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Function Study of Atrial Fibrillation-Linked Connexin Mutants and Heterotypic Docking Compatibility of Cardiac Connexins

Willy G. Ye

The University of Western Ontario

Supervisor
Dr. Donglin Bai
The University of Western Ontario

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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FUNCTIONAL STUDY OF ATRIAL FIBRILLATION-LINKED CONNEXIN MUTANTS AND HETEROTYPIC DOCKING COMPATIBILITY OF CARDIAC CONNEXINS

by

Willy G. Ye

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
Western University
London, Ontario, Canada

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Abstract

Gap junctions are critical for the propagation of action potentials in the heart. Many autosomal dominant Cx40 mutations have been linked to atrial fibrillation (AF) and have showed GJ impairments. However, others such as L221I and V85I showed apparently no GJ impairments. Cells expressing L221I or V85I alone showed increased propidium iodide uptake, which was also the case for cells co-expressing L221I and wildtype Cx40, suggesting increased hemichannel opening probability. A novel AF-linked Cx45 mutant (M235L) was found to impair GJ function. These alterations in hemichannel or GJ function might play a role in promoting AF. The atria express Cx40 and Cx43 abundantly with low levels of Cx45, and may form heterotypic GJs. However, wildtype heterotypic Cx40/Cx43 or Cx40/Cx45 GJs were not functional, unless a designed Cx40 structural variant (D55N) was used, suggesting these heterotypic GJs are unlikely to mediate coupling in the native heart.

Keywords

Connexin, hemichannel, gap junction, dye uptake, dual whole-cell patch clamp, atrial fibrillation, heterotypic, docking, compatibility
Co-Authorship Statement

The work presented in this thesis has been performed by me. Some of my work in Chapter 2 of my thesis has already been published as a joint manuscript as indicated in the chapter. Some of my work in Chapter 4 has also been included in a joint manuscript that is currently in review.
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Most importantly, I would like to thank my family for their continued support by providing me the sustenance I needed to keep on working and for always being there whenever I needed any help regardless of what it may be.
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<tr>
<td>AF</td>
<td>Atrial fibrillation</td>
</tr>
<tr>
<td>AV</td>
<td>Atrioventricular</td>
</tr>
<tr>
<td>CL</td>
<td>Cytoplasmic loop</td>
</tr>
<tr>
<td>CT</td>
<td>Carboxyl-terminus or C-terminal</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DCC</td>
<td>Divalent cation containing</td>
</tr>
<tr>
<td>DCF</td>
<td>Divalent cation free</td>
</tr>
<tr>
<td>E1</td>
<td>First extracellular loop</td>
</tr>
<tr>
<td>E2</td>
<td>Second extracellular loop</td>
</tr>
<tr>
<td>ECF/S</td>
<td>Extracellular fluid/solution</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>FFA</td>
<td>Flufenamic acid</td>
</tr>
<tr>
<td>GJ</td>
<td>Gap junction</td>
</tr>
<tr>
<td>Gj</td>
<td>Transjunctional conductance</td>
</tr>
<tr>
<td>HB</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>HC</td>
<td>Hemichannel</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical cancer cells</td>
</tr>
</tbody>
</table>
ICF  Intracellular fluid
I_j  Macroscopic transjunctional current
IRES  Internal ribosomal entry site
MCS  Multiple cloning site
MFQ  Mefloquine
ms  Millisecond
N2A  Mouse neuroblastoma cells
nS  Nanosiemens
NT  Amino-terminus or N-terminal
PI  Propidium Iodide
SA  Sinoatrial
SEM  Standard error of the mean
TM  Transmembrane domain
V_j  Transjunctional voltage
+V_j  Positive transjunctional voltage
-V_j  Negative transjunctional voltage
V_m  Transmembrane voltage
γ_HC  Hemichannel unitary conductance
γ_j  Gap junction unitary conductance
μm  Micrometer
Chapter 1: Introduction

1.1 Gap junction channels

Direct communication between apposed cells is mediated by intercellular channels known as gap junctions (GJs). These channels allow the passage of ions and various molecules less than 1 kDa in size and are important for a number of physiological processes such as development, metabolic cooperation, and tissue synchronicity (Evans and Martin, 2002).

