating in neuronal tissue, neuronal VGSC produce larger $I_{Na\,P}$ when expressed in mammalian cell lines. Measures of persistent sodium currents
under normal cell conditions, were larger from neuronal VGSC as compared with those from the cardiac-dominant Nav1.5 or the skeletal muscle-dominant Nav1.4 (Mantegazza et al, 2005). Moreover, electrophysiological studies in these cell lines suggest that one or more neuronal VGSC and not Nav1.5, is/are responsible for the increased $I_{Na,P}$ in cardiomyocytes in response to $H_2O_2$ (Ward & Giles, 1997). Protein kinase C (PKC) inhibition attenuated the APD prolongation with $H_2O_2$ (Ward & Giles, 1997), and PKC activation slows VGSC inactivation (Qu et al, 1994) in isolated cardiomyocytes. PKC activation also slows the inactivation of a neuronal VGSC expressed in mammalian cells (Numann et al, 1991); whereas PKC activation did not slow inactivation of Nav1.5 expressed in the same cell line (Qu et al, 1994). In addition, the increase in $I_{Na,P}$ and in APD in cardiomyocytes in response to $H_2O_2$, could not be replicated with repeat experiments in a mammalian cell line expressing Nav1.5 (Ward et al, unpublished). Involvement of Nav1.5 cannot be completely ruled out, however, as PKC effects, and therefore the required characteristics of expression systems to mediate these effects, have been shown to be isoform-specific (Qu et al, 1996; Murray et al, 1997).

1.5 Evidence for Neuronal Voltage-Gated Sodium Channels in the Heart

1.5.1 Voltage-Gated Sodium Channel Transcripts and Their Relative Abundance

With the exception of one study that used RT-PCR to report the presence of Nav1.3 in mouse ventricular myocytes (Zimmer et al, 2002a), all reports using RT-PCR to detect the presence of neuronal VGSC in the heart use RNA from whole heart. However, one of these papers, Haufe et al (2005b), present data that argues for the importance of working from ventricular myocyte cell isolations rather than from whole heart. They demonstrated, in mice, that neuronal isoforms make up only 4.9% of VGSC from ventricular myocyte cell isolations, as compared with 23.6% of VGSC from whole heart. Despite this, this same group (Haufe et al, 2005b) provides data that they suggest rules out or minimizes any impact of RNA contamination from other cell types in whole heart. Using RT-PCR of neuron-specific markers, they conclude that between <1% and 10% of the whole heart RNA came from contaminating nerve. Similarly, Schaller et al (1992) report that they were unable to RT-PCR a low-abundant transcript specific
for select forms of neurons, from their whole heart RNA. Neither group discusses the fact that fibroblasts are the most abundant cell type in the myocardium and that these are known to contain neuronal VGSC (Munson et al, 1979). Sills et al (1989) report similar RT-PCR results for one VGSC isoform amplified from whole tissue and from cultured ventricular myocytes. However, culturing the ventricular myocytes may change their VGSC makeup (Renauld et al, 1983; Maltsev et al, 2008), and changes in tissue innervation have been demonstrated to change the membrane expression of VGSC (Frelin et al, 1981).

Schaller et al (1992) used RT-PCR to examine the neuronal VGSC makeup of adult rat brain, whole heart and skeletal muscle. Primers for Na\textsubscript{v}1.1, Na\textsubscript{v}1.2 and Na\textsubscript{v}1.3 amplified sequence for Na\textsubscript{v}1.1, for its functional alternative splice variant (Na\textsubscript{v}1.1a –Noda et al, 1986a), and for Na\textsubscript{v}1.3. Schaller et al (1992) make the following two points regarding the VGSC make-up of the heart based on the screening of what they refer to as “several” clones, and based on RNase protection assay data that is not shown in the paper: they were unable to find any evidence for Na\textsubscript{v}1.2 in the heart, and the amount of Na\textsubscript{v}1.1 in rat heart is the same or slightly more than the amount of Na\textsubscript{v}1.3.

Haufe et al used two PCR methods to compare the transcript level of different sodium channel isoforms, in dog (2005a) and then in mouse (2005b) heart. In the dog, Haufe et al limited their examination of neuronal isoforms to Na\textsubscript{v}1.1, Na\textsubscript{v}1.2 and Na\textsubscript{v}1.3. In contrast with the rat data from Schaller et al, they provide restriction digest evidence for Na\textsubscript{v}1.2 in 7 of 8 cardiac tissues including the ventricles. Competitive PCR controlling for amplification efficiency (i.e. the rate of accumulation of full-length product) found that Na\textsubscript{v}1.3 was the most abundant and that Na\textsubscript{v}1.1 and Na\textsubscript{v}1.2 were of equal abundance, in cardiac ventricles. In their second study using mice, Haufe et al included an examination of the expression of the cardiac-dominant isoform Na\textsubscript{v}1.5 and of the skeletal muscle-dominant isoform Na\textsubscript{v}1.4, as well as of an additional neuronal isoform, Na\textsubscript{v}1.6. Some general statements apply to all findings from this second paper across whole heart from all developmental ages, limited data from isolated ventricular myocytes in young adult mice, and both competitive and real-time PCR. First, mRNA from Na\textsubscript{v}1.5 made up 84% of the VGSC transcript in mouse heart. Second, in mice the skeletal
muscle-dominant isoform $\mathrm{Na}_v1.4$ was as abundant as the most abundant neuronal isoform, $\mathrm{Na}_v1.3$. Additionally, $\mathrm{Na}_v1.2$ was found to be more prevalent, both in whole heart and in the ventricular myocyte cell isolations, than was either $\mathrm{Na}_v1.1$ or $\mathrm{Na}_v1.6$.

Zimmer et al (2002a), in the only paper with all RNA coming from cardiomyocyte cell isolations, sequenced the full-length mouse $\mathrm{Na}_v1.4$ and $\mathrm{Na}_v1.5$ sequence in overlapping segments. Competitive and real-time PCR, found $\mathrm{Na}_v1.5$ to be only 5X more abundant than $\mathrm{Na}_v1.4$. Moreover, in contrast with the conclusions of Haufe et al (2005b) from whole heart in the same species, competitive PCR using the same primer pair found $\mathrm{Na}_v1.3$ to be far less abundant than $\mathrm{Na}_v1.4$.

As well, all studies reporting relative amounts of specific VGSC isoforms in the heart (Schaller et al, 1992; Zimmer et al, 2002a; Haufe et al, 2002a & 2002b) amplify only a small segment (164 to ~1000 bp) of the full length (~6000 bp) coding sequences. However, over the last decade there has been an increasing awareness of the ubiquitous nature of alternative splicing, with more recent estimates suggesting that it is involved in the expression of greater than 90% of genes (Pan et al, 2008; Wang et al, 2008a). Stamm et al (2005) conclude from their literature review that most splicing events result in subtle changes in protein function. At the opposite end of the spectrum, they point out that selection of one of two mutually exclusive exons is the most common alternative splicing mechanism and that 25 to 35% of alternate exons introduce a premature stop codon. Moreover, Wang et al (2008a) provide examples of non-functioning sodium channel splice variants that are or appear to be the sole variant in a particular tissue.

1.5.2 Conflicting Results Provided by Isoform-Specific Antibodies

Maier et al (2002 & 2004) used immunocytochemistry to examine the distribution of $\mathrm{Na}_v1.5$ and of the three neuronal isoforms, $\mathrm{Na}_v1.1$, $\mathrm{Na}_v1.3$, and $\mathrm{Na}_v1.6$, within mouse cardiomyocytes. This data showed the neuronal isoforms limited to the same sharply defined pattern produced by an antibody to a z line protein, with z lines running alongside transverse t-tubules in muscle. In contrast, the authors interpret their $\mathrm{Na}_v1.5$ data as showing that this isoform is possibly exclusively located at the intercalated
discs. They sum up their findings as showing the “sharp complementary nature” of the distribution of Na\textsubscript{V}1.5 versus that of the neuronal sodium channels. In agreement, Malhotra et al (2001) using the same Na\textsubscript{V}1.1 antibody show immunohistochemistry with fluorescence limited to the distinct t-tubule pattern in rat neonatal myocardial tissue strips.

However, variations in these results have been reported, both for Na\textsubscript{V}1.5 and for the neuronal VGSC. In the case of the neuronal isoforms, these variations cannot be explained by differences in either the antibody used or the species investigated. Haufe et al show both in mouse (Haufe et al, 2005b) and dog (Haufe et al 2005a), that the same Na\textsubscript{V}1.1 and Na\textsubscript{V}1.2 antibodies also produce fluorescence at the intercalated discs.

Immunochemistry from Cohen (1996) using a second Na\textsubscript{V}1.5 antibody again suggests that this isoform is concentrated at the intercalated discs but also shows some presence in the t-tubule system in rat cardiomyocytes. Maier et al (2002) state that the Na\textsubscript{V}1.5 antibody used by Cohen has the potential based on the sequence that it was designed against, to cross react with Na\textsubscript{V}1.3. However, Cohen et al (1996) report but do not show that two additional antibodies provided the same results. Also, some validation is provided for all three antibodies in the form of immunoprecipitation from partially purified rat membrane proteins and immunoblot data showing bands of the expected size for Na\textsubscript{V}1.5 both before and after deglycosylation (Cohen & Levitt, 1993). Haufe et al (2005a) using a third Na\textsubscript{V}1.5 antibody against mouse cardiomyocytes (same species used by Maier et al, 2002), conclude that their findings agree with those of Cohen et al (1996). However while their data repeats the t-tubule distribution pattern shown by Cohen et al (1996), the concentration of this isoform at the intercalated discs is less dramatic than in the data presented by the other two groups.
1.5.3 Electrophysiological and Pharmacological Evidence for Neuronal Voltage-Gated Sodium Channels

Pharmacological differences between the cardiac-dominant VGSC and the neuronal isoforms have been used to provide evidence for functioning neuronal VGSC in the heart. The primary agent used to separate currents from different VGSC is TTX. This toxin allows a high degree of separation of currents from the neuronal as compared with the cardiac-dominant VGSC, based on a roughly 100-fold higher TTX-sensitivity of the former. This agent is not believed, however, to be able to completely separate neuronal versus cardiac VGSC currents (Brette & Orchard, 2006a). Maier et al (2002) calculate that the 6.3 µM $K_D$ for TTX blockade of the peak sodium current of the AP upstroke (Brown et al, 1981; Antoni et al, 1988), would translate into the 100 to 200 nM TTX used in these studies blocking 1.5 to 3% of the much larger current contribution from the cardiac-dominant Nav1.5.

Our lab now has extensive experience (unpublished) demonstrating the ability of the much smaller TTX concentration of 10 nM, to block persistent sodium currents in ventricular myocytes treated with H$_2$O$_2$. This level of TTX has been observed to eliminate all current in neuronal tissue with an estimated $EC_{50}$ of 0.3 nM (Roy & Narahashi, 1992), while the 100 nM $ED_{50}$ for TTX blockade of Nav1.4 in data from SB Drug Discovery (Glasgow, UK) suggests that only a small percentage of any Nav1.4 channels in ventricular myocytes would be affected. This represents the strongest available evidence both for functioning neuronal VGSC in ventricular myocytes, and for their role in producing the clinically significant persistent currents.

Electrophysiological differences between neuronal and the cardiac-dominant VGSC have been used as supportive evidence in pharmacological studies. Efforts to examine the function of individual isoforms in heterologous expression systems must accept the influence of the new cell environment (i.e. differences in protein isoforms, signaling pathways and channel auxiliary subunits). All studies presented here use one of two expression conditions shown to provide the best approximation of the electrophysiology of native cells. Channels are either expressed in mammalian cells (West et al, 1992;
Isom et al, 1995b), or are expressed in *Xenopus* oocytes with the electrophysiology “corrected” with coexpression of both β1 and β2 sodium channel auxiliary subunits (Isom et al, 1992; Isom et al, 1995a).

Two electrophysiological measures have been shown to differentiate between the cardiac-dominant and neuronal VGSC: the voltage-dependence of channel inactivation from a closed state, and the voltage-dependence of channel activation or opening. The cardiac-dominant VGSC performs both of these tasks at more hyperpolarized potentials when compared with the neuronal VGSC (Fozzard & Hanck, 1996; O’Leary, 1998; Lossin et al, 2002). Na\textsubscript{V}1.4, the skeletal muscle-dominant isoform, both inactivates from a closed state and activates at intermediate levels of membrane potentials (O’Leary, 1998; Zimmer et al, 2002a). As well, O’Leary (1998) provides electrophysiological data recorded from ventricular myocytes, neurons and skeletal muscle fibers by other groups, alongside his findings in expression cells for each of the cardiac-dominant Na\textsubscript{V}1.5, the skeletal muscle-dominant Na\textsubscript{V}1.4, and a common rat variant of the neuronal isoform Na\textsubscript{V}1.2. Overall, the reviewed studies support his findings in the expression cells.

Brette & Orchard (2006a) use both electrophysiological and pharmacological differences between Na\textsubscript{V}1.5 and the neuronal isoforms, to argue for functional neuronal VGSC in dissociated rat ventricular myocytes. O’Leary et al (1998) demonstrated in expression systems and through a review of the literature using isolated cardiomyocytes, that Na\textsubscript{V}1.5 activates with less depolarization stimulus than do either the neuronal or the skeletal VGSC (midpoint of activation -48 mV vs -22 mV or -28 mV, respectively). Brette & Orchard (2006a) demonstrate differences in the ability of TTX to block the peak current response to two levels of depolarization that are consistent with increased neuronal VGSC activation by the strongest depolarization stimulus. As well, they argue that the changes in gating characteristics with 100 nM TTX, are consistent with selective blockade of neuronal channels. Moreover, the widest separation between the two TTX-dose response curves (two sizes of depolarization stimulus) is between the doses of approximately 10 to 100 nM TTX, suggesting that this dose range is best able to preferentially block neuronal channels in their experiments. Based on calculations, they suggest that 100
nM TTX blocked 14% of Na$_{\text{V}}$1.5 as compared with 92% of neuronal channels, and that 8 nM TTX will block 50% of neuronal VGSC. From the dose response curves, they calculate that TTX-sensitive current (i.e. current blocked by 100 nM TTX) makes up 11% of total sodium current in ventricular myocytes. Support is provided by Haufe et al (2005a) who used dog ventricular myocytes and present TTX dose response curves showing that the impact of the level of depolarization is significant over the dose range from roughly 5 to 50 nM.

Haufe et al (2005a) used an additional approach to discover the relative contribution of neuronal and cardiac VGSC to peak sodium currents in dog cardiac myocytes. They capitalized on the location of a cysteine in the pore region of Na$_{\text{V}}$1.5 that confers its TTX-resistance and that is not found at the corresponding location within neuronal isoforms (Heinemann et al, 1992a). MTSEA (methanethiosulfonate reagents) binds covalently to the sulfhydryl groups of cysteine and is capable of physically blocking the channel pore. This group demonstrated selective block of Na$_{\text{V}}$1.5 and not of Na$_{\text{V}}$1.1, expressed individually in a heterologous expression system. In agreement with the estimates made by Brette & Orchard (2006a), MTSEA treatment of ventricular myocytes suggested that neuronal channels accounted for $10 \pm 5\%$ of peak sodium current in ventricular myocytes. This remaining current after MTSEA block was eliminated with 100 nM TTX. Furthermore, a recent paper by the Haufe et al group (Biet et al, 2012), uses MTSEA to demonstrate that non-cardiac VGSC in ventricular myocytes contribute almost half (estimated at $44 \pm 5\%$) of the I$_{\text{Na,P}}$ produced by isolated ventricular myocytes under normal cellular conditions.

These studies do not consider the potential role of currents from the skeletal muscle-dominant isoform Na$_{\text{V}}$1.4. One group reported cloning and sequencing the full-length transcript of this isoform in overlapping segments from the heart (Zimmer et al, 2002a). Na$_{\text{V}}$1.4 would be expected to respond to the voltage protocols used in the studies (O’Leary, 1998; Zimmer et al, 2002a) as well as to both of the pharmacological agents used (non-peer reviewed publication; Fozzard & Lipkind, 2010), in a manner closer to that of the neuronal VGSC.
1.6 Statement of the Problem and Overall Goal

Under experimental conditions simulating the myocardial ischemia and reperfusion of acute MI, VGSC in isolated ventricular myocytes take longer to settle into the closed state of inactivation allowing for small persistent inward sodium currents. Evidence links the degree of VGSC-mediated increase in myocardial sodium concentration in perfused hearts under conditions of ischemia and reperfusion, with each of the three important clinical outcomes, i.e. lethal cardiac arrhythmias, reversible contractile dysfunction or myocardial stunning, and cell death.

What is not known, however, is which VGSC isoforms are involved. Electrophysiological and pharmacological evidence makes a strong case for the presence of other VGSC alongside the cardiac-dominant isoform in cardiac myocytes. Data from studies using isoform-specific VGSC antibodies is widely-accepted. As well, RT-PCR of small segments of VGSC transcript from whole heart has been used to describe the VGSC makeup of ventricular cardiomyocytes.

However, discrepancies across studies using immunohistochemical and/or immunocytochemical techniques suggests problems with the design of antibodies capable of reliable identification of individual VGSC isoforms. As well, there is an increasing awareness of widespread alternative splicing, and evidence that a significant portion of these events results in transcript that is unlikely to produce functional proteins. Neither immunocytochemistry nor the RT-PCR studies, provide the full-length VGSC sequence required as evidence of transcript capable of producing functional channel. Finally, with the exception of one study using isolated cardiomyocytes RT-PCR has been performed on whole heart RNA. It has been long-recognized that even RNA from cardiomyocyte cell isolations may contain low level contamination from other cardiac cells that express VGSC.

We suggest that one or more neuronal VGSC are likely involved. Unpublished work from our lab demonstrates the ability of 10 nM TTX to turn off persistent sodium channel gating under conditions simulating ischemia/reperfusion. This TTX concentration is expected to have limited impact on any skeletal muscle-dominant VGSC, and would have negligible impact on the cardiac-dominant isoform. In
addition, persistent sodium currents have been shown to be a fundamental part of the function of the neuronal VGSC. The evidence is strongest for Nav1.1 and Nav1.6 producing large and functionally important persistent currents, and of these two, we have the most evidence for Nav1.1 in ventricular myocytes.

The overall goal of this thesis was, therefore, to begin the process of identifying any neuronal isoform(s) that exist in ventricular myocytes in a full-length or functional form. These VGSC isoforms are the strongest candidates for proteins involved in the translation of myocardial ischemia and reperfusion into the high death rates associated with ischemic heart disease.

1.7 Hypothesis and Research Objectives

1.7.1 Hypothesis

The full-length transcript for the neuronal voltage-gated sodium channel isoform Nav1.1, previously published from rat brain, exists in rat right ventricular myocytes.

1.7.2 Research Objectives

In order to test this hypothesis, we had the following research objectives:

1. to RT-PCR, clone and sequence overlapping segments of the full-length Nav1.1 transcript and of any of its splice variants, from enzymatic cardiomyocyte cell isolations; and

2. in order to rule out RNA contamination from other cardiac cell types, to demonstrate that RT-PCR band sizes produced from enzymatic cardiomyocyte cell isolations match those produced from the RNA of single cardiomyocytes when the same Nav1.1 primers are used.

As well, discrepancies have been reported between the two previously published full-length Nav1.1 sequences from rat brain. A secondary research goal was to determine the correct nucleotide
sequence for electrophysiological studies in a heterologous cell line. In order to achieve this, we had the following additional research objective:

3. to sequence a second clone from an independent RT-PCR as a means of ruling out cloning and sequencing error, whenever sequence differs from the original published sequence.
Chapter 2

Methods

2.1 Preparation of cDNA and Genomic DNA

2.1.1 Animals and Animal Care Ethics

All cells and tissue used in these experiments were harvested from adult male Sprague-Dawley rats (Charles River Laboratories International, Inc.). Procedures followed protocols approved by the University Animal Care Committee at Queen’s University, in accordance with the Animals for Research Act (Ontario) and the standards of the Canadian Council on Animal Care.

2.1.2 Isolation of Right Ventricular Myocytes

Cardiomyocytes were isolated from the right ventricle of 200-225g rats (approximately 8 weeks old) using a modified version of the method of Ward and Giles (1997). Animals were restrained using a decapicone (Braintree Scientific Inc., Braintree MA) and decapitated. The heart was then rapidly removed and cannulated via the aorta on a Langendorff-style perfusion apparatus. Hearts were perfused at 10 ml min\(^{-1}\) with a standard Tyrode solution containing (in mM) 140 NaCl, 5.4 KCl, 1 Na\(_2\)HPO\(_4\), 5 HEPES, 10 glucose, and 1 MgCl\(_2\), that was pH adjusted to 7.4 with 1 M NaOH and bubbled with 100% O\(_2\). For the first 5 minutes of perfusion, 1 mM CaCl\(_2\) was added to the perfusion solution, followed by 5 minutes of perfusion with Ca\(^{2+}\)-free Tyrode solution. Hearts were then digested by perfusion for 7 minutes with Tyrode solution containing 0.02 mg ml\(^{-1}\) collagenase (Yakult Co. Ltd., Japan) and 0.004 mg ml\(^{-1}\) protease (typeXIV, Sigma-Aldrich Canada Co., Oakville, ON). The heart was then removed from the cannula and the right ventricle was dissected and minced into 10 ml of low Ca\(^{2+}\) (10 µM CaCl\(_2\)) Tyrode solution with collagenase (0.5 mg ml\(^{-1}\)), protease (0.1 mg ml\(^{-1}\)), and bovine serum albumin (3 mg ml\(^{-1}\), Sigma-Aldrich), and put into a 36°C shaker bath. Liquid was collected from the bottom of the container with a Pasteur pipet (VWR International, Edmonton, AB) at 5 or 6 time points between
appearance of cells and the point where a number of cells had begun contracting. Care was taken to not remove visible non-dissociated tissue. Five to 8 drops of this liquid were added to a tube containing a modified KB solution containing (in mM): K-glutamate 100, K-aspartate 10, KCl 25, glucose 20, KH$_2$PO$_4$ 10, HEPES 5, MgSO$_4$ 2, taurine 20, creatine 5, and EGTA (ethylene glycol tetraacetic acid) 0.5 with bovine serum albumin (0.8 mg/ml), and pH adjusted to 7.2 with 1M KOH, and stored at 4°C. Cells were used the same day or frozen at -20°C overnight.