The connexin (Cx) is an integral membrane protein which constitutes the GJ channel. 21 genes in the human genome and 20 in the mouse encode connexins, and each connexin is named according to its predicted molecular weight in kDa from Cx23 to Cx62 (Sohl and Willecke, 2004). Connexins are predicted to have a topological structure of four transmembrane domains (TM1-TM4), two extracellular loops (E1-E2), one cytoplasmic loop (CL), and intracellular amino and carboxyl terminals (NT and CT, respectively) (Fig. 1.1A). These connexins oligomerize into hexamers to form the hemichannel (HC) (or connexon). Based on the connexin arrangement, these HCs can be homomeric or heteromeric (Evans and Martin, 2002).

HCs exist in nearly all cells that express connexins (Saez et al., 2010), and they should not be confused with pannexins, which are similar in topological structure (Sosinsky et al., 2011). When docked to form a full GJ channel, these docked HCs (or GJ channel) open to allow intercellular communication between cells. When undocked, however, HCs usually remain closed especially in cultured cells in resting conditions, but this may not be reflective of those in vivo, as various amounts of stimuli are present (Saez et al., 2010). The
open probability of HCs can be increased by various means such as membrane depolarization, mechanical stimulation, increased intracellular calcium ([Ca$^{2+}$]$_i$), or decreased extracellular calcium ([Ca$^{2+}$]$_o$ (Bennett et al., 2003; De Vuyst et al., 2006; Goodenough and Paul, 2003; Tong et al., 2007). When opened, HCs may appear to play a role in paracrine signaling due to their ability to leak out ions and signalling molecules such as ATP, Na$^+$, PGE$_2$, and Ca$^{2+}$ (Fig. 1.1B) (Kar et al., 2012). Unitary conductance of HCs (γ$_{HC}$) also tend to be approximately twice that of unitary transjunctional conductance (γ$_j$) as demonstrated in Cx26 (Gonzalez et al., 2006).

In order to form a GJ channel, each neighbouring cell contributes at least a HC, which dock head-to-head through non-covalent interactions, including hydrogen bonds. Hydrogen bonds are critical in docking as a reduction in the number of hydrogen bonds prevent docking and function altogether (Gong et al., 2013). As most cells express more than one connexin isoform, connexins can oligomerize in different combinations or arrangements as they form HCs. Based on connexin composition, the HCs can be classified as homomeric or heteromeric, and the GJ channels can be classified as homotypic or heterotypic (Fig. 1.1C) (Evans and Martin, 2002). Due to the differences in amino acid sequences of connexins, not all homomeric heterotypic combinations produce functional GJ channels (Bai and Wang, 2014). It is interesting to understand which residues are important for heterotypic docking of non-compatible connexins.

As previously mentioned, GJ channels provide electrical coupling between cells, which is critical for tissue synchronicity, such as in the heart. GJ channel function can be influenced by changes in potential difference between the cytoplasms of coupled cell-pairs (transjunctional voltage, V$_j$) and the absolute voltage between internal and external
environments of the cell (transmembrane voltage, \( V_m \)). While \( G_j \) of all vertebrate GJ channels is sensitive to \( V_j \), some GJ channels are influenced by both \( V_j \) and \( V_m \) (Gonzalez et al., 2007). Maximal \( G_j \) for homotypic GJ channels tends to occur when \( V_j \) is equal to zero. Moreover, at increasing positive and negative \( V_j \), symmetric decreases to low conductance values (\( G_{\text{min}} \)) occur to form a bell shape as demonstrated in amphibian blastomeres (Harris et al., 1981). The \( V_j \)-gating properties can be described by the Boltzmann function for each \( V_j \) polarity (Gonzalez et al., 2007). GJs of different connexins show different \( V_j \)-gating properties, which may be important for their unique function.
Figure 1.1 Topological structures of the connexin, hemichannels, and gap junction channels. 

A. A topological structure of a connexin, an integral membrane protein, which has four transmembrane domains (TM1-TM4), two extracellular domains (E1-E2), one cytoplasmic loop (CL), and intracellular amino and carboxyl terminals (NT and CT, respectively). 

B. A connexin hemichannel. A hexamer of connexin proteins, which can allow the passage of a variety of ions, metabolites, and signaling molecules. 