2.1.3 Harvesting Rat Brain

Brain tissue was harvested from rats ranging in weight from 200 through 400 g (approximately 8 to 18 weeks old). The skull cap was opened, the brain removed, all tissue connecting brain hemispheres cut, and brain tissue immediately submerged in Trizol (Invitrogen, Life Technologies) that had been pre-chilled on ice.

2.1.4 RNA Isolation

All procedures outlined above for the isolation of cardiomyocytes and removal of brain tissue, as well as all procedures outlined in this section for RNA isolation, were aimed at preserving RNA. Metal and glassware were baked overnight at 180 to 200 °C, all plastic containers were filled with 0.1N NaOH/0.1% ethylenediaminetetraacetic acid (EDTA) overnight, gloves and a face mask were worn, all surfaces were cleaned with RNase Away (Invitrogen, Life Technologies), and all water was DEPC-treated (diethylpyrocarbonate—Sigma-Aldrich). DEPC treatment involved addition of 0.1% DEPC to water, overnight incubation at room temperature, and then autoclaving to inactivate the DEPC.

RNA was isolated from rat right ventricular cardiomyocytes and from rat brain using the Trizol method and company-provided protocols. Cell lysis was achieved through manual homogenization of 1 brain hemisphere in 6 ml of Trizol, or repetitive pipetting of cardiomyocytes isolated from 4 right ventricles in 1 ml of Trizol. No record of which brain hemisphere was used was kept. The cardiomyocyte lysis was then centrifuged (12,000 X g, 10 min, 2 to 8°C) and the supernatant with RNA was moved to a fresh tube (recommended for RNA isolation from muscle). The cardiomyocyte sample or
one-sixth of the homogenized brain sample was incubated for 5 minutes at 15 to 30°C. Chloroform (0.2 ml) was then added to each sample, and the tubes vigorously shaken for 15 seconds, followed by a further incubation of 2 to 3 minutes at 15 to 30°C. Samples were then centrifuged 12,000 X g for 15 minutes at 2 to 8°C. The colourless upper aqueous phase containing the RNA was moved to a fresh tube, and 0.5 ml of isopropyl alcohol was added. Samples were then incubated at 15 to 30°C for 10 minutes and subsequently centrifuged at 12,000 X g for 10 minutes, at 2 to 8°C. The supernatant was aspirated and discarded. The RNA pellet was washed by adding 1 ml of 75 percent ethanol, vortexing, and centrifuging at 7,500 X g for 5 minutes, at 2 to 8°C. Care was taken to remove as much of the supernatant as possible with a pipettor. The RNA pellet was air dried for 5 to 10 minutes and then dissolved in 30 ml of DEPC-treated water by gentle pipette mixing. RNA was stored at -80°C.

RNA integrity was checked on a denaturing (MOPS buffer with 2% (v/v) of 37% formaldehyde) 1% agarose gel with 0.5 µg/ml ethidium bromide (EtBr). One µl of RNA sample was added to 2 µl of 5X RNA gel loading buffer and 7 µl of 1X MOPS buffer, vortexed briefly, incubated at 65°C for 10 minutes, set on ice for 5 minutes, and then collected through centrifugation at 8000 X g for 5 minutes at 4°C. The gel was run at 50 volts for 2 hours. The 260/280 ratio on a NanoDrop 2000 (Thermo Scientific, Burlington, ON) spectrophotometer provided a check on the purity of each RNA isolation.

2.1.5 Reverse Transcription (RT)

DNase I treatment of RNA and reverse transcription (i.e. first strand synthesis) were performed according to instructions provided by the Invitrogen Superscript III kit (Life Technologies), with minor modifications as indicated. For DNase treatment of RNA, 10 µl of RNA (500-5000 ng in DEPC-treated water) was incubated with 2.5 µl of 10X reaction buffer and 2.5 µl of DNaseI, for 30 minutes at 37°C. This was followed by the addition of 2.5 µl of EDTA and further incubation for 5 to 10 minutes, at 70°C. First strand synthesis begins with incubating 8 µl of the DNase-treated RNA, 1 µl of 50 µM oligo dT primers and 1µl of 10 mM dNTPs for 5 minutes, at 65°C, followed by immediate transfer to ice for at least 1 minute. Eleven µl of a master mix including 2 µl of 10X reaction buffer, 4 µl of 25 mM MgCl₂, 2
µl of 0.1 M DTT, 1 µl of RNase OUT and 2 µl of Superscript III reverse transcriptase (instead of the recommended 1 µl due to the large size of templates and a higher annealing temperature) was added (creating a total reaction volume of 21µl as directed by Invitrogen tech support), and the reaction annealed for 50 minutes, at 55°C (instead of the recommended 50°C due to secondary structures), followed by 5 minutes at 85°C. First strand product was then incubated with 1 µl of RNaseH for 20 minutes at 37°C and then stored at -20°C. All RNA was converted into cDNA within 24 hours of isolation. The multiple RT product tubes that were produced simultaneously were tested through positive control PCR with GAPDH primers (proprietary rat primers, catalogue # RDP-106, R & D Systems, Minneapolis, MN). PCR amplicons were run side-by-side on a gel and compared for both the strength and consistency of bands. These GAPDH primers will also produce an alternate sized band if genomic DNA is present in the cDNA (cDNA 265 bp versus genomic ~650 bp).

2.1.6 Isolation of Genomic DNA

Rat genomic DNA was isolated from the same Trizol phase separation as described above for the isolation of brain RNA. Care was then taken to remove all liquid in the upper aqueous phase before addition of 0.3 ml of 100 percent ethanol to each tube. Tubes were inversion mixed, incubated at room temperature (15-30°C) for 2 to 3 minutes, and centrifuged at 2000 X g for 5 minutes at 2 to 8°C. The supernatant was removed and the DNA pellet was washed twice in 0.1 M sodium citrate in 10% ethanol. Each wash involved incubation for 30 minutes in 1 ml of the wash solution at 15 to 30°C with periodic inversion mixing, and the wash solution was then removed after a spin at 2000 X g for 5 minutes at 2 to 8°C. The final pellet was then suspended in 1.5 to 2 ml of 75 percent ethanol, and incubated at 15 to 30°C for 10 to 20 minutes with periodic inversion mixing, before a final spin at 2000 X g for 5 minutes at 2 to 8°C. The DNA pellet was air dried for 5 to 15 minutes, and then dissolved in 400 µl of 8 mM NaOH. The tube was centrifuged at 12,000 X g for 10 minutes, the supernatant with the DNA was moved to another tube, and each tube was prepared for storage with the addition of 34.4 µl of 0.1 M HEPES, followed by the addition of 0.162 g of EDTA. Genomic DNA was stored at -20°C.
2.2 TA and Digestion Cloning

All PCR amplifications were performed using the Eppendorf Mastercycler ep gradient S PCR machine (Eppendorf International, Hamburg, Germany). Primers were designed with the use of the NCBI Primer BLAST (Basic Local Alignment Search Tool) tool for selection of specific primers (http://www.ncbi.nlm.nih.gov/tools/primer-blast). A positive and a negative control were run for all PCR in this thesis. Each RT (reverse transcription, i.e. first strand synthesis) included one tube with DNase-treated RNA and no additional reaction components. The product of this RT-PCR was then used as template in the negative control RT-PCR. Positive control PCR amplified GAPDH (proprietary rat primers, catalogue # RDP-106—cDNA 265 bp versus genomic ~650 bp, R & D Systems). Negative control PCR were also run with no template whenever a new or used RT or PCR kit was used for the first time.

All experiments involving “nested” PCR reactions used the Multiplex PCR system and protocol with minor modifications. The primer pair used in an initial PCR reaction is referred to as the “outer” primers (Table 1). A second PCR reaction used a primer pair nested, or set within, the targeted sequence amplified by the first. First round PCR used 5 µl of primer (2 µM of each forward & reverse), 5 µl of company-provided 10X “Q solution” (for templates with high GC content or with secondary structures), 6 µl of cDNA, 9 µl of H2O, and 25 µl of Multiplex PCR master mix (includes HotStar Taq polymerase, Multiplex PCR buffer, & dNTPs). Second round PCR used 4 µl of first round PCR product as template, 7.5 µl of primer (2 µM of each forward & reverse), and 8.5 µl of H2O. Reaction tubes were held at 95°C for 15 minutes (for enzyme activation), followed by 30 to 45 cycles of denaturation (30 s at 94°C), annealing (3 min at 57 to 63°C), and extension (90 s at 72°C), and a final extension period of 10 minutes at 72°C. One additional modification was made in select experiments as indicated.

PCR amplicons were purified before TA cloning, as well as both before and after each digestion step in preparation of the amplicons for digestion cloning (see below). Nine µl of PCR amplicons with both ends digested was run on a 1% agarose gel prior to the final purification. If the PCR produced a
single band in an otherwise clean lane, said amplicons were purified from the tube as in the previous steps. If the PCR produced multiple bands or any diffuse brightness at the bottom of the lane, the product was run on an EtBr-free gel, each band of interest was cut out without UV exposure, and the DNA was purified from the gel (Wizard Gel and PCR Clean-Up System, Promega Corp., Madison WI). Plasmids were purified without gel separation after each digestion step. Calf intestinal peptide (New England Biolabs, Ipswich, MA) was added (1.25 µl) to the final plasmid digestion reaction tube at the end of the digestion incubation, and the amplicons were incubated for an additional 1 hour at 37°C prior to final purification.

Negative ligation controls were performed with H$_2$O replacing insert DNA. All ligation reactions were performed overnight at 16°C. Two negative controls and one positive control were used for all transformation experiments. The negative controls transformed either 10 µl of the negative control ligation reaction product (i.e. no insert) or 10 ng of the company-provided linearized plasmid, while 10 ng of the company-provided circular plasmid was transformed as a positive control. The transformation efficiency of each batch of company-provided or lab-produced competent cells was tested using the company-provided circular plasmid. Xgal (800 µg/20 ml plate—Fisher Scientific, Ottawa, ON) was spread on TA cloning plates for blue/white identification of positive clones. EcoR1 digestion (2 µl EcoR1 buffer and 1µl of enzyme in a 20µl reaction) (New England Biolabs) of Miniprep-isolated plasmids (Qiagen) was used to check for expected size inserts prior to sending colonies for sequencing (ACGT Corp., Toronto, ON).

2.3 Cloning of Full-Length Nav1.1 in Overlapping Segments

The full-length rat Nav1.1 coding sequence, previously cloned from rat brain and published by Noda et al (1986a), was cloned and sequenced in overlapping segments from isolated rat right ventricular cells, first using TA cloning (primer pairs TA-1, TA-2, TA-3, TA-4, TA-5, & TA-6) and then digestion cloning (primer pairs D-1, D-2, D-3, D-4, D-5, D-6, & D-7) (Section 2.8, Table 1). NCBI blast searches
of both EST (expressed sequence tags) and RefSeq databases were performed for confirmation of select sequence variants.

PCR reactions used HotStar HiFidelity Polymerase (Qiagen) and the company-provided protocol with minor modifications. Reactions (50 µl) contained 6 µl of cDNA, 10 µl of 5X HotStar Buffer, 1 µl of the polymerase, 5 µl of 10 µM F primer, 5 µl of 10 µM R primer, and 23 µl of H₂O. The cDNA used as template was produced using 2000 ng of RNA in the first strand synthesis reaction. After enzyme activation for 5 minutes at 95°C, reactants went through 32 amplification cycles involving denaturation at 94°C for 15 s, annealing at 60°C for 1 minute and extension at 72°C for 1.5 minutes. The reaction was completed with a final extension step at 72°C for 10 minutes. All amplicons were run on a 1% agarose gel with 0.5 µg/ml EtBr.

TA cloning used a TA Cloning Kit including the pCR2.1 plasmid, T4 DNA ligase, ligase buffer, and One Shot INVαF (endonuclease-deficient (endA1), recombination-deficient (recA1), restriction minus (hsdR17), and gyrase-deficient (gyrA96)) chemically competent cells (Invitrogen, Life Technologies). The TA cloning kit ligation protocol called for 1 µl of 10X ligation buffer, 2 µl of 25 ng/µl pCR2.1 vector, ~10 ng of PCR amplicons, and 1 µl of T4 DNA ligase in a 10 µl reaction. This was followed for these reactions with insert sizes from 800 to 1200 bp, setting up an approximate 2:1 insert to vector molar ratio.

Digestion cloning used the pcDNA3.1V5HisB plasmid (Invitrogen, Life Technologies), New England Biolabs (NEB) restriction enzymes (Ipswich, MA), and NEB digestion protocols. Chemically competent DH5α cells (endonuclease-deficient (endA1), recombination-deficient (recA1), gyrase-deficient (gyrA96), and restriction minus (hsdR17)), were produced for digestion cloning using cold CaCl₂ solution. The tip of a pipette was touched to bacterial cells and grown overnight in Luria broth (LB, Invitrogen, Life Technologies), at 37°C, without shaking. Four ml of these cells were placed in 100 ml of LB broth and shaken at 37°C to an optical density of 0.2 (ie. absorbance of 10⁻⁰.²) at a wavelength of 550 nm (approximately 2 hr) (NanoDrop 2000 Model C, Thermo Scientific). Cells were placed on ice
and centrifuged at 7000 rpm for 15 minutes at 4°C, and then stored at -80°C in 15% glycerol and 25% LB broth. Digestion cloning used 20 µl ligation reactions with 2 µl of NEB ligation buffer, a total of 50 ng of vector DNA, a 2:1 insert to vector molar ratio, and 1 µl of NEB T4 DNA ligase.

All transformations used to clone the overlapping fragments (largest size <1.2 kb), used LB broth and the same transformation protocol. Transformation reactions began with the addition of 100 µl of competent cells to 10 µl of the ligation product in 1.5 ml Eppendorf tubes, followed by 30 minutes incubation on ice. Cells were heat shocked in 42.5°C H2O for 1 minute 40 s, placed on ice for ~3 minutes, and then pipette mixed gently with 800 µl of room temperature LB broth. Cells in broth were shaken (horizontal, 225 to 250 rpm) in 37°C for one hour and then centrifuged at 4000 rpm. Eight hundred µl of supernatant was removed, cells were resuspended in remaining buffer, and 50 to 200 µl was spread on agar plates. Bacterial plates were grown overnight at 37°C. Colonies were selected and grown overnight in tubes with 4 ml of LB broth at 37°C in a shaker (225-250 rpm), at 45°C.

2.4 Assembly of Nav1.1

All ligations required to assemble Nav1.1 were performed with 1 µl of T4 DNA ligase, 2 µl of ligase buffer, and 50 ng of vector (NEB). Figure 2A illustrates the digestion cloning strategy. The seven overlapping fragments covering the full-length transcript that had been individually cloned and sequenced, are labelled 1A, 1B, 2, 3A, 3B, 4A and 4B. The first step in assembling the full-length transcript in a single plasmid, involved digestion removal of fragments 1A, 3A and 4A from their plasmids. Plasmids containing fragments 1B, 3B and 4B were opened at the 5’ end of the insert with digestion (NEB restriction enzymes and protocols) preparation of both ends, producing modified vectors. These vectors were then ligated with their respective A fragment, using a 2:1 insert to vector molar ratio (Figure 2B). Complete fragments 1, 3 and 4 were cloned. Fragments 1, 2 and 3 were then digestion removed from their plasmids, and cut and purified from a gel. The plasmid with fragment 4 was opened (digestion preparation of both ends for cloning) at the 5’ end of the insert, and the size of this modified
Nav1.1
(6027 bp coding sequence plus a short 5' & 3' UTR)

Enzyme Sites Added to Insert
for Cloning

pcDNA3.1 V5HisB → Fragment 4B + Fragment 4A

pcDNA3.1 V5HisB → Fragment 3B + Fragment 3A

pcDNA3.1 V5HisB → Fragment 1B + Fragment 1A

pcDNA3.1 V5HisB → Fragment 2
Figure 2: Approach Used to Assemble the Full-Length Na\textsubscript{v}1.1 Transcript

A Strategy for digestion cloning of full-length Na\textsubscript{v}1.1. Primers were designed to clone the transcript in 7 segments (fragments 1A, 1B, 2, 3A, 3B, 4A & 4B). The full-length sequence includes a 251 bp 5’ UTR, a 6027 bp coding sequence, and an 86 bp 3’ UTR. Nucleotide positions are preceded by a +, signifying that the position is relative to the translation start site. Seven Na\textsubscript{v}1.1 enzyme sites were used (diamonds), and both the forward primer for fragment 1A and the reverse primer for fragment 4B, added enzyme sites (triangle (Kpn I) & square (Age I)). All 7 segments were individually cloned in a pcDNA3.1V5HisB plasmid.

B Illustration of the initial ligation steps in the process of assembling all 7 segments into one plasmid. Fragments 1A, 3A & 4A were removed from their plasmids. Fragments 1B, 3B & 4B were opened at the 5’ end of the insert, and ligated with their respective A fragments.

C Illustration of the assembly of all 7 segments into a single plasmid. Complete fragments 1, 2 and 3 were removed from their plasmids. The plasmid with the complete fragment 4 was opened at the 5’ end of the insert. Four piece ligation used the open plasmid with fragment 4 attached as vector, with fragments 1, 2, and 3 as inserts.
vector was checked on a gel. The final step involved a four-piece ligation with fragments 1 (~1807 bp), 2 (~560 bp) and 3 (2193 bp) as inserts, and the plasmid with fragment 4 attached (~7222 bp) acting as the vector (Figure 2C). In order to increase the chance of success, four-piece ligations were completed with a 3:1 insert to vector molar ratio for each insert and a total reaction DNA amount of 264 ng, as well as with a 2:1 insert to vector molar ratio for each insert and a total reaction DNA amount of 141 ng.

2.5 Cloning Full-Length and Near Full-Length Nav1.1 RT-PCR Amplicons

The full-length (~6.1 kb) Nav1.1 coding sequence was PCR amplified from rat right ventricle in one piece with use of the long-product protocol for HotStar HiFidelity Polymerase (Qiagen). The cDNA used as template was produced with 2000 ng of RNA in the 50 µl first strand synthesis reaction. Fifty µl PCR reactions contained 6 µl of cDNA, 5 µl of 10X buffer, 1.5 µl of dNTPs (10 mM), 10 µl of 5X Q solution, 2.5 µl of 10 µM digestion F primer pair #1 (Section 2.8, Table 1), 2.5 µl of 10 µM digestion R primer pair #7 (Section 2.8, Table 1), 1 µl (0.2 units) of HotStar HiFidelity polymerase, 1 µl (5 units) of Qiagen HotStar Taq polymerase, and 20.5 µl of H2O. Enzymes were activated at 95°C for 2 minutes, and then the reaction proceeded through 33 cycles of denaturation (94°C, 10 s), annealing (61°C, 1 min) and extension (68°C, 6.5 min). Approximate 6.1 kb cDNA was cut out of an EtBr-free 1% agarose gel without UV exposure, and purified (Wizard Gel and PCR Clean-Up System).

To amplify a near full-length (~4.3 kb) section of the transcript, the forward primer of the #1 digestion cloning primer pair and the reverse primer of the #5 digestion cloning primer pair (Section 2.8, Table 1) were used. The cDNA template was produced with 5000 ng of RNA in the first strand synthesis. LA Takara HS (hot start) polymerase (Clontech Laboratories Inc., Mountain View, CA) is marketed as an enzyme designed both for fidelity and for long product amplification, and was used according to the company-provided protocol. Fifty µl reactions contained 5 µl of 10X LA PC Buffer II (Mg2+ plus), 8 µl of dNTPs (2.5 mM), 3.6 µl of cDNA, 3µl of 10 µM F primer, 3 µl of 10µM R primer, 26.4 µl of H2O, and
1µl of 2.5 units/µl Takara LA Taq HS. After 1 minute at 94°C, the reaction was cycled through 33 cycles of denaturation at 98°C for 10 s and annealing at 68°C for 15 minutes, followed by extension at 72°C for 10 minutes. Approximate 4.3 kb cDNA was cut from a 1% agarose gel with 0.5 µg/ml EtBr, and purified (Wizard Gel and PCR Clean-Up System).

All ~6.1 and ~4.3 kb PCR amplicons were incubated with Qiagen HotStar Taq Polymerase and dNTPs for 1 hr at 72°C to add A overhangs prior to TA cloning. Ten to 15 µl reactions were set up with a total ng of PCR amplicons from 9 through 98 ng, no added H2O, and the remaining components adjusted accordingly from a 50 µl reaction protocol with 1 µl 10 mM dNTPs, 5µl 10X HotStar buffer, and 1U of the polymerase.

Both the ~6.1 and the ~4.3 kb NaV1.1 PCR amplicons were cloned using components of the TA Cloning Kit including the pCR2.1 plasmid, T4 DNA ligase, and the ligase buffer. Ligations used 50 ng of vector, 2 µl of 10X ligation buffer, and 1 µl of ligase in a 20 µl reaction. Unless indicated, ligations used a 1:1 insert to vector molar ratio. Sure2 restriction minus (mcrA, mcrCB, mcrF, mrr, hsdR), endonuclease-deficient (enda), and recombination-deficient (recB, recJ) bacteria (Agilent Technologies, Mississauga, ON), were used to clone the ~6.1 kb NaV1.1 transcript which includes the full-length coding sequence; whereas Stbl2 restriction minus (McrA), restriction-mutated (mcrBC, hsdRMS & mrr), recombination-deficient (recA1), gyrase-deficient (gyrA96), and endonuclease-deficient (enda1) bacteria (Invitrogen, Life Technologies), were used to clone the ~4.3 kb NaV1.1 transcript. Transformation began with the addition of 100 µl of competent cells to 10 µl of the ligation product, followed by a 30 minute incubation on ice. Cells were heat shocked in 42.5°C H2O for 45 seconds, placed on ice for ~3 minutes, and then pipette mixed gently with 450 µl of Super Optimal Broth with Catabolite repression (S.O.C) broth (Sigma-Aldrich), prior to 1 hour of horizontal shaking (225 to 250 rpm) in 37°C. Cells were then spun at 4000 rpm, 450 µl of supernatant was removed, cells were resuspended in remaining buffer and 50 to 200 µl was spread on agar plates. Bacterial plates were grown at 30°C or 16°C (cloning the ~6.1 kb transcript) or at room temperature (cloning the ~4.3 kb transcript), and were harvested as young colonies.
Overnight bacterial growth of individual colonies (4 ml of LB broth, tubes at 45° angle, shaken at 225 to 250 rpm) took place in 30°C (cloning the ~6.1 kb transcript) or at room temperature (cloning the ~4.3 kb transcript).

2.6 Single Cell PCR

The cytosol of single cardiomyocytes was harvested for single cell PCR using the whole cell patch clamping setup (Hamill et al, 1981). All solutions used in these experiments used DEPC-treated water (Invitrogen, Life Technologies) (see 2.2.4 RNA Isolation). Glass ware, utensils and glass pipetters were oven baked at 180 to 200 °C overnight.

Cells were superfused with Tyrode solution containing 1 mM CaCl₂ at room temperature. Borosilicate glass electrodes were pulled on a microprocessor-controlled, multiple stage puller (model P-97, Sutter Instruments, USA), and polished on a MF-200 micro-forge (World Precision Instruments, Sarasota, Florida, USA). Pipette resistance ranged from 1.5 to 2 MΩ when filled with pipette solution. Internal pipette solution contained (in mM) KCl 20, K-aspartate 110, EGTA 10, HEPES 10, MgCl₂ 1, K₂ATP 5, CaCl₂ 1, and NaCl 10, pH adjusted to 7.2 with 1M KOH. Gentle suction was applied to achieve a high resistance seal (10-100 GΩ) between the pipette tip and cell membrane. The membrane under the pipette tip was then ruptured to gain access to the cell. The maintenance of the seal between cell membrane and the wall of the pipette tip was monitored electronically (Axopatch ID amplifier, Axon Instruments and pClamp 10.0 data acquisition software) throughout the 9 minutes allowed for equilibration of the cell contents and the 10 µl internal pipette solution. The pipette was then gently withdrawn from the cell, sealing the pipette tip with a membrane patch in the “outside-out” configuration (Hamill et al, 1981) of cell membrane. The pipette tip was broken off and the pipette solution was air-forced into a 0.5 ml PCR tube for use as template for RT-PCR.

DNase-I treatment and RT were performed immediately after cytoplasm capture, according to the protocol described (see 2.2.5 Reverse Transcription (RT)) with one exception. The 10 µl of pipette
solution after washout of cell cytosol, replaced the 500 to 5000 ng of RNA in 10 µl.

2.7 Colony PCR

A toothpick was touched to a bacterial colony on an agarose plate, and then stirred in 30 µl of H₂O for 12 s. The 30 µl was placed in a 100°C heat block for 5 minutes, put on ice for 10 minutes, and then centrifuged for 1 s at 6000 X g. Released plasmids in the supernatant were used as template for PCR amplification and the PCR amplicons were sent for sequencing (ACGT Corp.).
### Table 1: Primers

All primers used in this thesis. Nested primer (NP) sets include both an outer (1st round) and inner (2nd round) primer pair. However, the primer set NP-4 uses the same reverse primer for both the outer and inner primer pair (i.e. hemi-nested, bolded).

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Expected Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA-1</td>
<td>5'-AGATGGAGCAACAGTGCTGTGACC-3′</td>
<td>5'-GCTCTGATGCGGGCCAATGTGCGCC-3′</td>
<td>1039 bp</td>
</tr>
<tr>
<td>TA-2</td>
<td>5'-GCTCTGATGCGGGCCAATGTGCGCC-3′</td>
<td>5'-GCTCTGATGCGGGCCAATGTGCGCC-3′</td>
<td>1039 bp</td>
</tr>
<tr>
<td>TA-3</td>
<td>5'-CTGCGGAGGAGGTGGCTGAGTCG-3′</td>
<td>5'-GTGACGAGGAGGTGGCTGAGTCG-3′</td>
<td>1006 bp</td>
</tr>
<tr>
<td>TA-4</td>
<td>5'-CTGCGGAGGAGGTGGCTGAGTCG-3′</td>
<td>5'-GACGAGGAGGTGGCTGAGTCG-3′</td>
<td>1097 bp</td>
</tr>
<tr>
<td>TA-5</td>
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<td>5'-GACGAGGAGGTGGCTGAGTCG-3′</td>
<td>1097 bp</td>
</tr>
<tr>
<td>TA-6</td>
<td>5'-CTGCGGAGGAGGTGGCTGAGTCG-3′</td>
<td>5'-GACGAGGAGGTGGCTGAGTCG-3′</td>
<td>1097 bp</td>
</tr>
<tr>
<td>TA-7</td>
<td>5'-TTGACGAGGAGGTGGCTGAGTCG-3′</td>
<td>5'-GACGAGGAGGTGGCTGAGTCG-3′</td>
<td>1097 bp</td>
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<td>TA-8</td>
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<td>5'-GACGAGGAGGTGGCTGAGTCG-3′</td>
<td>1097 bp</td>
</tr>
<tr>
<td>TA-9</td>
<td>5'-CTGCGGAGGAGGTGGCTGAGTCG-3′</td>
<td>5'-GACGAGGAGGTGGCTGAGTCG-3′</td>
<td>1097 bp</td>
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<td>5'-GACGAGGAGGTGGCTGAGTCG-3′</td>
<td>1097 bp</td>
</tr>
<tr>
<td>NP-1, Inner</td>
<td>5'-AGATGGAGCAACAGTGCTGTGACC-3′</td>
<td>5'-GCTCTGATGCGGGCCAATGTGCGCC-3′</td>
<td>1039 bp</td>
</tr>
<tr>
<td>NP-2, Outer</td>
<td>5'-CTGCGGAGGAGGTGGCTGAGTCG-3′</td>
<td>5'-GCTCTGATGCGGGCCAATGTGCGCC-3′</td>
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</tr>
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### 2.8 Primers

However, the nested primer (NP) sets include both an outer (1st round) and inner (2nd round) primer pair.
Chapter 3

Results

3.1 The Full-Length Rat Heart Na\textsubscript{v}1.1 Sequence from Overlapping Segments

3.1.1 Optimization of PCR Template Concentrations

The brain and heart RNA samples used in this thesis were checked for integrity and purity. Figure 3A shows distinct 28S and 18S ribosomal RNA bands, along with a roughly 2 fold greater intensity of the 28S band. The ratio of UV absorbance at 260 versus 280 nm for heart RNA was 1.75 (Ht-1) or 1.84 (Ht-2) nm, and for brain RNA was 2.06 (Br) nm. Ht-2 RNA was used in all gels in this thesis comparing Na\textsubscript{v}1.1 bands from brain and heart. GAPDH bands provided an indication of the success of individual RT-PCR. Figure 3B shows representative bands produced from the cDNA used in brain and heart comparisons.

Initial experiments attempted to amplify cDNA from rat right ventricular cardiomyocyte isolations using overlapping TA cloning primers covering the full-length Na\textsubscript{v}1.1 (TA-1 through TA-6, for primer details see Methods, Table 1). These experiments were first run using the lowest recommended quantity (ng) of RNA for RT-PCR and the lowest estimated cDNA volume recommended for PCR (HotStar HiFidelity polymerase). Rat brain was used as a positive control and produced bands of the expected sizes (883 to 1363 bp) (Figure 4A). In contrast, no bands were produced with repeated RT-PCR (N=6) from the cardiomyocyte isolations (e.g. Figure 4B). Stepped increases in template concentration did not produce bands from the cardiomyocyte isolations until the quantity of RNA used in the first strand synthesis was doubled (1000 ng) and the volume of cDNA used in the PCR was increased 3 fold to 6 µl (Figure 4C). Figure 4C shows that the bands produced from cardiomyocyte isolations with this elevated template concentration were not as strong as the bands produced from rat brain using the lowest recommended template concentration. The cardiomyocyte template concentration was then further
Figure 3: Comparison of the Quality of RNA Samples from Rat Heart and Brain

A Denaturing agarose gel electrophoresis of RNA from two different right ventricular cardiomyocyte isolations (Ht-1 & Ht-2) and from whole brain (Br). Bands consistent with those for 18S and 28S ribosomal RNA are labelled.

B RNA samples shown in panel A were reverse transcribed (RT) and PCR amplified using rat GAPDH primers. Panel B shows the resulting PCR bands from all cDNA used in this thesis comparing heart and brain. PCR bands from heart (Ht-2A & Ht-2B) were both produced from the same RNA sample (Ht-2). Size indicators, in kb, are marked on the ladder. The expected size for GAPDH bands from mRNA is 265 bp.

The gels shown in this figure and all gels in subsequent figures in this thesis, have been modified to remove lanes with data that is not important to the point being made.
Figure 4: Template Concentrations Required to Produce Na\(_v\)1.1 RT-PCR Bands

**A** RT-PCR of 6 overlapping segments that span the full-length coding sequence from rat brain (primers TA-1 through TA-6).

**B** Representative data from attempts to RT-PCR Na\(_v\)1.1 from rat heart, with the same template concentrations used for rat brain shown in panel A.

**C** RT-PCR of the first, second and third segments of Na\(_v\)1.1 transcript (primers TA-1, TA-2 & TA-3) from rat heart (Ht) and rat brain (Br). Heart PCR used cDNA produced with 2 fold ng of RNA and used 3 fold µl of cDNA in each reaction.

**D** RT-PCR of the first segment of Na\(_v\)1.1 transcript (primer TA-1) from rat heart after an additional doubling of the ng of RNA used in the production of cDNA, along with the 3-fold µl of cDNA in the PCR reaction.

Panels **A**, **C** & **D** include positive (GAPDH) and negative (no RT enzyme) controls. Panel **B** includes a negative (no template) control. All heart RNA is from right ventricular cardiomyocyte cell isolations. Size indicators, in kb, are marked on the ladder.
increased for all PCR reported in this thesis (e.g. Figure 4D). As compared with the lowest recommended levels, this selected template concentration involves a 4 fold increase in the quantity of RNA used in first stand synthesis (2000 ng) and a 3 fold increase in the volume of cDNA used in the PCR (6 µl).

3.1.2 Point Nucleotide Differences from the Rat Brain Published Sequence

Following optimization of PCR conditions, rat heart Na\textsubscript{v}1.1 segments were sequenced and compared to the previously published rat brain sequence (Noda et al, 1986a; Accession #NM_030875.1). We identified four point nucleotide differences: 1) c.2935A>G (Figure 5A); 2) c.3483G>A (Figure 5B); 3) c.5172G>C (Figure 5C); and 4) c.5373T>C (Figure 5D). All of these point nucleotide differences are silent with the exception of c.2935A>G, which would translate into an amino acid shift from arginine (AGG) to glycine (GGG) at amino acid position 979 (Arg979Gly).

To further explore the amino acid switch at position 979, a segment of the nucleotide sequence surrounding the nucleotide switch at position +2935 was PCR amplified from rat brain mRNA and from genomic DNA. Nested PCR (brain primer pairs NP-5 & genomic primer pairs NP-6, for primer details see Methods, Table 1) used the Multiplex PCR system reaction components common to all nested and hemi-nested PCR in this thesis with one exception. Two µl of dilute (1:10) Qiagen HotStar HiFidelity taq was added to improve the fidelity of nucleotide incorporation. Fifty µl first round amplifications used 6 µl of rat brain cDNA or rat genomic DNA as template, 20 amplification cycles and a high annealing temperature of 68\(^0\) C, while second round amplifications used 2 µl of first round PCR amplicons as template, 33 amplification cycles and an annealing temperature of 60\(^0\) C.

cDNA from 3 PCR (41 µl each) was superloaded in a double well. Two superloaded lanes for each of genomic and brain DNA were then cut from an EtBr-free gel without UV exposure, purified (Wizard Gel and PCR Clean-up System), and sent without cloning for sequencing (ACGT Corp.). Both showed consensus sequences for the same glycine codon (GGG) at amino acid position 979 that was found in rat heart mRNA.

To provide additional evidence for the amino acid switch, we conducted a BLAST search of the
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**Figure 5: Identification of Point Nucleotide Differences**

Sequencing results showing four nucleotide differences from the published rat brain sequence: A) c.2935A>G (N=2); B) c.3483G>A (N=4); C) c.5172G>C (N=2); and D) c.5373T>C (N=2). Sequence numbering is relative to the translational start site. N-values refer to the number of observations from independent RT-PCR experiments.
NCBI rat EST database (http://blast.ncbi.nlm.nih.gov) against the published Na\textsubscript{V}1.1 sequence. The search produced two matches that cover the transcript section of interest. GenBank accession numbers FM065514.1 and CB708982.1 are both rat EST’s with 86% identity to the published Na\textsubscript{V}1.1 sequence, suggesting that these sequences belong to VGSC. In both EST, the codon at the position corresponding to that for amino acid 979 in Na\textsubscript{V}1.1 is GGC. While the corresponding codon found in the present study is GGG, both code for glycine.

3.1.3 Deletion Variants

Cloning and sequencing the full-length Na\textsubscript{V}1.1 coding sequence in overlapping segments from rat right ventricular cardiomyocytes, first with TA cloning and then with digestion cloning, identified four potential deletions over three deletion sites. A 208 bp deletion involving conserved channel sequence (c.[del266_473]; Figure 6A) was found at the first deletion site. A 33 bp deletion that does not involve conserved channel sequence (c.[del2012_2044]; Figure 6B) was found at the second deletion site. Two possible deletions of 255 bp (c.[del4004_4258]; Figure 6C) or 282 bp (c.[del4003_4284]; Figure 6C) were found at the third deletion site, and both involved conserved channel sequence. It is important to note that each of the involved segments was also found in its expected form with no deletion.

The NCBI Reference Sequence for Na\textsubscript{V}1.1 (NM_030875.1) from rat brain was BLAST searched against the rat genomic sequence (GenBank RGSC v3.4) to examine exon/intron structure and to identify splice sites around the first and third deletion sites. Alignment results were used as a starting point to locate 5’intronic splice donor site consensus sequence (GT or GC) and 3’intronic splice acceptor site consensus sequence (AG), and the result was cross checked with the cDNA sequence.

All deletion variants at the first and the third deletion sites were coincident with splice sites with one exception. The rat genomic sequence suggests that the 3’end of c.[del4004_4258] is internal to exon 21. The consensus acceptor splice site AG, is not present at this location within the exon. NCBI BLAST searches were used to look for supporting evidence of an alternate splice acceptor site at this location within exon 21. A sequence composed of the 128 bp before and the 151 bp after the 255 bp
Figure 6: Deletion Variants

Sections of nucleotide sequence surrounding three deletion sites. **A** shows the deletion c.[del266_473] (N=2); **B** shows the deletion c.[del2012_2044] (N=1); and **C** shows two possible deletions: c.[del4004_4258] (N=2) (black underlined) & c.[del4003_4284] (N=1) (both black & red underlined). Sequence numbering is relative to the translational start site. N values refer to results from independent RT-PCR experiments.
c.[del4004_4258] deletion, was searched against the NCBI EST and RefSeq databases for each of human, rat and mouse. No matches were found. A repeat of these searches after removal of the entire exon 21 (c.[del4003_4284]) from the query sequence, also did not produce a match. Finally, the full-length Nav1.1 Reference Sequence was searched against these same databases. This process produced a high number of sequence matches covering the third deletion site, however, none of these sequences showed a deletion at this site. Of note, none of the sequence matches were from heart.

After the initial finding of the first deletion site (c.[del266_473]), additional TA cloning primer pairs were designed for RT-PCR around this site (TA-7, TA-8 & TA-9) (for primer details see Methods, Table 1). One primer pair from the digestion cloning experiments (D-1) also amplified around this site. Figure 7 provides representative examples of the multiple band patterns produced by each of 11 PCR. This pattern was observed using primer pairs TA-1 (Figure 7A), D-1 (Figure 7B), TA-7 (Figure 7C), TA-8 (Figure 7C), and TA-9 (Figure 7C). The band at the expected size for transcript (i.e. no deletion) was predominant in the results of 6 of 11 PCR, including the 3 PCR results shown in Figure 7C. All bands shown were sequenced and confirmed to be either the expected product (i.e. no deletion) or transcript with c.[del266_473], with the exception of the smaller band produced by TA-9. This band was not sequenced, however, its size is consistent with the presence of the deletion.

The third deletion site was sequenced both with c.[del4004_4258] and c.[del4003_4284]. However, the initial experiments using overlapping TA cloning primer pairs covering the full-length Na\textsubscript{v}1.1 did not produce double bands with amplifications of this segment of Na\textsubscript{v}1.1 (Figure 8). The sequence used for both the design of the TA-4 reverse and the TA-5 forward primers is internal to the deleted region. Figure 8A shows the PCR amplicons of the TA-1 through TA-6 primer pairs covering the full-length Na\textsubscript{v}1.1 transcript. Double bands are produced with amplification around the first deletion site (TA-1 primer pair), as compared with single bands produced with both the TA-4 and the TA-5 primer pairs. Figure 8B shows the results of a second PCR using TA-4 through TA-6 primer pairs, and again showing single bands produced by both the TA-4 and TA-5 primer pairs.
Figure 7: RT-PCR Around the First Deletion Site.

Representative samples of RT-PCR around the c.[del266_473] deletion site using different TA cloning and digestion cloning primer pairs.

A  Primer pair TA-1 with an expected product size of 1039 bp.
B  Primer pair D-1 with an expected product size of 897 bp.
C  Primer pair TA-7 with an expected product size of 1363 bp, TA-8 with an expected product size of 883 bp, and TA-9 with an expected product size of 1215 bp.

RNA was from right ventricular cardiomyocyte cell isolations. Size indicators, in kb, are marked on the ladder.
Figure 8: RT-PCR Using Overlapping TA Cloning Primers

A RT-PCR of the full-length Na\textsubscript{v}1.1 transcript (primer pairs TA-1 through TA-6).

B RT-PCR of the second half of the Na\textsubscript{v}1.1 transcript (primer pairs TA-4 through TA-6).

RNA was from right ventricular cardiomyocyte cell isolations. Size indicators, in kb, are marked on the ladder.
Seven PCR amplifications were completed around the c.[del4004_4258]/c.[del4003_4284] deletion site using the digestion cloning primer pair D-5. All 7 of these gels displayed at least two bands, with one matching the expected size and another at a size consistent with transcript with one of the two deletions (Figures 9A-C). Transcript with and without a deletion was sequenced from PCR amplicons shown in Figures 9A and 9C, as well as from PCR amplicons shown in lane 1 of Figure 9B. The deletion sequenced from PCR amplicons shown in Figure 9A and lane 1 of Figure 9B was c.[del4004_4258]; whereas the deletion sequenced from PCR amplicons shown in Figure 9C was c.[del4003_4284]. In contrast with PCR results at the c.[del266_473] site, the more predominant bands in these gels are consistent with the size of transcript with a deletion.

Nested primer pairs were designed around each of the c.[del266_473] and c.[del4004_4258]/c.[del4003_4284] deletion sites for use with the lower template concentrations in single cell PCR. Prior to use in single cell PCR experiments, these primer pairs were optimized against cDNA from cardiomyocyte isolations using systematic changes in template concentration, the number of second round amplification cycles, and annealing temperatures. Along with the use of different primers, these optimization experiments used the Multiplex System with Taq polymerase, as compared with the HotStar HiFidelity polymerase and reaction components. The resulting data (Figure 10) provides an additional look at the relative ability of RT-PCR to amplify expected size transcript versus transcript with a deletion from isolated cardiomyocytes. With stepped reductions in template concentration, one of two optimization experiments (Figure 10B with higher annealing temperatures versus Figure 10A) showed that bands consistent with the size of transcript with c.[del266_473] were lost prior to the loss of bands at the expected size for transcript with no deletion. In contrast, bands at the expected size for transcript with no deletion at the c.[del4004_4258]/c.[del4003_4284] deletion site, were lost prior to the loss of bands at a size consistent with this segment of transcript with one of the two possible deletions (Figures 10A & 10B). Figure 10C provides data from an additional optimization experiment around c.[del4004_4258]/c.[del4003_4284]. A third optimization condition, the number of second round
Figure 9: RT-PCR Around the Third Deletion Site.
Representative gels of PCR amplifications performed around the c.[del4004_4258]/c.[del4003_4284] deletion site using primer pair D-5.
A & B Illustrate the double band pattern with an expected product size of 1006 bp.
C The band pattern from PCR amplicons that has been digested for cloning with an expected size for digested amplicons of 897 bp.

RNA was from right ventricular cardiomyocyte cell isolations. Size indicators, in kb, are marked on the ladder.
Figure 10: RT-PCR Around the c.[del266_473] and c.[del4004_4258]/c.[del4003_4284] Deletion Sites

Optimization of nested primer pairs around the c.[del266_473] deletion site (A & B—NP-2, expected band size 883 bp vs size of transcript with the deletion 675 bp) and around the c.[del4004_4258]/c.[del4003_4284] deletion site (A, B & C—NP-3, expected band size 727 bp vs size of transcript with a deletion 445 or 472 bp). Experiments used the Multiplex System containing Taq polymerase, as opposed to the HotStar HiFidelity enzyme used in the experiments reported above (Figures 7 & 9).

A, B & C The effect of graded dilutions of cDNA (A & B—1:10 through 1:3000; C—1:5 through 1:100).

A vs B The effect of changes in annealing temperature (A, 1st round—68°C 5 cycles, 63°C 15 cycles, 2nd round—60°C; B, 1st round—70°C 5 cycles, 65°C 15 cycles, 2nd round—65°C).

C The effect of increases in the 2nd round cycle number (30 through 45 rounds) across different annealing temperatures (lanes 1 through 14—68°C for both 1st & 2nd round amplifications; lanes 15 through 18—70°C for the first 5 cycles of the 1st round PCR, with 65°C for all remaining amplification cycles).