C. The four types of gap junction channels based on different possible connexin rearrangements. The nomenclature on hemichannels and GJ channels are indicated below each channel.
1.2 Cardiac connexins

Gap junctions are required for the rapid conduction of electrical signals for synchronized contractions in the heart. Under physiological conditions, action potentials originate from the sinoatrial (SA) node, which propagates to the atria, and slows down in the atrioventricular (AV) node. From there, it travels through the Purkinje-His conduction system and finally from the apex of the heart to the ventricles. Any disruption in the rapid propagation of action potentials in the heart may lead to complications which may result in ventricular and atrial fibrillation (AF), as examples (Hagendorff et al., 1999).

Three major connexins, located in specific regions of the heart, are responsible for the heart’s electrical synchronicity (Fig. 1.2). Connexin45 (Cx45) resides in the nodal regions and in the Purkinje-His conduction system, including the upper and lower bundle branches, of the heart. Connexin40 (Cx40) resides in the atria and in the Purkinje-His conduction system including the upper and lower bundle branches, and connexin43 (Cx43) resides primarily in the atria and ventricles with some expression in the Purkinje fibres and lower bundle branches. (Severs et al., 2008). My thesis will focus on two of these cardiac connexins, namely Cx40 and Cx45.
Figure 1.2 Typical expression of the three major cardiac connexins: Cx40, Cx43, and Cx45. (Adapted from Severs et al., 2008)
1.2.1 Connexin40

Cx40 is one of the three major connexins expressed in cardiac tissue. It is encoded by the gap junction protein alpha 5 (GJA5) gene on chromosome 1 in humans (Willecke et al., 1990). It has also been shown to be expressed in other tissues such as smooth muscle cells and endothelial cells (Gabriels and Paul, 1998; Little et al., 1995).

Cx40 is highly expressed in the atria and tends to co-localize with Cx43 (Gemel et al., 2014). The possibility of the existence of heteromeric Cx40 and Cx43 HCs have also been postulated through the use of co-immunoprecipitation. This introduces a lot of possible variability in GJ function as multiple single channel current amplitudes were observed through functional studies (He et al., 1999). This provides a contrast to homomeric heterotypic Cx40 and Cx43 GJ channels as studies show conflicting results in regards to the presence of function, which may be due to docking compatibility (Bruzzone et al., 1993; Lin et al., 2014; Valiunas et al., 2000).

The importance of Cx40 has been shown in a number of animal models (Chaldoupi et al., 2009). In ECG recordings of Cx40 knockout (KO) mice there were no differences in heart rate, but interval prolongation in each tested ECG parameter was observed, which suggests a decrease in conduction velocity. In addition, arrhythmia was also observed in these mice, which highlights the importance of the presence of Cx40 in cardiac tissue for fast and proper electrical conduction (Hagendorff et al., 1999; Kirchhoff et al., 1998).
1.2.2 Connexin45

Cx45 is one of the principal connexins expressed in the heart, and is expressed in specific regions such as the nodal tissues and conduction system. It is encoded by the gap junction protein gamma 1 (GJC1) gene on chromosome 17 (Kanter et al., 1994). Cx45 can also be found in a variety of tissues such as the lung, brain, and skin (Hennemann et al., 1992).

In the early stages of the fetus, Cx45 is highly expressed and plays a major role in heart development. It has been shown that Cx45 knockout mice died as early as E9.5, as after the initiation of vasculogenesis, blood vessel formation and smooth muscle layer development in the arteries were not able to proceed (Kruger et al., 2000). In adult mice, however, cardiomyocyte-restricted Cx45 KOs were viable but showed AV nodal conduction impairment. This was shown by a prolonged PQ interval on the electrocardiogram and prolonged AH (atria to His bundle) interval on the intracardiac electrogram, which highlights its importance in fetal development, especially in the heart (Frank et al., 2012).

1.3 Atrial fibrillation

Atrial fibrillation (AF) is a sustained cardiac arrhythmia which affects more than 2.5 million Americans and 350,000 Canadians (Fuster et al., 2006). This number is expected to almost triple by the year 2050, which will lead to an increased burden on the health care system (Go et al., 2001). In most cases, AF is believed to be secondary to systemic disorders or heart diseases since it will lead to structural changes in the heart which promote AF. These diseases include: diabetes, hypertension, coronary artery disease, and myocardial infarction (Benjamin et al., 1998). In approximately 30% of cases, however,
AF occurs on its own without association with cardiovascular diseases; this is called idiopathic or lone AF. The study of lone AF provides insight into the possible etiology and pathophysiology of AF without confounding factors that may be present when systemic or cardiovascular disease exists.