RNA was from right ventricular cardiomyocyte cell isolations. Size indicators, in kb, are marked on the ladders.
amplification cycles, is included. Across all PCR conditions, there is a band at a size consistent with transcript with one of the two deletions at this deletion site, and in each RT-PCR result this band is either the only band or it is the predominant band.

3.2 Single Cell Experiments

For heart experiments reported so far, we used isolated cardiac myocytes. Although the isolation procedure preferentially selects for cardiac myocytes, contamination of our RNA samples with RNA from other cardiac cells cannot be ruled out. To address this concern, we used the technique of single cell PCR. Nested primer pairs and the Multiplex PCR system including the HotStar taq polymerase, were used to amplify around the c.[del266_473] (NP-1 & NP-2) and the c.[del4004_4258]/c.[del4003_4284] (NP-3) deletion sites (for primer details see Methods, Table 1). Multiplex PCR protocol parameters used for nested PCR are described in Methods, and modifications used for this set of experiments are described here. First round 100 µl single cell PCR (i.e. outer primers) used 10 µl of first strand product as template, an annealing temperature of 60°C, and 20 amplification cycles. Second round 50 µl amplifications (i.e. inner primers) used 4 µl of first round PCR product as template, an annealing temperature of 55°C, and 35 amplification cycles. Two variations in the second round amplifications were used in some experiments. Amplifications around c.[del266_473] shown in Figure 11B used the HotStar HiFidelity polymerase, primers and protocol as described in Methods. As well amplifications around each of the c.[del266_473] and c.[del4004_4258]/c.[del4003_4284] deletion sites, were repeated with 40 amplification cycles (Figures 11B & 12B). All gels used to display single cell amplicons contained 1.0 vs 0.5 µg/ml EtBr.

Ten of 11 cells used for single cell PCR amplification around the c.[del266_473] deletion site (Figure 11) and all 8 cells used for single cell PCR amplification around the c.[del4004_4258]/c.[del4003_4284] deletion site (Figure 12), produced at least one band at a size consistent with one of the sequenced versions of the NaV1.1 segment. However, these bands were made
Figure 11: RT-PCR of Na\textsubscript{v}1.1 Transcript from Single Cardiomyocytes: the c.[del266_473] Deletion Site

RNA was isolated individually from each of 10 cardiomyocytes, reverse transcribed immediately upon harvesting, and PCR amplified. PCR used nested primer pair NP-1 (panels A & B—expected band size for transcript with no deletion is 1039 bp), or nested primer pair NP-2 (panel C—expected band size for transcript with no deletion is 883 bp).

PCR were performed with (I) or without (II) Qiagen’s “Q solution”. Size standards (panel C) are PCR bands produced using the same nested primer pairs against cDNA produced from cardiomyocyte isolations. RT-PCR bands from cardiomyocyte isolations were chosen for use as size standards based on the level of clarity of both bands (i.e. the expected size band and the band consistent with transcript with the deletion). Panel B used HotStar HiFidelity polymerase vs HotStar taq polymerase, and 40 vs 35 2\textsuperscript{nd} round amplification cycles. 37.5 (panel A), 12.5 (panel B) or 50 µl (panel C) of the single cell PCR amplicons were loaded per lane on a gel with 1.0 µg/ml EtBr. The rat brain positive control in panel B used a 1:1000 dilution of cDNA as template.

Size indicators, in kb, are marked on the ladders.
Figure 12: RT-PCR of Na\textsubscript{v}1.1 Transcript from Single Cardiomyocytes: the c.[del4004_4258]/c.[del4003_4284] Deletion Site

RNA was isolated from individual cardiomyocytes, reverse transcribed immediately upon harvesting, and PCR amplified. The cDNA produced from cells 4 through 11 in Figure 11C, was PCR amplified using nested primer pair NP-3 with an expected band size for transcript with no deletion of 727 bp.

Size standards are PCR bands produced with the same nested primer pairs against cDNA from cardiomyocyte cell isolations. Experiments in panel B used 40 vs 35 2\textsuperscript{nd} round PCR cyles. 9 µl/lane were loaded of the standards, in comparison with 50 (panel A) or 60 (panel B) µl/lane of the single cell PCR amplicons.

Size indicators, in kb, are marked on the ladder.
visible by superloading the gel with the PCR amplicons and the cDNA concentrations obtained (i.e. 0.13 ng/µl) were too low for ligation and sequencing.

The strongest bands in Figure 11 (panels A, B & C) are produced from cells 1, 2, 6, 7, 8 and 9, and in all 6 of these cells the band at a size consistent with the presence of c.[del266_473] is either the only band or is the predominant band. Weaker bands produced from three additional cells (cells 5, 10 & 11) support the predominance of transcript with this deletion. Only one cell (cell 3) produced a more predominant band at the expected size for no deletion.

Despite two attempts, only 5 of 8 cells produced at least one strong band with amplification around the c.[del4004_4258]/c.[del4003_4284] deletion site (Figures 12A & 12B). These 5 cells were roughly split in terms of which size of band was the only or the most predominant band. Both the predominant band from one cell (cell 10, Figure 12A) and the only band from another cell (cell 9, Figure 12B), match the expected size for Na\textsubscript{V}1.1 with no deletion. On the other hand, 3 cells (cells 5, 6 & 7, Figure 12B) produced a single band consistent with transcript with c.[del4004_4258] or c.[del4003_4284].

### 3.3 Rat Brain Experiments

To determine whether the deletions found in rat heart were also present in rat brain, additional experiments were conducted. Initially, the full-length rat brain Na\textsubscript{V}1.1 transcript was PCR amplified using the TA overlapping set of primers pairs (TA-1 through TA-6, for primer details see Table 1 in Methods). As discussed above, these primers are unable to detect Na\textsubscript{V}1.1 transcript with c.[del4004_4258] or c.[del4003_4284]. However, Figure 13A shows only a single band of the expected size for transcript without c.[del266_473] produced from rat brain with the TA-1 primer pair. Figure 13B using the same primer pair (TA-1) and Figure 13C using three additional primer pairs that amplify around the c.[del266_473] deletion site (TA-7, TA-8 & TA-9), show side-by-side results for RT-PCR from brain and heart. With the exception of a possible second band at a size consistent with the presence of the
Figure 13: Comparison of Brain and Heart RT-PCR Around the c.[del266_473] Deletion Site

A & B  Panel A shows RT-PCR bands from brain produced with overlapping TA cloning primer pairs covering the full-length of Na\textsubscript{v}1.1 transcript (TA-1 through TA-6). Panel B shows side-by-side RT-PCR bands from heart and brain, produced with overlapping TA cloning primer pairs covering roughly the first half of Na\textsubscript{v}1.1 transcript (TA-1, TA-2 & TA-3). The TA-1 primer pair (boxes) amplifies the segment of transcript containing the c.[del266_473] deletion site, and has an expected size for transcript with no deletion of 1039 bp.

C  Side-by-side RT-PCR bands from brain and heart produced with 3 additional primer pairs designed to amplify the segment of transcript containing the c.[del266_473] deletion site. Expected sizes for transcript with no deletion are 1363 bp for primer pair TA-7, 883 bp for primer pair TA-8, & 1215 bp for primer pair TA-9.

Brain PCR used 2 µl of cDNA produced with 500 ng of RNA: heart PCR used 6 µl of cDNA produced with 1000 ng of RNA (panel B) or 6 µl of cDNA produced with 2000 ng of RNA (panel C).

Size indicators, in kb, are marked on the ladder.
deletion in Figure 13B, single bands matching the expected size for transcript with no deletion are produced from brain. All four primer pairs produced double bands from the heart (Figures 13B & 13C). These gels also show more predominant bands matching the expected size for transcript with no deletion from brain as compared with from heart. All brain RT-PCR in Figure 13 used 500 ng of RNA in the RT and 2 µl of cDNA in the PCR; whereas heart RT-PCR used 1000 or 2000 ng of RNA in the RT and 6 µl of cDNA in the PCR. The bands from brain in Figure 13 were not sequenced, however, all three gels were produced with the same primer pairs that were used to produce the rat heart RT-PCR amplicons shown above in Figures 7A and 7D (TA-1 in Figures 7A, 13A & 13B; TA-7 in Figures 7D & 13C; TA-8 in Figures 7D & 13C; & TA-9 in Figures 7D & 13C). All rat heart bands in Figures 7A and 7D that were sequenced (7 of 8 bands), were Na\textsubscript{v}1.1 with or without c.[del266_473].

Figure 14 shows the results of brain RT-PCR around the c.[del266_473] deletion site and also around the c.[del4004_4258]/c.[del4003_4284] deletion site, using digestion cloning primer pairs D-1 and D-5 (for primer details see Methods, Table 1). There is a possible second band at a size consistent with the presence of c.[del266_473], when the template concentration is the same as that used in the brain RT-PCR of the previous experiment (500 ng & RNA 2 µl cDNA, solid arrow). This band shadow becomes a distinct band with the first step increase to the lowest template concentration previously shown capable of producing Na\textsubscript{v}1.1 bands from the heart (Figure 4, 1000 ng RNA & 6 µl cDNA). RT-PCR around the c.[del4004_4258]/c.[del4003_4284] deletion site produced 2 faint bands at sizes consistent with the presence of c.[del4004_4258] and c.[del4003_4284] in addition to a band at the expected size for transcript with no deletion, when the lowest template concentration (500 ng RNA & 2 µl cDNA, solid arrow) was used. Beginning at the second template concentration (1000 ng of RNA & 6 µl of cDNA), an additional faint band appears. This band represents transcript that is smaller than expected for no deletion, and larger than the size of transcript with either c.[del4004_4258] or c.[del4003_4284].

The band matching the expected size for transcript with no deletion is more predominant in RT-PCR from the brain as compared with in the RT-PCR from the heart presented above (Figure 7) at the
Figure 14: RT-PCR Around the c.[del266_473] and the c.[del4004_4258]/c.[del4003_4284] Deletion Sites With Graded Increases in Brain Template

Template concentrations used were 500 ng of RNA and 2 µl of cDNA (solid arrows), 1000 ng of RNA and 6 µl of cDNA, 1500 ng of RNA and 6 µl of cDNA, 2000 ng of RNA and 6 µl of cDNA (arrow outlines), and 2000 ng of RNA and 10 µl of cDNA. Primer pair D-1 was used to amplify around the c.[del266_473] deletion site (expected size with no deletion 897 bp: expected size with deletion 689 bp). Primer pair D-5 was used to amplify around the c.[del4004_4258]/c.[del4003_4284] deletion site (expected size with no deletion 1006 bp: expected size with a deletion 751 or 724 bp).

Size indicators, in kb, are marked on the ladder.
c.[del266_473] deletion site. This predominance is also observed at the c.[del4004_4258]/c.[del4003_4284] deletion site where the RT-PCR from the heart presented above (Figure 9) show these bands to be of equal or lesser strength as compared with bands matching the size of transcript with a deletion. The bands in Figure 14 were not sequenced. However, previous experiments suggest that the digestion cloning primers are specific for Na\textsubscript{v}1.1. All clones sequenced from amplicons of both D-1 (c.[del266_473] deletion site) and D-5 (c.[del4004_4258]/c.[del4003_4284] deletion site) primer pairs were Na\textsubscript{v}1.1, and these clones were products of two (D-1, e.g. Figure 7B) or three (D-5, Figures 9A, 9B & lane 1 of 9C) different RT-PCR.

3.4 Cloning Full-Length and Near Full-Length Na\textsubscript{v}1.1

As the deletions found at the c.[del266_473] and c.[del4004_4258]/c.[del4003_4284] deletion sites remove conserved segments of the transcript, experiments were designed to discover whether Na\textsubscript{v}1.1 transcript exists in the heart with no deletion at both of these sites. Initial efforts to answer this question involved the cloning of two long RT-PCR amplicons: the full-length and a near full-length Na\textsubscript{v}1.1 transcript.

Three unrelated experimental results described here were considered in the experimental design of the long product cloning experiments. Firstly, efforts to build a Na\textsubscript{v}1.1 clone for expression and patch clamp studies, produced an unusual result. pcDNA3.1V5HisB plasmids sequenced to show successful ligation of 1 of 4 Na\textsubscript{v}1.1 segments, were digestion opened and used in a four-piece ligation with the other 3 Na\textsubscript{v}1.1 segments. A total of 4 clones appeared on the sample plates of 3 ligation/transformation experiments. Figures 15A and 15B show the plasmids from these 4 clones run out on a gel. The plasmids from each of the 4 clones from sample plates were smaller than the “control” plasmids containing 1 of 4 Na\textsubscript{v}1.1 segments.

Secondly, digestion cloning and sequencing of the full-length Na\textsubscript{v}1.1 transcript in overlapping segments produced one result that is reported above with the complete exon 21 missing (Figure 16). This
Figure 15: Ligation of Full-length Na\textsubscript{v}1.1

A four-piece ligation was used to assemble the full-length Na\textsubscript{v}1.1 transcript. Panels A and B show uncut plasmids from all 4 clones on sample plates from 3 ligation/transformation experiments. Control plasmids in these experiments are pcDNA3.1V5HisB with 1 of 4 segments of the full-length Na\textsubscript{v}1.1 transcript inserted.

Size indicators, in kb, are marked on the ladder.
Figure 16: Recombination Events in a Clone with the Complete Exon 21 Removed

One unconfirmed cloning and sequencing result with complete removal of exon 21 (blue) and evidence of three recombination events. This sequencing result begins at +3487 relative to the translational start site. The c.[del4003_4284] deletion is followed by:

first, the 22 nucleotides in the published (Noda et al, 1986a) sequence that follow exon 21;

second, an upstream section of the cloned Na\textsubscript{v}1.1 segment (purple);

third, the reverse complement of the section of the cloned Na\textsubscript{v}1.1 expected to follow the 22 nucleotides from the published sequence (green) including the sequence for the reverse primer used to produce the insert (underlined); and

fourth, 109 bp with 100\% identity to a section of the expression vector pIB-NC-EGFP (GenBank A cc. # KF111246.1) (red).
sequencing result also contained evidence of three recombination events. Two of these events involved parts of the Na\textsubscript{v}1.1 transcript. The third involved a sequence that is not part of the pcDNA3.1V5HisB plasmid or of the Na\textsubscript{v}1.1 insert.

Finally, amplification of a segment of Na\textsubscript{v}1.1 sequence that includes the point nucleotide difference at position +2935, both from genomic DNA (PCR) and from brain mRNA (RT-PCR), produced an amplicon that appears to form secondary structures (Figure 17). The genomic sequence includes a 381 bp intron. Amplicons produced 4 band patterns that were slightly different, depending on the DNA template (Fig. 17A—genomic DNA; Fig. 17B—brain mRNA). In both gels, the band that travels the furthest within each lane matches the expected size. The PCR reactions contained Qiagen’s Q solution (according to the provided protocol 10 µl in 50 µl reactions) marketed for templates with high GC content or secondary structures (Figures 17A & 17B). Removal of Q solution did not change the band pattern produced (Figure 17B). However, replacing the Q solution and 7 µl of H\textsubscript{2}O with the maximal recommended amount of betaine (17 µl of a 5M solution in a 50 µl reaction), another agent marketed for use with templates with high GC content or secondary structures (Sigma Aldrich; see Rees et al, 1993), reduced the multiple band pattern to a single band at the expected size (Figure 17B).

RT-PCR of the full-length ~6.1 kb transcript (5871 to 6367 bp depending on the deletions present) in one piece (D-1 forward and D-7 reverse primer pairs, for primer details see Methods, Table 1) provided two results consistent with successful Na\textsubscript{v}1.1 amplification. Neither result was repeatable. The strongest ~6.1 kb RT-PCR result is shown in Figure 18, and suggests two bands within a size range of 6 to 6.2 kb. This RT-PCR amplicon was then reamplified using the same primer pairs and cut from a gel. Three ligation/transformation experiments produced a total of 2 colonies, both with plasmids matching the size of the negative control plasmid.

Given the problems working with the full-length transcript, the digestion cloning primers D-1 forward and D-5 reverse were then used to PCR amplify a smaller segment (~4.3 kb) that included both the c.[del266_473] and c.[del4004_4258]/c.[del4003_4284] deletion sites (for primer details see Methods,
Figure 17: Amplification of Nav1.1 Genomic DNA and Brain mRNA Around the Coding Sequence Nucleotide Position +2935

Amplification of a segment of genomic DNA (panel A, PCR) and of brain mRNA (panel B, RT-PCR) around the point nucleotide difference from the NCBI rat brain reference sequence at position +2935 relative to the translation start site (+2751 through and including +3431). Expected band size for genomic DNA is 999 bp: expected band size for brain cDNA is 681 bp. PCR for panels A and B are performed with Q solution (10 µl per 50 µl reaction), no additive, or with the maximal amount of betaine (17 µl of 5M in a 50 µl reaction).

Size indicators, in kb, are marked on the ladder.
Considering that transcript may have the 33 bp deletion (c.[del2012_2044], second deletion site), the expected size of transcript with no deletion at the c.[del266_473] and the c.[del4004_4258]/c.[del4003_4284] deletion sites is 6334 or 6367 bp. Transcript with c.[del266_473]) and no deletion at the third deletion site would be 6126 or 6159 bp. In the reverse, transcript with c.[del4004_4258] or c[del4003_4284] and no deletion at the first deletion site, would be 6052 or 6079 with the 33 bp deletion, as compared with 6085 or 6112 bp without the 33 bp deletion. Transcript with a deletion at both the first and the third deletion sites would be 5871 or 5904 bp.

RNA was from right ventricular cardiomyocyte cell isolations. Size indicators, in kb, are marked on the ladder.
Table 1). These RT-PCR produced either 1 (2 reactions) or 2 (3 reactions) bands. Twenty-nine µl of the ~4.3 kb amplicons from each of 2 RT-PCR that produced double bands were combined and run on a 0.7% extra-long agarose gel (25 cm long Subcell Model 192 Cell, BioRad Laboratories (Canada) Ltd., Mississauga, ON) with EtBr. Three bands showed on the gel (Figure 19), with sizes of ~4075 bp, ~4200 bp and ~4500 bp. These were run at 30 V in a cold room for 60 hours for separation. The gel was repetitively exposed to UV light in order to monitor band migration, and to cut the bands individually from the gel.

With a low concentration of DNA recovered from the ~4500 bp band and no adjustment in the amount of vector used, ligation of this band used a 1:3 insert to vector molar ratio as compared with the 1:1 insert to vector molar ratio used for all other ~6.1 and ~4.3 kb band ligations. Ligation and transformation of each of the three ~4.3 kb bands produced white colonies (X-gal blue/white screening), however, digestion (NEB restriction enzymes—EcoRI and XhoI) results were inconclusive and fourteen clones (3 from the ~4075 bp band, 2 from the ~4200 bp band & 9 from the ~4500 bp band) were sent for sequencing.

No insert sequence was found in the multiple cloning site of any of the 14 colonies. Figure 20 shows representative sequencing results from two colonies. Both results show loss of plasmid sequence, including loss of one EcoRI digestion site in the first sequencing result and loss of both in the second. The second result includes evidence of a recombination event with addition of 23 unidentified nucleotides. Sequence specific primers (nested primer pair NP-2 and hemi-nested primer pair NP-4) against the plasmids were used to check for insert sequence outside of the multiple cloning site (for primer details see Methods, Table 1). No bands were produced from any of the 14 colonies.

3.5 Further Exploration of Full-Length Nav1.1 Transcript in the Heart

To further investigate the three ~4.3 kb bands that were individually cut from a gel (see above), two additional approaches were used. Copy DNA from each band was explored around the c.[del266_473] and the c.[del4004_4258]/c.[del4003_4284] deletion sites in two sets of experiments. The
Figure 19: RT-PCR of a Segment of Na\textsuperscript{+}1.1 Spanning All Three Deletion Sites

PCR used digestion cloning primers D-1 forward and D-5 reverse. The estimated size of the resultant bands are 4500 bp, 4200 bp and 4075 bp. Considering that transcript may have the 33 bp deletion (c.[del2012_2044]), these sizes approximate the expected size of transcript with no deletion at either the c.[del266_473] or the c.[del4004_4258] deletion site (4571 or 4604 bp), with one deletion across these two sites (4396 or 4363 bp with only c.[del266_473]; 4349 or 4316 bp with only c.[del4004_4258]; 4322 or 4289 bp with only c.[del4004_4258]), and with a deletion at both the first and the third deletion sites (4141 or 4108 bp—c.[del4004_4258]; 4114 or 4081 bp—c.[del4003_4284]).

RNA was from right ventricular cardiomyocyte cell isolations. Amplicons were run on a 0.6% agarose gel. Size indicators, in kb, are marked on the ladder.
The multiple cloning site of the pCR2.1 plasmid used in TA cloning of ~4.3 kb \(Na_v 1.1\) RT-PCR bands (middle), along with 2 of 14 sequencing results (top & bottom). Both sequence results begin (vertical arrow on plasmid sequence) with two nucleotides of the T7 promoter and read in the direction of the horizontal arrow. One sequencing result (top) begins with the plasmid sequence shown in the yellow box on the plasmid map (highlighted in the sequencing result) which includes an EcoR1 digestion site (underlined in sequencing result), and then picks up immediately after the second EcoR1 digestion site with no insert sequence. The other sequencing result shows loss of most of the plasmid sequence in the yellow box including the first EcoR1 digestion site (shorter highlighted sequence in sequencing result), and after a 23 nt unidentified sequence, also continues immediately after the second EcoR1 digestion site.

**Figure 20: Representative Sequencing Results: Cloning the ~4.3 kb PCR Bands**

The multiple cloning site of the pCR2.1 plasmid used in TA cloning of ~4.3 kb \(Na_v 1.1\) RT-PCR bands (middle), along with 2 of 14 sequencing results (top & bottom). Both sequence results begin (vertical arrow on plasmid sequence) with two nucleotides of the T7 promoter and read in the direction of the horizontal arrow. One sequencing result (top) begins with the plasmid sequence shown in the yellow box on the plasmid map (highlighted in the sequencing result) which includes an EcoR1 digestion site (underlined in sequencing result), and then picks up immediately after the second EcoR1 digestion site with no insert sequence. The other sequencing result shows loss of most of the plasmid sequence in the yellow box including the first EcoR1 digestion site (shorter highlighted sequence in sequencing result), and after a 23 nt unidentified sequence, also continues immediately after the second EcoR1 digestion site.
bands were first used as template for PCR reamplifications around these two deletion sites, and where these PCR were successful, the amplicons were cloned and sequenced. Secondly, the cloning of each of the three individual ~4.3 kb bands was repeated, that is previously produced ligation product (see above) was retransformed. However, blue/white identified positive colonies were heat lysed according to directions used for colony PCR rather than being grown overnight in bacterial cultures. The reamplification PCR were then repeated against the resulting plasmids.

The Multiplex PCR system reaction components and nested or hemi-nested PCR (primer pairs NP-2 for amplification around the c.[del266_473] and NP-4 for amplification around the c.[del4004_4258]/c.[del4003_4284] deletion sites, for primer details see Methods, Table 1) were used for both series of PCR reamplification experiments with some exceptions. Two µl of dilute (1:10) Qiagen HotStar HiFidelity taq was added to improve the fidelity of nucleotide incorporation. Also PCR reamplifications of plasmids from blue/white-identified positive colonies for the ~4.3 kb inserts, were first performed with the Qiagen-provided Q solution (10 µl in the 50 µl reactions) as described above for all Multiplex PCR in this thesis, and then repeated with replacement of the Q solution with betaine (17 µl of 5M solution in 50 µl reactions). PCR reamplifications of the individual ~4.3 kb RT-PCR bands cut from a gel, used 20 amplification cycles for the first round PCR and 35 amplification cycles for second round PCR. A higher number of amplification cycles, i.e. 25 first round cycles and 40 second round cycles, was used for PCR reamplification of plasmids released from blue/white-identified positive colonies for the ~4.3 kb NaV1.1 inserts. All PCR reamplification experiments used an annealing temperature of 60° C for both rounds of PCR.

The PCR reamplification products from the ~4.3 kb bands were cloned and sequenced. Eighteen µl of each tube of PCR reamplification product from the individually cut ~4.3 kb RT-PCR bands, were run on a gel. The remaining 32 µl of all PCR reamplification products producing even a faint band or bands, were combined and superloaded on an EtBr-free gel (128 µl in one lane). A piece of gel which would capture all possible PCR reamplification product given the sequencing results from experiments
reported above, whether producing a visible band or not, was cut out without UV exposure and purified (Wizard Gel and PCR Clean Up System). Recovered cDNA was ligated using the NEB T4 DNA ligase and NEB protocol, and TA cloned using the pCR2.1 plasmid and One Shot INVαF (see Methods for genotype). The NEB ligation protocol calls for 2 μl of 10X NEB ligation buffer, 2 μl of 25 ng/μl pCR2.1 vector, and 1 μl of NEB T4 DNA ligase in a 20 μl reaction. Ligation reactions for these experiments used 40 ng of the PCR reamplification product, setting up roughly a 3:1 insert to vector molar ratio. Transformations were performed as described in Methods for TA cloning and digestion cloning of the full-length transcript in overlapping segments.

3.5.1 Reamplification of ~4.3 kb RT-PCR Bands

Figures 21A and 21B show a sample of the PCR bands produced with reamplification of the ~4075 bp band around the c.[del266_473] deletion site. All 14 PCR reactions that produced bands showed a band consistent with Nav1.1 transcript with c.[del266_473], i.e. 675 bp. Four of these reactions also produced a second faint band that approximated but did not appear to match, the expected size for Nav1.1 transcript with no deletion, i.e. 883 bp. Two of these showed a band that appears to be just over 800 bp, and a third gel (not shown) shows two PCR results with a second faint band that appears to be just under 800 bp. The remaining PCR amplicons from reactions producing bands, was combined, superloaded on a gel, and then cut from the gel in a manner that would capture all previously sequenced Nav1.1 deletion variants for cloning. Plasmid digestion released 21 bands at a size consistent with Nav1.1 transcript with c.[del266_473], and 1 band at the expected size for Nav1.1 transcript with no deletion. Both versions of the transcript (1 colony each) were sequenced. Attempts to reamplify the transcript around the c.[del4004_4258]/c.[del4003_4284] deletion site did not produce bands.

Figure 22 shows a sample of PCR bands from reamplification of the ~4200 bp band around the c.[del266_473] (Figure 22A) and around the c.[del4004_4258]/c.[del4003_4284] (22B & 22C) deletion sites. Figure 22A shows 5 of a total of 12 bands produced with amplification around c.[del266_473], with all 12 at a size (~675 bp) consistent with Nav1.1 transcript with this deletion. Each of the 12 bands
Figure 21: Reamplification of the ~4075 bp Band Around the c.[del266_473] Deletion Site

Seven results across panels A (lanes 2, 3 & 5) and B (lanes 1, 3, 4 & 6) from a total of 14 reamplifications. Nested primer pair NP-2 was used with an expected size for NaV1.1 with no deletion of 883 bp, and an expected size for NaV1.1 with c.[del266_473] of 675 bp.

Size indicators, in kb, are marked on the ladder. For primer details see Methods, Table 1.
Figure 22: Reamplification of the ~4200 bp Band Around the c.[del266_473] and the c.[del4004_4258]/c.[del4003_4284] Deletion Sites

A Shows 5 of 12 bands produced with reamplification of the ~4200 bp band around c.[del266_473] (lanes 1 (faint), 2, 4, 5 & 6). Nested primer pair NP-2 was used with an expected size for Na\(_V\)1.1 with no deletion of 883 bp, and an expected size for Na\(_V\)1.1 with c.[del266_473] of 675 bp.

B and C Show the 3 strongest of 5 bands produced with reamplification of the ~4200 bp around the c.[del4004_4258]/c.[del4003_4284] deletion site (panel B, lane 1; panel C, lanes 1 & 4). Nested primer pair NP-4 was used with an expected size for Na\(_V\)1.1 with no deletion of 727 bp, an expected size for Na\(_V\)1.1 with c.[del4004_4258] of 472 bp, and an expected size for Na\(_V\)1.1 with c.[del4003_4284] of 445 bp.

Size indicators, in kb, are marked on the ladder. For primer details see Methods, Table 1.
appeared in otherwise clean lanes; no bands were produced at the expected size (883 bp) for Na\textsubscript{v}1.1 transcript with no deletion in any of the results. The remaining PCR amplicons from reactions producing bands, was combined, superloaded on a gel, and then cut from the gel in a manner that would capture all previously sequenced Na\textsubscript{v}1.1 deletion variants for cloning. Plasmid digestion released 7 bands, all matching the size of Na\textsubscript{v}1.1 transcript with c[del266_473], and this was confirmed through sequencing (1 colony).

Figures 22B and 22C show the three strongest of a total of five bands at a size consistent with Na\textsubscript{v}1.1 transcript with either c.[del4004_4258] (472 bp) or c.[del4003_4284] (445 bp). Each of the 5 bands appeared in otherwise clean lanes; no bands were produced across at the expected size (727 bp) for Na\textsubscript{v}1.1 transcript with no deletion in any of the results. The remaining PCR amplicons from reactions producing bands, was combined, superloaded on a gel, and then cut from the gel in a manner that would capture all previously sequenced Na\textsubscript{v}1.1 deletion variants cloning. Plasmid digestion released 10 bands, all matching the size of Na\textsubscript{v}1.1 transcript with c[del4004_4258] or c.[del4003_4284], and c.[del4004_4258] was confirmed through sequencing (1 colony).

Figure 23 shows a sample of the PCR bands resulting from reamplification of the ~4500 bp band around the c.[del266_473] deletion site. Five PCR produced bands at a size (~675 bp) consistent with Na\textsubscript{v}1.1 with c.[del266_473]. In comparison, two PCR produced a PCR band at the expected size (~883 bp) for Na\textsubscript{v}1.1 with no deletion. Remarkably, no lane in either gel showed bands at both potential product sizes. Lane 7 with the band at the expected size for Na\textsubscript{v}1.1 with no deletion, also showed the appearance of what may have been a band at around 575 bp (arrow). The remaining PCR amplicons from reactions producing bands, was combined, superloaded on a gel, and then cut from the gel in a manner that would capture all previously sequenced Na\textsubscript{v}1.1 deletion variants for cloning. Plasmid digestion released 39 bands at the expected size for Na\textsubscript{v}1.1 with no deletion, and 1 band at a size consistent with Na\textsubscript{v}1.1 with c.[del266_473]. Both versions of the transcript (1 colony each) were sequenced. No bands were produced with attempts to reamplify the transcript around the c.[del4004_4258]/c.[del4003_4284]
Figure 23: Reamplification of the ~4500 bp Band Around the c.[del266_473] Deletion Site

One of 2 almost identical gels produced with amplification of the ~4500 bp band around the c.[del266_473] deletion site. Nested primer pair NP-2 was used with an expected size for Na\textsubscript{v}1.1 with no deletion of 883 bp, and an expected size for Na\textsubscript{v}1.1 with c.[del266_473] of 675 bp. The arrow indicates a faint ~575 bp band.

Size indicators, in kb, are marked on the ladder. For primer details see Methods, Table 1.
deletion site.

3.5.2 Reamplification of ~4.3 kb Plasmid Inserts

Ligation products used in the previous attempt to clone the three ~4.3 kb bands cut individually from a gel (see above), were repeat transformed. Again white colonies (blue/white screened) appeared on plates; 25 from the ~4075 bp band, 9 from the ~4200 bp band, and 4 from the ~4500 bp band. Colonies taken off the plates were heat lysed using the colony PCR technique, and serial dilutions of the plasmid DNA were PCR reamplified around the c.[del266_473] and the c.[del4004_4258]/c.[del4003_4284] deletion sites. Despite extensive optimization of the reactions and again the use of the nested/hemi-nested primers, only two distinct bands at a size consistent with Na\textsubscript{V}1.1 were produced (Figure 24A, lane 3 & Figure 24B, lane 5). Both of these bands were produced from the ~4075 bp band, both were produced with reamplification around the c.[del266_473] deletion site, and both were at the expected size (~675 bp) for Na\textsubscript{V}1.1 transcript with c.[del266_473]. Reamplification around the c.[del266_473] deletion site also produced a possible band at the same size (Figure 24B, lane 6), and a possible ~750 kb band (Figure 24A, lane 2 black box). The expected size for Na\textsubscript{V}1.1 with no deletion is 883 bp.

3.6 Alignment of Sequence Results for a Section of Transcript Around c.[del266_473]

The reamplification experiments reported above provided seven sequencing results covering the section of transcript from 367 bp before to 164 bp after c.[del266_473]. Alignment of these sequencing results showed that the nucleotide at position 254 relative to the translations start site is thymine (T) in transcript without this deletion (N=2) and, with one exception, is guanine (G) in transcript with this deletion (N=5, 4 of 5 are G vs T) (Figure 25). GKL-9 is the only sequencing result with the presence of c.[del266_473] that has thymine at position +254, and this clone had two basepairs different from all other clones at positions +136/137 (AG vs GA—not shown). Consistent with this, the full-length transcript reported by Noda et al (1986a) has T at this position. As only one of the three ~4.3 kb bands produced reamplification bands around the c.[del4004_4258]/c.del[4003_4284] deletion site, and these
Figure 24: Reamplification of the ~4075 bp Plasmid Insert Around the c.[del266_473] Deletion Site
Ligation product used in the previous attempt to clone the ~4075 bp band was repeat transformed.
Blue/white screened colonies were taken off the plates and heat lysed using the colony PCR technique. Serial dilutions of the plasmid DNA were PCR reamplified around the c.[del266_473] deletion site. Nested primer pair NP-2 was used with an expected size for Nav1.1 with no deletion of 883 bp, and an expected size for Nav1.1 with c.[del266_473] of 675 bp.

Size indicators, in kb, are marked on the ladder. Black box indicates location of band not visible in document. For primer details see Methods, Table 1.
Figure 25: Alignment of Sequencing Results With and Without c.[del266_473]

Alignment of sequencing results for a segment of transcript that contains the c.[del266_473] deletion site. GKL-9 through GKL-15 are sequences obtained from the reamplification of ~4.3 kb bands cut individually from a gel (see above). “Published” refers to the rat brain sequence published by Noda et al (1986a). Blue highlights basepair position 254 relative to the translation start site. Purple is sequence that is removed by this deletion.
reamplification bands all show the presence of c.[del4004_4258] or c.[del4003_4284], sequence alignments to search for nucleotide predictors of deletion at this site could not be performed.

3.7 Nav1.1 5΄ Untranslated Region from Rat Heart

Seven clones produced with the digestion cloning primer pair D-1 (for primer details see Methods,Table 1) were compared with the published 251 bp 5΄ untranslated region (UTR) (Noda et al, 1986a). The present experiments used the same forward primer used by Noda et al (1986a). Four ‘of 7 clones exactly matched the published sequence (Figure 26, top); while 2 sequencing results were missing the 5΄ section of the published 5΄ UTR sequence including both the forward primer sequence and the 27 bp imperfect repeat. The only portion of the final cloning result (Figure 26, bottom) that matched the published sequence was the sequence of the forward primer. This cloning result contains a 95 bp imperfect repeat. NCBI blast searches were used to look for a match to the 95 bp imperfect repeat section of this result. The EST and the RefSeq database for each of human, rat and mouse, were searched against the first, second and third 30 basepair section of this repeat. One match was found with the 5’UTR of an unrelated rat gene. The 27 bp imperfect repeat sequence originally published by Noda et al (1986a) is at position -227 (A of ATG=+1). As no portion of the coding sequence was found in the cloning result with the 95 bp imperfect repeat, we do not know where this is in relation to the translation start codon.
Figure 26: Nav1.1 5’ Untranslated Region Sequences in Rat Heart

Top  The published (Noda et al, 1986a) 251 bp 5’ UTR from rat brain. The green section of this sequence is the forward primer sequence used by Noda et al (1986a) and in the present study; the red section is the remainder of the published 5’ UTR; and the underlined section is a 27 bp imperfect repeat sequence beginning at position -227 (A of ATG=+1). The sequence of 6 of 7 rat heart clones suggested that they had the same 5’ UTR.

Bottom  The sequence of 1 of 7 rat heart clones from the present study. This sequence showed no remnant of the published sequence other than the complete sequence of the forward primer (green). Instead, this clone contained an unidentified sequence (purple) that includes a 95 bp imperfect repeat (underlined section).

RNA for these RT-PCR and cloning experiments was from right ventricular cardiomyocyte cell isolations.
Chapter 4
Discussion

4.1 Summary of Findings: Cloning and Sequencing of Full-Length Nav1.1 mRNA in Rat Right Ventricle in Overlapping Segments

This thesis continues work aimed at identifying the neuronal VGSC isoform(s) that is/are present in ventricular cardiomyocytes. Electrophysiological/pharmacological evidence suggests that at least one neuronal VGSC exists in a functional form in these cells, and the author has argued (see Sections 1.3, 1.4 & 1.5 of the Introduction) that neuronal VGSC are the strongest candidates for proteins that play a role in the development of all three clinical outcomes associated with MI (Pike et al, 1990; Haigney et al, 1992 & 1994; Pike et al, 1995; Ju et al, 1996; Ward et al, 1997; Imahashi et al, 1999 & 2005; reviewed in Bers, 2008). Of the neuronal isoforms with evidence suggesting their presence in cardiomyocytes, functionally important persistent gating has been best demonstrated for Nav1.1 and Nav1.6 (Llinas & Sugimori, 1980; Raman & Bean, 1997; Raman et al, 1997; Vega-Saenz de Miera et al, 1997; Smith et al, 1998; Maier et al, 2002; Maier et al, 2004; Mantegazza et al, 2005). Therefore, the goal of the present work was to document the full-length Nav1.1 sequence and any of its alternative splice variants present in rat right ventricular myocytes through cloning and sequencing of overlapping fragments.

Figure 27 shows the Nav1.1 coding sequence and its deletion variants sequenced from cardiomyocyte isolations. Three nucleotide differences from the rat brain NCBI Reference Sequence (Noda et al, 1986a) are silent, with the fourth involving a change from large, charged arginine to small, uncharged glycine (c.2935A>G, Arg979Gly) (Table 2). Glycine at this position was previously reported from rat brain by Smith & Goldin (1998) (Table 2). The novel c.[del266_473] deletion removes exons 2 and 3, a total of 208 bp, and would therefore introduce a frameshift (Table 3). The 33 bp
c.[del2012_2044] deletion has previously been reported from human (Lossin et al, 2002) and rat (Noda et al, 1986a) brain, as well as from rat whole heart (Schaller et al, 1992) (Table 3). Two variants were cloned and sequenced at the third deletion site (Table 3). One, c.[del4004_4258], removes all of exon 21 except for the first and the 26 most 3’ nucleotides (255 bp); whereas the second, c.[del4003_4284], removes the complete exon 21 (282 bp). c.[del4004_4258] is a novel variant. c.[del4003_4284] is the only nucleotide difference or deletion variant that was not confirmed through the sequencing of a second clone from a separate RT-PCR experiment, however this variant was previously reported by Blechschmidt et al (2008). This group examined VGSC isoforms in human, pig, rat and mouse whole heart, stating that variants were sequenced once and then confirmed through restriction digest. The radical amino acid switch along with all three deletion variants involving more than 200 nucleotides (c.[del266_473; c[del4004_4258]; c.[del4003_4284]), involve conserved sequence segments of the NaV1.1 transcript (Table 2 & Table 3).

4.2 Nav1.1 Displays Characteristics of Unstable Inserts

RT-PCR and cloning experiments produced results consistent with Nav1.1 transcript forming secondary structures and, perhaps related to this, being “unstable” as plasmid inserts when exposed to bacteria through the cloning process. We were unable to clone either the full-length Nav1.1 transcript (~6 kb) or the minimal fragment that spans all three deletion sites (~4.3 kb). In both cases results provided evidence suggesting that the bacteria was cutting out the inserts. One group (Clare, 2006) with extensive experience cloning VGSC expands on the characteristics of unstable inserts. They report that unstable transcripts such as VGSC also undergo point mutations, large deletions, and recombination events with exposure to bacteria during cloning. Our single cloning result with sequence missing the complete exon 21, c.[del4003_4284], includes evidence suggesting multiple recombination events.

Some unstable inserts are believed to be targeted by the bacteria’s recombination and repair systems because they contain secondary structures. The use of mutations aimed at disabling these bacterial
Figure 27: The Nav1.1 Coding Sequence and Its Deletion Variants in Rat Right Ventricle

Nucleotides are numbered relative to the translational start site, and those that are different from the rat brain NCBI Reference Sequence NM_030875.1 are shown in lower case, bold.

A switch from adenine to guanine at position +2935 changes the codon from AGG to GGG (2935A>G, Arg979Gly), with the remaining three point nucleotide differences silent (3483G>A, 5172G>C, 5373T>C). Underlined segments were present in some RT-PCR amplicons and absent in others. Arrowheads (<> ) bracket exons involved in deletions. c.[del266_473] removes exons 2 and 3; c.[del2012_2044] removes the 5΄ end of exon 11. Two variants were cloned at the third deletion site. c.[del4003_4284] removes almost the entire exon 21 with the exception of the first and the 26 most 3΄ nucleotides (black underlined); c.[del4003_4284] removes the complete exon 21 (both black & red underlined).
<table>
<thead>
<tr>
<th>Point Nucleotide Differences from the NCBI Reference Sequence</th>
<th>NCBI Reference Sequence</th>
<th>Previously Published?</th>
<th>Predicting Impact on Channel Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. c.2935A&gt;G, Arg979Gly</td>
<td>Acc # NM_030875.1, rat brain</td>
<td>Smith &amp; Goldin, 1998 --rat brain</td>
<td>Radical Amino Acid Switch Conserved Sequence Segment</td>
</tr>
<tr>
<td>2. c.3483G&gt;A</td>
<td>Acc # NM_030875.1, rat brain</td>
<td>Novel</td>
<td>Silent</td>
</tr>
<tr>
<td>3. c.5172G&gt;C</td>
<td>Acc # NM_030875.1, rat brain</td>
<td>Novel</td>
<td>Silent</td>
</tr>
<tr>
<td>4. c.5373T&gt;C</td>
<td>Acc # NM_030875.1, rat brain</td>
<td>Novel</td>
<td>Silent</td>
</tr>
</tbody>
</table>

Table 2: The Nav1.1 Nucleotide Sequences in Rat Right Ventricle
<table>
<thead>
<tr>
<th>Deletion Variant</th>
<th>NCBI Reference Sequence</th>
<th>Previously Published?</th>
<th>Predicting Impact on Channel Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.[del266_473]</td>
<td>Acc # NM_030875.1, rat brain</td>
<td>Novel</td>
<td>Conserved Sequence Segment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Introduces a Frameshift</td>
</tr>
<tr>
<td>c.[del4004_4258]</td>
<td>Acc # NM_030875.1, rat brain</td>
<td>Novel</td>
<td>Conserved Sequence Segment</td>
</tr>
<tr>
<td>c.[del4003_4284]</td>
<td>Acc # NM_030875.1, rat brain</td>
<td>Blechschmidt et al, 2008 --whole heart rat, mouse, human or pig</td>
<td>Conserved Sequence Segment</td>
</tr>
</tbody>
</table>

Table 3: Nav1.1 Deletion Variants in Rat Right Ventricle
systems has achieved some success with encouraging bacteria to retain plasmid inserts with tandem and inverted repeats capable of forming secondary structures (Ishiura et al, 1989; Hanahan et al, 1991). As well, at least one group working with VGSC focused their efforts on identifying and modifying these types of repeat sequences in their cloning inserts (personal communication with F. Steiner, Genionics, Switzerland). Figure 17 provides an example of our results that would be consistent with the ability of Na\textsubscript{v}1.1 transcript to produce secondary structures. PCR of a small segment of rat genomic DNA and RT-PCR of the corresponding segment of rat brain mRNA, produced a band at the expected size along with two or three additional bands with reduced migration on the gel. Addition of betaine sold for the purpose of reducing secondary structures in DNA (Rees et al, 1993), reduced these multiple bands to a single band at the expected size. The genomic and brain mRNA amplified sequences share a 618 bp sequence, and therefore this transcript segment may contain sequence capable of forming secondary structures.

4.3 The Na\textsubscript{v}1.1 Sequence in Rat Heart

Two groups have previously published the full-length Na\textsubscript{v}1.1 sequence, with both reports from rat brain (Noda et al, 1986a, Acc #NM_030875.1; Smith & Goldin, 1998). Overlapping clones from cDNA libraries that were sequenced by Noda et al (1986a), would translate into more than 50 % of the Na\textsubscript{v}1.1 coding sequence being sequenced two or three times (see Noda et al, 1986b). On the other hand, Smith & Goldin (1998) PCR amplified and directly sequenced the full-length coding region in two overlapping cDNA fragments. Smith & Goldin (1998) report finding 11 nucleotide point variants in comparison with the sequence of Noda et al (1986a), however they only provide details of the impact of these differences on the amino acid sequence. No mention is made of any efforts to distinguish true nucleotide variants from PCR (e.g. Lundberg et al, 1991) or cloning copy error.

The Na\textsubscript{v}1.1 amino acid sequence obtained in the present work from rat heart agreed with the amino acid at position 979 contained within one (Smith & Goldin, 1998) but not the other (Noda et al, 1986a) previously reported sequences from rat brain (Table 4). Glycine as opposed to arginine was
Table 4: The Codon at Amino Acid Position 979 of the Nav1.1 Sequence

Present findings from rat cardiomyocyte isolations, brain, and genomic DNA (black box), as compared with the previously published sequences from rat brain (top), human genomic DNA (bottom), and genomic DNA of a patient with a severe form of epilepsy (bottom).
confirmed through sequencing of an additional clone from a separate RT-PCR. Additional support for glycine at this position was obtained with a BLAST search that showed two GenBank EST aligning with this section of the NaV1.1 sequence. Both had 86% identity to the NaV1.1 sequence, suggesting that they are from one or two other VGSC isoforms. Furthermore, the present work extends the initial report of glycine as opposed to arginine at position 979 (Smith & Goldin, 1998), by showing this to be the consensus sequence (i.e. direct sequencing) in both brain and genomic DNA (Table 4).

The ability of single amino acid switches to change the electrophysiology of VGSC is well-documented, with much of what is known about the important functional components of these channels coming from studies using experimental point mutations (e.g. Stuhmer et al, 1989). The likelihood that this point difference translates into an important functional difference is increased by its radical nature involving a change from large, charged arginine to small, uncharged glycine, along with its location within an S6 transmembrane segment (DIIIIS6) believed to line the inner pore of the channel (reviewed in Catterall, 1995 & Fozzard & Hanck, 1996). An example from the literature provides support for this. The majority of patients with severe forms of epilepsy (e.g. 30 of 35 patients in Fujiwara et al, 2003) have a mutation in the NaV1.1 gene, and the only NaV1.1 mutation in 39% of patients (total of 102 patients studied) with the most severe form of epilepsy is a missense or point mutation (reviewed in Mulley et al, 2005). In fact, the human genomic NaV1.1 sequence has glycine at amino acid position 979 (Escayg, 2000, GenBank P35498, Table 4). Arginine as opposed to glycine at this position (G979R) was reported as the only NaV1.1 mutation in a patient with one of the more severe forms of epilepsy (Fujiwara et al, 2003, Table 4).

As well, the point has been made in the literature that “silent” nucleotide changes may play important roles in directing alternative splicing (Woolfe et al, 2010). We report three silent point nucleotide differences from the Noda et al (1986a) sequence within the NaV1.1 coding sequence (Figure 27). Our sequence results at these three locations were confirmed in at least one additional RT-PCR experiment, and were present in any additional sequencing results.
In addition, Noda et al (1986a) provide a list of three positions in the Na\textsubscript{v}1.1 sequence (+1441, +1780, & +2467) where the nucleotide appears to “flip flop” between two nucleotides across transcripts. They also suggest that a link may exist between these variations and the presence or absence of the 33 bp deletion in the first intracellular loop (c.[del2012_2044]). Alignment of multiple sequencing results for a segment of transcript that includes a deletion site, can be used to search for this phenomenon. Our limited look at roughly 600 bp surrounding c.[del266_473], found a novel nucleotide position that appears to “flip flop” between two bases and that may be predictive for the presence or absence of this novel deletion (Figure 25). Later sections of this chapter consider the possibility that this phenomenon represents RNA editing, suggest that c.[del266_473] would devastate channel function, and present a case for this and other features of Na\textsubscript{v}1.1 to be part of evolved systems for tight control of VGSC gene expression.

4.4 Emerging Evidence of Extensive Alternative Splicing of Voltage-Gated Sodium Channels

4.4.1 Alternative Splicing of Voltage-Gated Sodium Channels

The number and the prevalence of VGSC alternative splice variants (eg. Raymond et al, 2004), underscore the fact that alternative splicing is a fundamental part of the expression of these channels. Moreover, a recent review of all documented mammalian Na\textsubscript{v}1.5 splice variants (Schroeter et al, 2010) reports that 3 of 8 alternative splicing sites produce variants that do not produce functional channels in heterologous expression systems.

Four of 5 functional Na\textsubscript{v}1.5 splice variants involve small (3 bp) or larger (53 or 120 bp) deletions within the less conserved DII/III linker (Gersdorff et al, 2001; Zimmer et al, 2002a: Ou et al, 2005; Camacho et al, 2006; Blechschmidt et al, 2008), and all produce altered gating in at least one heterologous expression system (Zimmer et al, 2002a; Makeilski et al, 2003; Comacho et al, 2006; Kerr et al, 2007). As well Na\textsubscript{v}1.5, along with the neuronal isoforms Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, Na\textsubscript{v}1.6 and Na\textsubscript{v}1.7
have a developmentally-regulated splice site that incorporates one of two mutually exclusive exons. The alternate exons cover the extracellular tip of DIS3, the S3/S4 extracellular linker and the majority of the highly conserved DIS4, but differ by only 1 to 7 amino acids depending on the VGSC isoform. Functional differences have been demonstrated in a heterologous expression system between the two versions of Na\textsubscript{v}1.2 (Xu et al, 2007) and between the two versions of Na\textsubscript{v}1.5 (Onkal et al, 2008).

In comparison, Na\textsubscript{v}1.5 splice variants that do not produce functional channels in heterologous expression systems involve deletions within highly conserved channel components. One variant involves the loss of almost half of a transmembrane segment thought to line the inner channel pore (DIIIS6), along with three quarters of the less conserved DII/III loop (Zimmer et al, 2002a). Another nonfunctional variant involves the loss of 54 bp of the pore loop (Schroeter et al, 2010). The final example consists of a splice site that produces three truncated (i.e. loss of >1000 bp) splice forms, with the truncation beginning in DIVS3 or S4 (Shang et al, 2007).

Furthermore, three groups have documented neuronal VGSC splice variants that introduce a premature stop codon in the first intracellular loop, roughly in the middle of the transcript, or just past the middle of the transcript. These variants involve an extension of an exon (Schaller et al, 1992—Na\textsubscript{v}1.3), an addition of one or more exons (Kerr et al, 2008—Na\textsubscript{v}1.2 & Na\textsubscript{v}1.3 & Na\textsubscript{v}1.7), or a set of two mutually exclusive alternative exons (Plummer et al, 1997—Na\textsubscript{v}1.6). Involved sequences are believed to play an important functional role as they are conserved across mammalian, bird and fish genomes (Plummer et al, 1997; Kerr et al, 2008), and expression of these sequences is regulated showing both developmental and tissue-specific expression patterns (Plummer et al, 1997).

Prior to the beginning of the present study, only two alternative splice sites had been reported for Na\textsubscript{v}1.1. The first is the location of the 33 bp deletion c.[del2012_2044] that was found in the present study and has been labelled Na\textsubscript{v}1.1a (Noda et al, 1986a—rat brain; Schaller et al, 1992—rat brain & heart; Lossin et al, 2002—human brain). A version of this deletion with 84 bp missing has also been
reported (Lossin et al, 2002—human brain). Schaller et al (1992) used RNase protection assays to demonstrate a 9.5 fold higher concentration of Na\textsubscript{\textit{\textit{V}}}1.1a over Na\textsubscript{\textit{\textit{V}}}1.1 in rat brain. While they do not show the whole heart and skeletal muscle data, they report that Na\textsubscript{\textit{\textit{V}}}1.1a is roughly 3 fold more abundant than the full-length version of this section of the channel in these tissues. Both Schaller et al (1992) and Lossin et al (2002) conclude from examination of the intron exon structure and of splice site consensus sequences in this region, that these splicing events involve selection of alternate 5΄ splice donor sites within the intron. Limited evidence suggests that Na\textsubscript{\textit{\textit{V}}}1.1a produces a functional channel (Noda et al, 1987).

As well, both versions of the mutually exclusive exons involving DIS3, DIS4 and their extracellular loop as described above and with a total of 3 amino acid differences in Na\textsubscript{\textit{\textit{V}}}1.1, have been reported from adult human brain (reviewed in Gazina et al, 2010). However the mouse gene only contains the exon that is more highly expressed in adulthood (Gazina et al, 2010), we report this exon in both clones from separate RT-PCR experiments in adult rat heart.

4.4.2 Na\textsubscript{\textit{\textit{V}}}1.1 Alternative Splicing in Rat Heart

The level of coincidence between splice sites and the deleted sections in the present study, suggests that the deletions are the result of alternative splicing events in rat heart. The deletions at the first and the third deletion sites describe two forms of alternative splicing, exon skipping and the use of an alternate 3΄ splice acceptor site within the neighbouring exon (Figure 28). Exon skipping is the most common form of alternative splicing (Miriami et al, 2003; Sugnet et al, 2004; Schroeter et al, 2010). c.[del266_473] removes both exon 2 and exon 3; whereas c.[del4003_4284] removes exon 21. The novel deletion variant c.[del4004_4258] describes the use of an alternate 3΄ splice acceptor site within exon 21.

The intronic splice site consensus sequences (Burset et al, 2000), GT or GC at the 5΄ splice donor site and AG at the 3΄ splice acceptor site, are present in all introns surrounding the exons shown in Figure 28 with one exception. The consensus sequence AG is not found in the 3΄ end of the intron between exons 2 and 3. Of note the 5΄ end of this intron has the GC consensus splice donor site sequence as
Figure 28: Schematic Diagram of the Exonic/Intronic Structure of Rat Na_1.1 at Alternative Splice Sites
opposed to the canonical GT found in the remaining intronic splice donor sites in these sections of the NaV1.1 gene. The splicing mechanisms involved in removal of multiple exons such as the removal of both exon 2 and 3 with c.[del266_473], are unknown (Desmet & Beroud, 2012). However, removal of both exons allows use of the canonical GT splice donor site at the 5’ end of the intron between exons 1 and 2, along with the canonical AG splice acceptor site at the 3’ end of the intron between exons 3 and 4.

We cannot rule out the possibility that our deletion variants are artifacts of the RT-PCR process. A wide range of experimental approaches have been used to support the existence of this phenomenon (Pallansch et al, 1990; Cariello et al, 1991; Cocquet et al, 2006), with perhaps the strongest evidence from Cariello et al (1991). This could occur if the reverse transcriptase or the polymerase skipped a section of the template in the copying process through intramolecular template switching (see Cocquet et al, 2006). Factors thought to promote this are direct repeat sequences (i.e. homology-dependent process—reviewed in Cocquet et al, 2006), secondary structures (Pallansch et al, 1990; Cariello et al, 1991; Henke et al, 1997; Cocquet et al, 2006), and either a reverse transcriptase (Superscript II, Cocquet et al, 2006) or a polymerase (Taq, Cariello et al, 1991) with low processivity.

However, limited data from Cocquet et al (2006) suggests that published canonical splice sites (i.e. intronic splice sites GT and AG) are not deletion artifacts, and that only rarely (~1%) can published noncanonical splice sites be explained as deletion artifacts. Splice sites were selected from a splice site database based on the presence of ≥ 8 nucleotide direct repeats surrounding the deleted sections, and tested through repeat RT with a highly processive enzyme (Thermoscript). Examination of the deleted sequence for one gene positive for a deletion artifact suggested a possible stem loop structure capable of bringing the direct repeat sequences closer together, and cDNA with the deletion had only one copy of the repeat sequence. Similarly, Miriami et al (2003) examined examples of exon skipping from another alternative splicing database and report complementary sequence motifs in the surrounding introns that would be predicted to form a stem loop. The exon to be removed is at the head of the structure and the two surrounding exons are drawn together. The presence of canonical splice sites sequences was
confirmed for all examples. Again the suggestion is that reports of exons skipping represent true alternative splicing events: literature is reviewed showing alternate function within the cell of the abbreviated variant for three of the examined genes.

Work by Henke et al (1997) provides evidence that deletion artifacts are also produced in PCR by secondary structures in cDNA template. They examine the effect of the addition of betaine to the PCR on the relative strength of bands representing full-length amplicons and their shorter splice variant (alternate splice donor site within an exon). Betaine works to improve the amplification of GC-rich template and template with secondary structures by reducing the DNA melting temperatures (Rees et al, 1993). The idea that sites of true alternative splicing events may also be prone to deletion artifacts through intramolecular template switching around a secondary structure, is demonstrated when this work is considered along with a report of RNase protection assay data suggesting that the same abbreviated sequence is also a product of true alternative splicing (Su et al, 1995).

Alternatively and as discussed above, large deletions found in unstable inserts such as Na\textsuperscript{v}1.1 transcript may be an artifact of the cloning process caused by the bacterial recombination and repairs systems. In the present work, one clone with c.[del4003_4284] that removes the entire exon 21 also showed evidence of multiple recombination events.

The case for alternative splicing is more difficult to argue for c.[del4004_4258] which describes the use of an alternate splice acceptor site within exon 21. Use of alternate 5´ or 3´ splice sites within exons normally removes only a small portion of the exon (Sugnet et al, 2004), whereas this deletion variant removes all of exon 21 with the exception of the first and the 26 most 3´nucleotides. As well, the consensus intronic splice acceptor site sequence AG is not found within the exon to support an alternate splice acceptor site at position +4257 to +4258. Perhaps the strongest argument that c.[del4004_4258] describes a splicing event is the fact that both the present work and another group (Bleichschmidt et al, 2008) sequenced Na\textsuperscript{v}1.1 from the heart with loss of the entire exon 21 (c.[del4003_4284]). This variant involves the common alternative splicing form of exon skipping with little existing evidence for loss of
complete exons as the result of RT or PCR artifact. Of note, Blechsmidt et al (2008) show RT-PCR bands from human, pig, rat and mouse whole heart that suggest a single deletion variant at this deletion site. On close inspection the band sizes shown more closely match the size for our novel variant c[del4004_4258] (114 bp) than for c.[del4003_4284] (87 bp).

4.5 Na\textsubscript{v}1.1 Transcript does not Appear to Exist in Rat Right Ventricle in a Form that Would Code for Functional Channel

4.5.1 Sequence Results from Overlapping Segments Posed a New Question: Does Na\textsubscript{v}1.1 Transcript that Codes for Functional Channel Exist in Rat Right Ventricle?

When transcripts are cloned and sequenced in overlapping segments as in the present study, we are in effect working with tunnel vision. Using this approach we found three deletion sites in the Na\textsubscript{v}1.1 transcript (Noda et al, 1986a; Smith & Goldin, 1998) in rat right ventricle. Deletions at each of these three sites exist is some but not all Na\textsubscript{v}1.1 transcript in this tissue. These results, therefore, do not confirm any full-length version of Na\textsubscript{v}1.1 transcript.

Describing full-length Na\textsubscript{v}1.1 transcript in the heart becomes more critical given that the existence of any one of the three deletions found across the first and the third deletion sites (c.[del266_473]; c.[del4004_4258]; c.[del4003_4284]), would be expected to be devastating to channel function (Figure 29). The novel c.[del266_473] in Domain I of the channel involves a frameshift that would be predicted to scramble the coding sequence of almost the entire channel. The presence of either c.[del4004_4258] or c.[del4003_4284] at the third deletion site in Domain III of the channel, involves loss of the entire S5 transmembrane segment thought to line the inner channel pore, along with the initial 57 or 63 % of the highly conserved pore loop thought to make up the channel’s outer pore region (Heinemann et al, 1992; Catterall et al, 2005). Loss of a small section (~15% of pore loop) roughly central within the 3’ half of the Na\textsubscript{v}1.5 DIII pore loop (see Schroeter et al, 2010), results in Na\textsubscript{v}1.5 channels that do not
Figure 29: Segments of the Nav1.1 Channel Lost with Deletions at the First and Third Deletion Sites

A The orientation of the predicted 4 domain, 24 transmembrane segment, voltage-gated sodium channels within the cell membrane (modified from Goldin, 2002).

B c.[del266_473] removes approximately one third of the amino terminal, the entire S1, the entire S1/S2 extracellular loop, and roughly 5% of S2 in Domain I. c.[del4004_4258] and c.[del4003_4284] remove approximately three quarters of the S4/S5 intracellular loop, the entire S5, and 57% or 63%, of the pore loop in Domain III. The orientation of the remaining transmembrane segments and the location of the termini with respect to the cell membrane, is unknown for channels with any of these three deletions.
produce current in an expression system (Wang et al, 2009). Additionally, the loss of a complete transmembrane segment with any of c.[del266_473], c.[del4004_4258], or c.[del4003_4284] would be expected to reverse the orientation of downstream transmembrane segments within the cell membrane (Plummer et al, 1997), making the channel nonfunctional. The loss of exon 21 (c.[del4003_4284]) is reported as the sole Na\textsubscript{V}1.1 mutation in a patient with the severe myoclonic infantile epilepsy syndrome (Mulley et al, 2006). Roughly half of this patient group has Na\textsubscript{V}1.1 mutations that are missing large sections of the channel, suggesting that this condition reflects loss or major disruption of Na\textsubscript{V}1.1 function (Mulley et al, 2005).

The novel c.[del266_473] introduces a frameshift, beginning early in the coding sequence (nt +266). Figure 30 suggests that the correct reading frame and amino acid sequence for the majority of the channel (Noda et al, 1984 & 1986a), cannot be preserved by the use of an alternate start codon. The only ATG that could achieve this in either the published 251 bp 5’ UTR or in the 265 bp section between the published ATG and c.[del266_473], would be met almost immediately by the first of a series of stop codons. Work by others (Martin et al, 2007—human and mouse brain Na\textsubscript{V}1.1) and our preliminary results (rat right ventricle), show differences in the 5’ UTR across Na\textsubscript{V}1.1 transcripts within a tissue. However, any alternate ATG provided by a different 5’ UTR that would allow translation of the correct amino acid sequence after c.[del266_473] would be expected to meet one of the seven stop codons that are spread throughout the 265 bp section of the coding sequence that precedes this deletion. The published coding sequence is supported by amino acid sequencing of the eel *Electrophorus* channel (Noda et al, 1984).

In fact, Figure 31 shows that use of an alternate reading frame will not produce Na\textsubscript{V}1.1 transcript with c.[del266_473] that does not contain a premature stop codon. Use of the reading frame dictated by the published start codon, will result in a stop codon just before the middle of the coding sequence in all transcript with this deletion. The third reading frame contains a number of start codons both in the published 5’ UTR and in the portion of the amino terminal before c.[del266_473]. Considering that an
Published ATG

Section Removed by c.[del266_473]

Only ATG that Would Result in Use of Correct Reading Frame After c.[del266_473]

Stop Codons with Use of Alternate ATG

Figure 30: The Start and Stop Codon Makeup of the Na\textsubscript{v}1.1 5\textquotesingle UTR and Coding Sequence Upstream of c.[del266_473]
Figure 31: Premature Stop Codons in The Two Remaining Reading Frames of Transcript with c.[del266_473]

Published Reading Frame

**Published ATG**

Novel c.[del266_473] 208 bp Deletion with Frameshift

Third Reading Frame

Start Codons in Third Reading Frame

Stop Codons in Third Reading Frame

Published ATG

TGA at +2627 of a 6027 bp Published Coding Sequence (not shown)
alternate 5’ UTR could be used and depending on the choice of start codon, this reading frame will contain a premature stop codon upstream of the published amino terminal or just after c.[del266_473].

The author would argue, then that it is unlikely that transcript with a deletion at either or both of the first and third deletion sites has the potential to be translated into functional channel. A “functional channel” would be capable of passing electrical current across a membrane. The present study does not rule this out. The impact of changes to ion channel transcript is examined through expression in a heterologous expression system, and electrophysiological testing. This has not been done for any of the three deletion variants across the first and the third deletion sites. MacArthur et al (2012) refer to gene variants predicted to have a serious impact on protein function, as “loss-of-function” variants. Some, but not all of these variants, are predicted to lead to a complete loss of the protein function. In this thesis the term nonfunctional transcript is used to refer to transcript with any one or more of c.[del266_473], c.[del4004_4258] or c.[del4003_4284].

4.5.2 An Alternative Approach to Describing Full-Length NaV1.1 Transcript in Rat Heart

We were unable to clone either the full-length NaV1.1 transcript (~6 kb) or the minimal fragment that includes both the first and the third deletion sites (~4.3 kb). Three bands were produced when we RT-PCR amplified the minimal fragment spanning both of these deletion sites (~4.1 to ~4.6 kb, Figure 18), and we capitalized on this in order to learn about the make-up of individual full-length NaV1.1 transcripts. We achieved strong separation of the three ~4.3 kb bands and cut them individually from the gel. Attempts were made to PCR reamplify the sections of these bands around the first and then around the third deletion site. When successful, the resulting PCR amplicons were cloned and sequenced. The results from these experiments confirmed the presence of NaV1.1 transcript with a deletion at both the first and the third deletion site in rat heart, and when considered with the relative strength of bands representing transcript with and without a deletion at these two sites produced both from the cytoplasm of single ventricular myocytes and from cell (ventricular myocyte) isolations, they provide strong data to suggest that only nonfunctional variants of NaV1.1 exist in rat heart.
Because deletions at the first and the third deletion site were roughly the same size, the possibility exists for two Na\textsubscript{\textit{v}}1.1 transcript versions, each with a deletion at the first or the third deletion site, to be represented by a single ~ 4.3 kb band. Our approach to these experiments, therefore, was to be persistent in attempts to clone and sequence any possible PCR band produced by the reamplifications. We were successful in cloning sequences that matched the size of all PCR reamplification bands with one exception. Figure 32 shows representative PCR bands resulting from reamplification of the largest of the three ~ 4.3 kb bands around the c.[del266_473] site. We cannot explain our finding that lanes had either the large or the small expected size band, but not both. Overexposure of this gel produced a possible band at ~ 590 bp (expected sizes 675 & 883 bp) below the weeping band at the expected size for sequence without c.[del266_473]. We screened 40 colonies from the combined product of this set of PCR, and all inserts were correct for Na\textsubscript{\textit{v}}1.1 sequence with or without c.[del266_473]. The faint band was either a very low abundant variant of unexpected size, or was artifact associated with streaking of the large amount of expected size DNA.

A summary of the sequenced reamplification bands from each of the ~4.3 kb bands is provided in Figure 32. The strength of the evidence for all Na\textsubscript{\textit{v}}1.1 having at least one devastating deletion, comes from the fact that we were able to PCR reamplify the mid-sized band around both the first and the third deletion sites. Eight PCR produced strong or sharply defined bands matching the expected size for transcript containing c.[del266_473] in otherwise clean lanes. Four PCR produced strong or sharply defined bands matching the expected size for transcript containing either c.[del4004_4258] or c.[del4003_4284] in otherwise clean lanes. Four additional PCR produced very faint bands matching the expected size for transcript with c.[del266_473], and one additional PCR produced a faint band matching the expected size for transcript with either c.[del4004_4258] or c.[del4003_4284]. Overexposure of gels on the UV reader did not show any additional bands, and cloning and sequencing provided the expected results.
Figure 32: Summary of Results: Reamplification of the ~4.3 kb Nav1.1 Bands Around the First and the Third Deletion Sites

RT-PCR of the minimal Nav1.1 section spanning the first and the third deletion sites (~4.3 kb) produced three bands that were maximally separated and cut individually from the gel. Nested (first deletion site) or hemi-nested (third deletion site) PCR were then used in attempts to reamplify each of these three bands around these deletion sites.

Both PCR amplicons with and without c.[del266_473] were sequenced from the largest ~4.3 kb band. We cannot rule out contamination from the nearby mid-sized ~4.3 kb band as an explanation for the reamplification band cloned and sequenced to show the presence of c.[del266_473] (?). The mid-sized ~4.3 kb band is Nav1.1 transcript with a deletion at both the first and the third deletion sites. The faint bar representing transcript without c.[del266_473] reamplified from the smallest ~4.3 kb band, represents a relatively weak showing across 14 successful PCR.
If the mid-sized ~4.3 kb band represents two versions of the transcript, each with a deletion at one of these two deletion sites, then the PCR were unable to amplify the sections with no deletion at not one but at both deletion sites. Moreover, we would have to accept that our PCR selectively amplified one section but not the other of the same transcripts. The author concludes that the mid-sized band in Figure 32 represents the version of Na\textsubscript{v}1.1 transcript with a deletion at both the first and the third deletion sites.

We were unable to reamplify the smallest and largest ~4.3 kb bands around the third deletion site. The ~4.3 bp PCR amplicons did not allow for design of two reverse primers around the third deletion site, and this may have played a role. That bands both with and without c.[del266_473] are amplified, cloned and sequenced from the largest ~4.3 kb band, suggests that this band is composed of two versions of the transcript, each with one deletion across the two deletion sites. Contamination from the neighbouring mid-sized band cannot be ruled out as an explanation for the version of the transcript with c.[del266_473]. However, careful analysis of the log ladder in Figure 32 suggests that the largest ~4.3 kb band is 250 to 350 bp larger than the mid-sized band with deletions at both sites. When we consider the other 33 bp deletion (c.[del2012_2044]) that can be in or out of any transcript, this size separation better fits our expectation of 175 to 284 bp if template in the largest ~4.3 kb band has one deletion across the first and third deletion sites, than it does with our expectation of 430 to 496 if the largest band represents functional template with no deletion at either of these sites.

Results for the smallest ~4.3 kb band that is roughly 100 bp smaller than the mid-sized band that we showed has a deletion at both sites, are not easily explained. Moreover, there is some evidence for a low-abundant version of the transcript within this smallest band that is without c.[del266_473]. We considered that our forward primers located within the 5´ UTR could have amplified different versions of this Na\textsubscript{v}1.1 untranslated region, however, alignments of reamplification band sequences showed that the 5´ UTR sections of these PCR amplicons are identical. We suggest that there is only one possible explanations for an ~4.3 kb band that is smaller than the mid-sized band that we know has a deletion at both the first and the third deletion sites. There may be an additional deletion or alternatively spliced site
encoded by the smallest ~ 4.3 kb band beyond the section that we sequenced. This would locate the potential sites for addition transcript variation within nucleotide positions +854 to +4308 of the coding sequence. The estimated size of any additional novel deletion would be 100 bp if the small band exists in two versions, each with a deletion at either the first or the third deletion sites, as suggested by our data.

As well on one occasion we obtained a RT-PCR band for full-length (i.e. 5871 to 6367 bp depending on the number of deletions) template that was strong enough to run out on a gel (Figure 18). The author suggests that the result does not support RT-PCR amplicons of the minimal 6334 bp band size (6367 if no 33 bp deletion) for functional Na\textsubscript{V}1.1 transcript, but appears to show two bands, one at or just below the 6 kb mark and the other not larger than 6.2 kb.

**4.5.3 Evidence Supporting the Conclusion that Transcript Represented by The Largest ~4.3 kb Band Does Not Code For Functional Na\textsubscript{V}1.1**

Conclusions made regarding the makeup of the three ~4.3 kb bands are supported by two other data sets. The original set of cloning and sequencing experiments of the entire Na\textsubscript{V}1.1 coding sequence in overlapping segments from ventricular myocyte cell isolations, first with TA and then with digestion cloning, provided a number of gels showing the relative predominance of bands matching the expected size for transcript with and without deletions at the first and the third deletion sites. Sequencing confirmed that the bands from these experiments represent versions of Na\textsubscript{V}1.1 with and without these deletions. As well nested or hemi-nested PCR were completed around these two deletion sites, both against cDNA from cardiomyocyte cell isolations and against cDNA produced from the cytoplasm of single rat right ventricular myocytes. All of these PCR produced bands at one or at both of the expected sizes. Bands were not cloned and sequenced. However, the nested/hemi-nested primer pairs used for these experiments in effect select transcript twice and are, therefore, both more sensitive and more specific.

All PCR in the TA and digestion cloning experiments used 32 amplification cycles, and visual inspection was the only method used to compare the end-point band strengths across the deletion variants.
in all experiments in this thesis. While the primers used to amplify the different deletion variants are the same, no measures were made of the relative efficiency of amplification (i.e. the rate of accumulation of full-length product) of the different deletion variants. As well, multiple factors are believed to play a role in the slowing and then plateau phase of PCR product accumulation and evidence suggests that non-specific binding of the polymerase to the increasing level of product plays a role (Morrison & Gannon, 1994; Kainz, 2000). The possibility that any of these factors may have a larger effect on the production of select splice variants cannot be ruled out.

McCulloch et al (1995) demonstrated that reducing the size of a PCR template such as with our deletions, has the potential to increase the efficiency of the reaction. Furthermore, the level of this effect increased with increasing absolute size of the PCR amplicons. Data presented by this group would translate into between a 10 and 30 percent increase in PCR efficiency for our variants with one of the large (>200 bp) deletions. Data supporting this conclusion is reported by one group (Arezi et al, 2003); whereas another group reports no effect of these types of differences in product length (Blechschmidt et al, 2008). The impact of changes in product size on PCR efficiency would be expected to change across studies that all use different PCR conditions. In particular this can be influenced by the efficiency or processivity of the polymerase that was used (Arezi et al, 2003).

On the other hand, smaller PCR amplicons produce less fluorescence on an ethidium bromide gel. Molar equivalents were calculated for all PCR amplicons and considered in examination of the gels with multiple bands. However, given the non-quantitative nature of these experiments, data is discussed only in terms of gross differences in the strength of bands representing Na\textsubscript{V}1.1 deletion variants.

Deletion variants reported in the present work, may also differ in their ability to produce secondary structures and the sequence removed from the smaller variants may have additional specific characteristics that change the overall efficiency of transcript copying. Mytelka & Chamberlin (1996) demonstrate the effect of template sequence on the strength of PCR bands. As well, literature reviewed
above suggesting the ability of secondary structures to produce deletion artifacts also show that the yield of PCR amplicons both with and without the deletion increases with experimental manipulations that reduce the artifacts (Cariello et al, 1991; Henke et al, 1997; Cocquet et al, 2006).

As a whole, the single cell data supports the ubiquitous nature of the splicing events at these two deletion sites in rat right ventricle. With the exception of one cell, RT-PCR bands representing transcript with c.[del266_473] are predominant, or are the only bands produced (total number of cells =11). While the RT-PCR around the third deletion site were generally less successful and the limited data suggests more variability across cells, overall this data again suggests that most Na\textsubscript{v}1.1 transcript has one of the two deletions at this site (c.[del4004_4258] or c.[del4003_4284]). Furthermore, considering only those cells where RT-PCR produced clear bands with amplification around both the first and the third deletion sites (3 of 8), cells 6 and 7 show no evidence of the full-length version at either of the deletion sites while the majority of transcript in cell 9 appears to have the a deletion at the first but not at the third deletion site (for reference see Figures 11C & 12B).

The case for the majority of Na\textsubscript{v}1.1 transcript in rat heart also having c.[del4004_4258] or c.[del4003_4284], is made more strongly by the RT-PCR bands that were produced from the ventricular myocyte cell isolations. Bands from all 8 RT-PCR support the predominance of transcript with a deletion at this site, with these bands appearing at least 4 fold stronger in 7 of 8 gels. Blechschmidt et al (2008) concludes from quantitative RT-PCR, that just over 90% of Na\textsubscript{v}1.1 from rat whole heart has a deletion at this site.

In contrast with the single cell results, however, the predominance of bands from ventricular myocyte cell isolations suggest that transcript with c.[del266_473] is either as abundant or is less abundant than transcript without this deletion. On the one hand, the data from the 11 single cells presents an almost uniform message in terms of the predominance of transcript with c.[del266_473]: on the other hand, measures of low-abundant transcript from single cells might be expected to be more variable and therefore to provide a less accurate overall picture of Na\textsubscript{v}1.1 transcript in the rat right ventricle.
The strength of the single cell data set comes from the fact that we can get an indication of the predominant form of transcript at both the first and the third deletion site within the same cell. This form of data, therefore, has the potential to provide insight into the makeup of different full-length versions of Nav1.1 transcript in the heart. For example, cell number 9 shows a clear band at the expected size for the version of the transcript that does not have c.[del4004_4258] or c.[del4003_4284], with no clear band representing transcript with a deletion at this site (Figure 12B). This same template source (cell number 9) amplified around c.[del266_473] shows a more predominant band representing transcript with the deletion (Figure 11C), suggesting that the predominant form of transcript in cell number 9 has c.[del266_473] but not c.[del4004_4258] or c.[del4003_4284]. We cannot draw conclusions from a sampling of 3 cells with data from both the first and the third deletion sites. However, the relative predominance of bands matching expected size for transcript with versus without a deletion at these sites from ventricular myocyte cell isolations, supports the idea of Nav1.1 transcript with a deletion at one of the two deletion sites. More specifically, the large number of gels examining these relationships suggest that a significant portion of Nav1.1 transcript in the rat right ventricle must have either c.[del4004_4258] or c.[del4003_4284] but not c.[del266_473].

This last point is particularly significant when considered along with the results of our reamplification experiments described above. Our interpretation of these rested on our cloning and sequencing both versions of the first deletion site from the large ~4.3 kb band, along with our clear demonstration that the mid-sized ~4.3 kb band represents one version of transcript containing both c.[del266_473] and c.[del4004_4258] or c.[del4003_4284]. This data suggests that the largest ~4.3 kb band represents two different versions of the transcript, each with a deletion at one of the deletion sites. Furthermore, as our reamplification results from the mid-sized ~4.3 kb band are conclusive, any suggestion that the largest ~4.3 kb band might represent functional (i.e. no deletion at either the first or the third deletion site) channel would have to accept that there is no significant transcript within the cell with a deletion at one but not the other of these deletion sites.
One additional point needs to be made with regard to the relative strength of bands of the expected size for transcript with and without c.[del266_473]. The author has argued that, irrespective of reading frame, all transcript with c.[del266_473] will have a premature stop codon and this premature stop will be located somewhere in the first half of the Na\(\text{V}_1.1\) coding sequence. Several lines of evidence reviewed in a later section of this Discussion, suggest that transcript with premature stop codons is targeted for a process called nonsense-mediated decay (NMD) after initial rounds of translation (Stoilov et al, 2004; Chang et al, 2007; Barash et al, 2010). This would suggest that the strength of our RT-PCR bands at the expected size for transcript with c.[del266_473] underestimates the frequency of this splicing event, and that the relative band strengths at this deletion site are biased towards the makeup of transcript without c.[del266_473].

4.6 Why Would a Cell Transcribe Message Not Destined to Be a Functional Protein?

Ong & Corces (2011) use the words complex, intricate and precise to describe the required interactions between proteins and DNA sequences that control the transcription of eukaryotic genes. In vitro experiments are used to identify the minimal set of elements or short gene sequences required for the initiation of transcription. This core promoter includes the start codon and DNA elements normally found within the 500 bp directly upstream of the start site. Transcription factors bind both sequence elements in the DNA and the eukaryotic RNA polymerase. Furthermore, transcription may be encouraged or silenced through interaction with DNA sequence elements distant from the core promoter. The current state of knowledge is in the early stages of identifying these distant enhancer and silencer elements and of developing an understanding of the mechanisms used by these elements to control what is and is not expressed.

Control of transcription is not the only means available to the cell of regulating the amount of functional protein. Transcribed message will need to move through a number of steps before translation into protein. These include addition of a 5’ cap and a 3’ polyadenylated tail, splicing, and transport from
the nucleus. As well, the amount of transcribed message present at any point in time in the cell can be regulated through decay processes (Wu & Brewer, 2012). Each of these steps and processes provides additional opportunities for isoform-specific and cell-specific regulation of the amount of functional protein in a cell. Moreover, Morris et al (2010) argue that sites of regulation do not act individually. They propose that control mechanisms acting at multiple levels of transcript processing act in a coordinated fashion to set the spatial (cell-specific) and temporal (developmental) gene expression patterns.

Our results invite questions regarding the rationale for production of $\text{Na}_\text{V}1.1$ mRNA that is unlikely to translate into functional channels. There is a belief that cells do not retain processes over time if those processes do not play a beneficial role (Dr. Paul Young, Queen’s University, personal communication). However, a recent paper (MacArthur et al, 2012) makes the point that the rapid loss of gene variants from a population, is dependent upon the variant threatening the animal’s survival or reproduction, i.e. negative selection. This leaves two possibilities: expression of nonfunctional $\text{Na}_\text{V}1.1$ transcripts in the heart is leftover from another time and does not bring significant benefit or harm to the animal, or as MacArthur et al (2012) argue, “loss-of-function” genes variants, including splice variants resulting in large deletions and frameshifts, may serve an alternate and beneficial role in terms of survival or reproduction, and therefore be positively selected for in the population. We argue from our data and its fit with emerging areas of research, that the $\text{Na}_\text{V}1.1$ alternative splicing events at the first and the third deletion sites are likely a part of complex VGSC, and perhaps in particular $\text{Na}_\text{V}1.1$, systems evolved for finely controlled, cell and condition-specific control of expression.

### 4.6.1 Demonstrated Partnership Between Alternative Splicing and Nonsense-Mediated Decay

For a number of years alternative splicing events leading to modulated or alternate functions focused attention on the ability of this process to increase protein diversity (Modrek & Lee, 2002). However, Lewis et al (2003) make the point that early attempts to describe alternative splicing in different tissues ignored splicing that would lead to the introduction of premature stop codons. These events were dismissed as errors or the result of random mutation. Nonsense-mediated decay (NMD) eliminates transcripts with
premature stop codons introduced ≥ 50 nucleotides upstream of the final exon/exon border (Maquat, 2002). The transcripts go through an initiating round of translation and are then targeted for degradation (Stoilov et al, 2004; Chang et al, 2007). The general view was that this system prevented a toxic level of buildup of otherwise nonfunctional protein (e.g. Gudikote & Wilkinson, 2002).

Lewis et al (2003) considered that the introduction of premature stop codons through alternative splicing may serve a functional role, and point to reports in the literature of two genes whose expression levels decrease with increases in the production of alternatively spliced transcripts containing premature stop codons (reviewed in Lewis et al, 2003). Lewis et al (2003) went on to examine 3,127 RefSeq mRNA sequences (i.e. 3,127 genes) that undergo alternative splicing, to test their hypothesis that the introduction of premature stop codons through alternative splicing may be a common, and therefore likely a functionally important, phenomenon. Canonical splice sites were determined by aligning human genomic sequences with the published RefSeq sequence with the greatest number of exons. Expressed alternative splicing variants were examined through alignment of EST database sequences with these RefSeq sequences. They report that roughly one third of EST-suggested transcripts appear to contain a premature stop codon, and that roughly three quarters of these transcripts fit the demonstrated criteria for transcripts targeted by NMD. Furthermore, the point has been made that the short lifespan of targeted transcripts would be expected to result in their underrepresentation in EST databases (Wollerton et al, 2004; Stamm et al, 2005).

This suggested partnership between alternative splicing and NMD is also found in Na\textsubscript{v}1.2, Na\textsubscript{v}1.3 and Na\textsubscript{v}1.6 (Plummer et al, 1997; Kerr et al, 2008). In these VGSC examples, the premature stop is introduced through the extension of an exon, the addition of one or two exons, or the selection of a mutually exclusive exon containing a premature stop. While the bulk of this work was performed in rodents, a search for mutually exclusive exons in human VGSC genomic sequence suggests the use of these as a means for the introduction of premature stop codons (Plummer et al, 1997). There are also multiple examples in the literature of alternative splicing acting through a frameshift to introduce a premature stop codon (Gudikote
& Wilkinson, 2002; Lewis et al, 2003; Wollerton et al, 2004). However, our novel c.[del266_473] in Na\textsubscript{V}1.1 is the first documented VGSC example.

A relatively recent examination of this phenomenon in each of a family of proteins, strongly suggests the functional importance of the partnership between alternative splicing and NMD (Lareau et al, 2007). All members of the SR family of splicing regulators produce splice variants with premature stop codons, and the section of the transcript involved in these splicing events is considered to be either “highly conserved” or “ultra-conserved”. Moreover, these conserved sequences suggest that individual members of the protein family have independently evolved the ability to use alternative splicing for the introduction of the premature stop codons.

The pairing of VGSC splicing and NMD also displays characteristics of a trait that has evolved to perform an important function. VGSC splicing events introducing premature stop codons are differentially regulated across tissues and developmental stages (Plummer et al, 1997; Kerr et al, 2008); these events are conserved across species (Plummer et al, 1997; Kerr et al, 2008); and the similarity between involved side by side mutually exclusive exons in human sodium channels and \textit{Drosophila melanogaster} calcium channels, suggests that they evolved independently in different species (Copley, 2004).

It is interesting that both of the VGSC examples of partnership between alternative splicing and NMD in the literature (Plummer et al, 1997; Kerr et al, 2008) along with the novel c.[del266_473] reported in the present work, all involve the introduction of premature stop codons around the middle or just past the middle of the coding sequence. Argument has been made for the development of the four domain calcium and sodium ion channels through gene duplications, beginning with the single domain potassium channel proteins and also involving intermediary two domain calcium and sodium channel proteins (Hille, 1989). Single domain potassium channel proteins (i.e. monomers) must combine in sets of four (i.e. tetramers) in order to form a functioning channel. The question then is whether a premature stop around the middle of VGSC transcript could produce half channels that are capable of combining in sets of two
(i.e. dimers) to form a complete and functioning VGSC. However, Stuhmer et al (1989) present data that suggests that each of the similar but unique sodium channel domains is required to form functional sodium channels, and any 4 domain VGSC produced according to this hypothesis would have be made up of two DI and two DII.

### 4.6.2 Proposed Dominant-Negative Impact of “Nonfunctional” Alternative Splice Variants on Functional Protein Expression

The conserved, regulated and prevalent pairing of alternative splicing with NMD, might suggest that alternative splicing acts to exert control on gene expression simply by diverting pre-mRNA from the formation of functional channel. However, early papers (Plummer et al, 1997; Tucker et al, 1998) suggested that the production of nonfunctional mRNA transcript and/or the protein product of this transcript, may interfere with the functional expression of the wild-type or full-length protein through dominant-negative effects. There are now a number of papers documenting this phenomenon. Most of this work it seems investigates the impact of transcript variants with premature stop codons (e.g. Stasiv et al, 2001; Bakker et al, 2005; Leung et al, 2007). However, we were able to find one example involving a splice variant with a 231 bp in-frame deletion (Oda et al, 1998). Fewer papers document the mechanism behind the dominant-negative effect (Stasiv et al, 2001; Leung et al, 2007). Shehata (2009) makes the point that nonfunctional splice variants may exert these effects through interference with any of the steps involved in wild-type channel expression or through direct effects on the wild-type channel protein. Examples provided by Shehata (2009) include competition for cellular components involved in channel processing and trafficking, as well as direct interference with channel assembly, gating, or its interactions with other cellular components important to function such as second messenger systems, auxiliary subunits or cellular/extracellular structural proteins.

Our data suggests that all Na_v1.1 transcript versions in rat heart have at least one deletion across the first and third deletion sites, and we have argued that the presence of a deletion at either of these deletion sites precludes translation into a functional channel. All versions of transcript including c.[del266_473]
would include a premature stop codon, thought to lead to NMD after initial rounds of translation (reviewed in Chang et al, 2007). However, substantial levels of nonfunctional transcript are maintained within the cell (Kang et al, 2009). We are not aware of any mechanism that would target the degradation of transcript with only c.[del4004_4258] or c.[del4003_4284].

Changes in the primary sequence with alternative splicing events may also result in misfolding of the protein product. Misfolding may expose signals for retention within the endoplasmic reticulum (Zarei et al, 2001; Arniges et al, 2006) and/or for intracellular degradation (reviewed in Kopito, 1997). These mechanisms may apply to Na\textsubscript{v}1.1 transcript with a deletion at either or both of the first and third deletion sites. Data examining the impact of premature stop codons on transcript translation and protein processing is limited. However while two papers suggest mechanisms for reduced truncated protein products (Kang et al, 2009; Zhang et al, 2010), a recent paper reports a stable protein product of a transcript with a premature stop codon (Tian & MacDonald, 2012).

Shang et al (2007) provide an example of the association between disease and the cell’s expression of nonfunctional VGSC capable of exerting a dominant-negative effect. The explanted hearts of heart failure patients showed elevated levels of expression of two nonfunctional Na\textsubscript{v}1.5 transcripts and decreases in expression of the wild-type, functional Na\textsubscript{v}1.5. Furthermore, they show that these are likely not independent phenomenon. Coexpression of the nonfunctional transcript in a heterologous expression system, reduces the expression of the wild-type channel.

4.7 Tissue-Specific Expression of “Nonfunctional” Alternative Splice Variants of Voltage-Gated Sodium Channels

RT-PCR bands of the expected size for our deletion variants were also observed with amplification of rat brain RNA (Figure 14). Bands produced at template concentrations similar to those used to produce Na\textsubscript{v}1.1 bands in the heart, suggest that transcript with c.[del266_473] may be less abundant in the brain whereas transcript with c.[del4004_4258] or c.[del4003_4284] may be at
comparable concentrations in both tissues. What sets these two tissues apart however, is the relative strength of bands consistent with functional (i.e. no deletion across the first and third deletion sites) versus variant (i.e. at least one deletion across the first and third deletion sites) transcript. As expected, RT-PCR of this neuronal VGSC in the brain at each of the first and the third deletion sites produced bands at the expected size for no deletion (i.e. functional) that were 10 to 20X stronger than those at the expected size for transcript with a deletion. Both full-length Na\textsubscript{v}1.1 sequences published to date came from rat brain (Noda et al, 1986a; Smith & Goldin, 1998), and there are many reports in the literature providing evidence (immunohistochemistry, microarray & RT-PCR of partial Na\textsubscript{v}1.1 transcript) that Na\textsubscript{v}1.1 is one of the predominant VGSC in the brain (Westenbroek et al, 1989; Gong et al, 1999; Whitaker et al, 2001; Raymond et al, 2004). This is in stark contrast with the relative bands strengths produced with RT-PCR of heart RNA around the first and the third deletion sites. Bands at the expected size for transcript with and without a deletion are either of roughly equal strength (c.[del266_473] deletion site), or the bands matching the expected size for transcript without a deletion are almost always at least three 3 fold weaker (c.[del4004_4258]/c.[del4003_4284] deletion site).

As well, at the first template concentration level that brought out Na\textsubscript{v}1.1 bands in the heart, there is the faint appearance of two intermediate-sized bands in the brain results for RT-PCR around the c.[del4004_4258]/c.[del4003_4284] deletion site (Figure 14). We cannot rule out non-specific priming. However, this primer set also produced a third band in 2 of 8 RT-PCR of heart RNA (Figures 9A & 9B), and all four clones produced from the heart with this primer set were correct for Na\textsubscript{v}1.1. Support for different splice variants being produced at this site is provided by one of the four clones sequenced from the heart. The complete exon 21 was missing in this clone as opposed to our result, confirmed in a second clone, where the splicing event does not remove the first and the 26 most 3’ nucleotides of exon 21 (i.e. alternate use of 3’ splice acceptor site).

A similar study demonstrated that the cardiac-dominant Na\textsubscript{v}1.5 appears to distribute its functional and nonfunctional splice variants across the brain and heart in what could be considered a direct reversal
of our findings with the neuronal isoform Na\textsubscript{v}1.1. Wang et al (2008c) identified a novel Na\textsubscript{v}1.5 splice variant missing the 54 amino acid exon 24 in the DIII pore loop, and later (Wang et al, 2009) demonstrated that expression of this variant in a heterologous expression system does not produce current. Wang et al (2008c) use software to estimate DNA content of the RT-PCR bands and report that the functional variant is preferentially expressed 4:1 in the heart, as compared with roughly equal expression of the functional and nonfunctional splice variants in the brain. Similarities between this finding and the present data include the involvement of a large deletion in the DIII pore loop; splice variants demonstrated to, or in our study expected to, devastate channel function; and the involvement of exon skipping in forming the splice variants.

The number of similarities between this data and our findings, tempts the author to speculate. The primary difference between our data and data presented by Wang et al (2008c) is the finding of two Na\textsubscript{v}1.1 sites versus one Na\textsubscript{v}1.5 site where alternative splicing devastates or likely devastates channel function. This point is made more important given our data suggesting that all of the channel expressed in one of its non-dominant tissues (i.e. neuronal Na\textsubscript{v}1.1 in heart) is nonfunctional. Wang et al (2008c) identified their novel splice variant through cloning and sequencing 10 overlapping segments of the full-length rat brain Na\textsubscript{v}1.5 transcript. However, the first walk over the full-length Na\textsubscript{v}1.1 transcript in the present study with only 6 primer pairs missed the deletions at the third deletion site. If the author had not found deletions at this site, it might have been assumed that RT-PCR bands showing the absence of c.[del266_473] were part of a functional channel.

4.8 Mechanisms for Tight Regulation of Tissue, Cell and Condition-Specific Expression of Functional Na\textsubscript{v}1.1

The point has been made and supported by mathematical modeling (Luo & Rudy, 1991) that the cell must tightly regulate membrane currents and the electrochemical gradients across membranes that they control. VGSC are highly spliced (Lin et al, 2009; Schroeter et al, 2010) and a substantial proportion of
VGSC splice variants do not produce (Plummer et al, 1997; Kerr et al, 2008; Schroeter et al, 2010) or are unlikely to produce (Plummer et al, 1997), currents in expression systems. It has been suggested that controlling the level of functional protein within a cell through alternative splicing allows a faster response than would be possible with control of channel transcription (Lewis et al, 2003; Copley, 2004). In this scenario, Na\textsubscript{V}1.1 transcript would remain nonfunctional but “at the ready”. This section of the thesis reviews literature, supported by secondary findings in our data, that paints a picture of complex VGSC control of expression, and furthermore provides the potential means for cell and condition-specific regulation of Na\textsubscript{V}1.1 alternative splicing.

Lu et al (1998) draw a contrast between the high level of VGSC coding sequence similarity across both isoforms and species, with the complexity of and low levels of similarity across isoforms of, their 5΄ UTR’s. Martin et al (2007) make the point that Na\textsubscript{V}1.1 demonstrates distinct tissue, tissue region and cell-type expression patterns. This isoform has a particularly complex upstream untranslated region (Martin et al, 2007; Long et al, 2008). One feature of the complexity of VGSC 5΄ UTR’s is the appearance of different untranslated exons (UTE’s), and these are found to change both for Na\textsubscript{V}1.1 as well as for other VGSC across tissues, tissue regions and cell culture conditions (Martin et al, 2007; Long et al, 2008). Additionally, evidence has been presented for multiple VGSC promoters within the same 5΄ UTR in a cancer cells (Na\textsubscript{V}1.2; Maue et al, 1990), humans (Na\textsubscript{V}1.2; Shade & Brown, 2000) (Na\textsubscript{V}1.1; Long et al, 2009), and rodent (Na\textsubscript{V}1.5; Shang & Dudley, 2005).

Martin et al (2007) labeled the different Na\textsubscript{V}1.1 upstream UTE’s in human and mouse. Their examination of a large number of clones (human N=150; mouse N=31) found versions of the upstream segment present in > 50 percent of clones, and documented the appearance of rare (i.e. in 1 of 150 clones; not represented in 31 clones although suggested by genomic sequence) UTE’s. Our cloning and sequencing results for the initial segment of the Na\textsubscript{V}1.1 transcript document a novel 5΄ UTR in rat heart (Figure 26). The published rat Na\textsubscript{V}1.1 5΄ UTR (Noda et al, 1986a) contains a 21 nucleotide long segment

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of imperfect repeats: our novel 5’ UTR found in 1 of 7 clones, contains a novel 95 nucleotide long segment of imperfect repeats.

Repeat sequences and resulting secondary structures near the core promoter region are believed to have the potential to influence binding to the ribosome (Dr. Donald Forsdyke—Queen’s University, personal communication). Evidence for this has been published for glutamate receptor 2 transcript (Myers et al, 2004). This transcript has different translation start sites and therefore different lengths of the 5’ UTR. Expression of recombinant versions of the in vivo 5’ UTR in expression systems, showed that glutamate receptor 2 transcripts including a 5’ UTR imperfect 34 to 42 nucleotide repeat expected to form a secondary structure in vivo, had lower translation efficiency. Furthermore, this characteristic of the 5’ UTR stood out with little or no effect of many other UTR characteristics believed to play an important role such as the number of alternate start sites. Transcripts both with and without the repeat are found both in human and rat, and transcripts lacking this repeat were more likely to be associated with the ribosome (rat cortex).

Moreover, the use of multiple versions of 5’ UTR sequences with differences in translation efficiency may provide a mechanism for cell- and condition-specific VGSC alternative splicing. Myers et al (1998) report that the relative amount of the glutamate receptor-2 transcripts with the long 5’ UTR’s containing the repeat sequence varies across brain regions. Mancl et al (2005) take this one step further. They demonstrate two different interferon regulatory factor-5 5’UTR’s with different promoter regions and transcription start sites. Furthermore, these upstream features were uniquely associated with different coding sequence functional splice variants, each with different cell-type distributions and responsive to different cell conditions. Pursuit of these lines of research may show that specific NaV1.1 5’ UTR’s and promoters are associated with the expression of nonfunctional splice variants, and that in concert with tissue and cell condition-specific splicing factors (Hanamura et al, 1998; Diss et al, 2004), these provide tight control over the levels of functional channel expression.

Finally, our data and a previous paper (Noda et al, 1986a), introduce the idea of VGSC using RNA
editing to control functional sodium channel expression. Noda et al (1986a) report three nucleotide positions within the Na\textsubscript{V}1.1 coding sequence from rat brain where the nucleotide “flip flops” across transcripts between adenine and guanine (+1441, +1780, & +2467), and suggest a possible association between these variants and the presence or absence of the 33 nucleotide deletion in the first intracellular loop. Our limited look at approximately 600 nucleotides surrounding the novel c.[del266_473] deletion site, suggests both that the nucleotide at position +254 of the Na\textsubscript{V}1.1 coding sequence may be edited by the cell (guanine versus thymine), and that this process may influence the cell’s decision to keep or remove this section of the transcript.

While RNA editing was until recently believed to be extremely rare in mammals, recent work suggests that this is a widespread and non-random phenomenon in humans (Li et al, 2011). Six papers document RNA editing in insect VGSC (e.g.’s Song et al, 2004; Olson et al, 2008). Furthermore, one of these papers linked RNA editing with alternative splicing and important VGSC electrophysiological changes (Song et al, 2004). In this context, it is interesting that RNA editing has also been linked with the level of activity of the NMD pathway in humans, a process that is likely involved in translating the cell’s response to our novel Na\textsubscript{V}1.1 splice variant (Agranal et al, 2008).

4.9 The Search for the Voltage-Gated Sodium Channel Isoform(s) that Play an Important Role in Ischemia/Reperfusion Injury

Indiscriminate sodium channel blockade in the 1980’s (CAST antiarrhythmic drug trial—Tsuneaki & Ogawa, 1994) highlighted the importance of respecting the critical role of VGSC function in the heart, and overriding statements can be found in the literature suggesting that more specific cellular targets will be required (Ju et al, 1996; Nattel, 1998) to manage the clinical outcomes associated with ischemic heart disease. Two groups (Haufe et al, 2005a; Brette & Orchard, 2006a) using different experimental methods, have concluded that “non-cardiac” VGSC contribute approximately 10% of peak sodium currents in cardiac myocytes under normal cellular conditions. As well, one of these groups used
the same technique to examine the contribution of these channels to $I_{Na,P}$ (Biet et al, 2012). They conclude that non-cardiac VGSC contribute almost half of these currents under the same cellular conditions. This thesis presents data suggesting that all Nav1.1 in the ventricular myocytes of normal, healthy adult rats, is rendered nonfunctional through alternative splicing. Therefore, one or more other non-cardiac VGSC contribute these currents and, more importantly, are candidates for the channel(s) that produce the increases in $I_{Na,P}$ in response to $H_2O_2$ application (Ward & Giles, 1997) or during hypoxia (Ju et al, 1996) that are thought to play a key role in the clinical outcomes associated with MI (Haigney et al, 1994; Pike et al, 1995; Imahashi et al, 1999 & 2005; Song et al, 2006; Bers, 2008). Unpublished pharmacological evidence from our lab suggests that neuronal VGSC are important players in the increased persistent sodium currents that are central to the clinical outcomes of ischemia/reperfusion. This is consistent with the important role of persistent sodium currents in neuronal tissues (Llinas & Sugimori, 1980; Aracri et al, 2006).

Next steps in identifying the VGSC isoforms mediating ischemia/reperfusion injury and lethal arrhythmia in heart disease, will need to consider emerging evidence showing that VGSC are highly spliced with a significant portion of alternative splicing events rendering the channel nonfunctional (Schaller et al, 1992; Plummer et al, 1997; Zimmer et al, 2002a; Stamm et al, 2005; Kerr et al, 2008; Wang et al, 2008c; Schroeter et al, 2010). The latter point is underscored by the present work suggesting that a VGSC expressed in ventricular myocytes does not exist in these cells in a functional form. Neither antibody binding to a roughly 20 amino acid section of a neuronal VGSC (Cohen et al, 1996; Malhotra et al, 2001; Maier et al, 2002; Maier et al, 2004; Haufe et al, 2005a; Haufe et al, 2005b), nor published sequence for segments ($\leq 1528$ bp of Nav1.1, Nav1.2, Nav1.3; Schaller et al, 1992 & Blechschmidt et al, 2008) of transcript, provides evidence for functional neuronal VGSC in the heart. Zimmer et al considered this, using a single primer pair to demonstrate three full-length versions of the cardiac-dominant Nav1.5 coding sequence in mouse heart. One of three failed to produce functional channel when the transcripts were expressed individually in an expression system. They searched for but did not
find, deletion variants of skeletal muscle-dominant Na\textsubscript{V}1.4 RT-PCR amplicons produced by 4 overlapping PCR primer pairs covering the full-length transcript.

As well, examination of the contribution of different VGSC to persistent gating under normal conditions (Maltsev et al, 2008; Biet et al, 2012; Yang et al, 2012) is one step removed from understanding the role of these channels in producing the increased I_{Na,p} that leads to cardiac arrhythmia, either in response to H\textsubscript{2}O\textsubscript{2} application or in a patient experiencing cardiac ischemia. Cellular conditions act through cell signaling mechanisms to modify VGSC inactivation (West et al, 1991; Abriel et al, 2007). More specifically protein kinase C (PKC) inhibition, shown to act through both shared and isoform-specific mechanisms (Qu et al, 1996, Murray et al, 1997), delayed and attenuated the action potential prolongation with H\textsubscript{2}O\textsubscript{2} application to ventricular myocytes (Ward & Giles, 1997). In the same way, models of increased persistent gating that are created through unknown mechanisms or mechanisms unrelated to the conditions seen by ischemic heart (e.g. sea anemone toxin—Yang et al, 2012), are difficult to interpret in terms of the involved channels or mechanisms in heart disease.

In addition, while Na\textsubscript{V}1.1 transcripts expressed under normal conditions in rat heart are unable to pass sodium current across the cell membrane, the author suggests that the cellular work to produce these transcripts serves some purpose. Na\textsubscript{V}1.1 has all the hallmarks of a gene that has evolved the ability to respond to changes in cell conditions with changes in expression level. There are striking similarities between our findings and those of Wang et al (2008c), who performed in effect the reverse of our study looking for splice variants of the cardiac-dominant VGSC in the brain. As well the introduction of a premature stop codon through alternative splicing that is demonstrated in the present study, has been shown to be a conserved and regulated process (Plummer et al, 1997; Lewis et al, 2003; Kerr et al, 2008).

As neuronal channels are the most likely candidates for channels that produce currents responsible for lethal cardiac arrhythmia and ischemia/reperfusion injury, it is of great interest to consider how the splicing patterns of Na\textsubscript{V}1.1 in the heart might change within a background of ischemic heart disease. It has been estimated that 29 to 50 percent of patients experience anginal symptoms prior to their
first MI (Feil, 1937 and Sampson & Eliaser, 1937 reviewed in Mounsey, 1951; Mounsey, 1951), and these statistics do not include the estimated 30% of adults with cardiovascular risk factors but no documented disease that experience silent ischemia (Anand et al, 2004). As well, while the majority of deaths due to arrhythmia occur in the setting of myocardial infarction (MI), there is an elevated risk for these arrhythmias over the month post-MI (Henkel et al, 2006). It could be argued then that experimental conditions designed to mimic ventricular myocyte conditions in these clinical settings would provide more insight into the VGSC isoforms involved in the clinical outcomes of ischemia/reperfusion.

In a paper (Shang et al, 2007) reviewed in an earlier section (4.6.2 Proposed Dominant-Negative Impact of “Nonfunctional” Alternative Splice Variants on Functional Protein Expression), it was shown that heart failure is associated with a higher expression of nonfunctional Na\textsubscript{v}1.5 splice variants and reduced expression of the wild-type channel. Huang et al (2001) provide evidence that they suggest argues for a larger contribution of neuronal sodium channel currents in rat ventricular myocytes one month post experimental MI. Macropatch currents (roughly 50 channels) supported an increase in persistent gating post-MI. Moreover, post-MI rats have significantly longer APD’s and 100 nM TTX expected to completely block TTX-sensitive channels with limited impact on Na\textsubscript{v}1.5, preferentially shortens the APD of the post-MI as compared with the sham-operated rats. This group shows blot hybridization data suggestive of increases in Na\textsubscript{v}1.1, however this probe would equally bind the functional and nonfunctional Na\textsubscript{v}1.1 transcript variants reported in the present work.

Finally, secondary findings in the present work may be consistent with emerging knowledge regarding mechanisms controlling gene expression and these areas may have potential for elucidating novel approaches to pharmacological treatments. Noda et al (1986a) state that three positions in the rat brain Na\textsubscript{v}1.1 sequence flip-flop between two different nucleotides, and suggest that these variants may be linked with the 33 bp deletion produced in some transcript through alternative splicing. Data from the present study may be consistent with another nucleotide position that exists in two versions in rat heart having the ability to predict the novel c.[del266_473] deletion, i.e. nonfunctional transcript. Furthermore,
it appears from the literature (Meyers et al, 1998; Mancl et al, 2005; Martin et al, 2007; Long et al, 1998), that variable VGSC 5’ UTR regions may dictate expression levels and transcript splicing in a cell and condition-specific manner. Our sequencing of 7 clones, documents a novel Na\textsubscript{v}1.1 5’ UTR with a 95 bp repeat in rat heart. In one paper (Myers et al, 2004), the 5’ UTR characteristic that was most strongly linked with expression levels was the presence of imperfect repeat sequences. Systematic examination of these phenomena may hold promise in terms of pharmacological approaches that can target the expression of individual VGSC isoforms in disease states.

4.10 Conclusions and Next Steps

The seminal finding from this work is that a VGSC isoform, widely accepted to have been shown to be in rat heart, does not exist in rat ventricular myocytes in a functional form under our experimental conditions. VGSC are known to be highly spliced, with a significant proportion of alternative splicing events producing transcript that does not code for functional protein. Despite this, the predominant approach in the literature has been the use of isoform-specific antibodies against dissociated ventricular myocytes. Antibodies bind to a roughly 20 amino acid section of channels and, therefore, do not distinguish between the protein products of functional versus nonfunctional splice variants. We have shown evidence suggesting that under normal cellular conditions, all versions of Na\textsubscript{v}1.1 transcript in rat ventricular myocytes have one or two deletions that individually are likely capable of rendering the channel unable to pass current. We are not the first to report the inability to detect a functional version of a VGSC isoform using RT-PCR, in a tissue expressing a nonfunctional version of the channel (Wang et al, 2008c). However, we are the first to show that this splicing pattern is a part of VGSC expression in the heart.

Next steps will pursue two avenues. For the reasons outlined above a role for Na\textsubscript{v}1.1 in the clinical outcomes of ischemia/reperfusion in different patient groups cannot be ruled out. Examination of the relative strength of Na\textsubscript{v}1.1 bands matching the expected size for transcript with and without a deletion at the first and third deletion sites, will provide a screen for identifying any time points over the month or
two post-MI that are associated with changes in Na\textsubscript{v}1.1 splicing. Data suggesting a switch to expression of functional Na\textsubscript{v}1.1 transcript can be followed up using the techniques in this thesis for identification of the full-length Na\textsubscript{v}1.1 versions being expressed. Animal models mimicking long-term myocardial ischemia without myocardial infarction would need to be developed for use in these experiments in order to understand the involved channels in the acute and early post MI periods for a large proportion of the patient population.

In addition, S1 nuclease digestion used by Noda et al (1986a) to confirm c.[del2012_2044], would be used to obtain evidence for c.[del4004_4258] and to examine the relative abundance of the two deletion variants at this site. As well research would examine the full-length versions and any splice variants of Na\textsubscript{v}1.6 in rat right ventricle under normal cellular conditions. The literature suggests that this is the next likely candidate for channels involved in the increased persistent gating demonstrated in isolated myocytes in response to the introduction of conditions simulating myocardial ischemia/reperfusion.
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