Patients with AF exhibit an irregular and erratic P-wave pattern on an electrocardiogram, which translates into improper atrial conduction, and subsequently, contraction. Consequently, this may affect the electrical conduction to the ventricles, which would negatively affect ventricular contraction as shown by a reduced QRS-complex (Wakili et al., 2011). These patients would have a six-fold increase in risk for embolic stroke and a two-fold increase in risk for death (Wolf et al., 1991).

1.4 Rationale and Objectives

GJA5, the gene encoding Cx40, has been suspected to be an important player responsible for lone AF. In a previous study, 4 out of 15 patients had mutations in GJA5 and showed early onset of lone AF (Gollob et al., 2006). Two recent germline GJA5 mutants, L221I and V85I, were discovered by Yang and colleagues, which were shown to be autosomal dominant with 100% penetrance (Fig. 1.3) (Yang et al., 2010). In our lab, we have demonstrated that L221I and V85I showed similar Gj as wildtype Cx40, which was unusual since most, if not all, Cx mutants show GJ impairment (Fig 1.4). When we assessed the potential hemichannel activity of these mutants through the dye uptake assay, we saw pronounced hemichannel activity. Since these mutants are autosomal dominant, it suggests that one allele is enough to trigger AF. In the atria, Cx40 and Cx43 are co-expressed and provide the possibility that these mutants interact with either Cx40 or Cx43. It will be
interesting to determine whether or not any differences in hemichannel activity exist when these mutants are present with wildtype Cx40 or Cx43.

As previously mentioned, differences in certain amino acid residues in the extracellular domains may be responsible for the incompatibility in heterotypic docking observed between different Cxs. Controversial data exists for the docking of Cx40 and Cx43, so it may be interesting to determine which residue(s) will be responsible for compatibility. In addition, previous studies on the heterotypic docking of mouse Cx40 and Cx45 showed coupling (Rackauskas et al., 2007). However, there were no studies on the heterotypic docking between human Cx40 and Cx45. This might be important for action potential propagation from the SA node to the atria and from the AV node through the ventricular conduction system, which is enriched with both of these connexins.

In summary, the objectives of this study is to characterize the hemichannel function of Cx40 mutants co-expressed with wildtype Cx40 or Cx43, and subsequently understand their possible role(s) in atrial fibrillation; characterize the GJ channel function of the Cx45 mutant and understand their possible role(s) in atrial fibrillation; and functionally study the role of the extracellular domains in the docking compatibility of cardiac connexins.
Figure 1.3 Pedigrees of the two Cx40 mutants. In the case of both (A) L221I and (B) V85I atrial fibrillation (denoted by the coloured shapes) co-segregated with each mutant (denoted by +) regardless of male (square) or female (circle). The probands are identified by the arrow. (Adapted from Yang et al., 2010)
AF-linked Cx40 mutants

Germline mutations are denoted in red and somatic mutations are denoted in green. (Adapted from Bai, 2014)
1.5 Hypotheses

1. I hypothesize that the co-expression of the AF-linked Cx40 mutants, L221I and V85I, with wildtype Cx40 or Cx43 will show an increased function compared to Cx40 hemichannels or heteromeric wildtype Cx40/Cx43 hemichannels.

2. I hypothesize that the Cx45 mutant, M235L will alter GJ function in homotypic GJs or heterotypic GJs with Cx45 (or Cx43). I also hypothesize that this mutant will show a difference in hemichannel activity.

3. I hypothesize that engineered variant of Cx40, D55N, will increase GJ coupling and GJ conductance in the docking of Cx43 and Cx45 compared to wildtype Cx40.
1.6 References


Chapter 2: Functional study with propidium iodide uptake on cells co-expressing atrial fibrillation-linked connexin40 mutants with connexin40 or connexin43

2.1 Introduction

Direct cell-cell communication via gap junctions (GJs) is critical for the rapid propagation of action potentials in the heart to synchronize the beating of the atria and ventricles. Gap junction channels are formed by a head-to-head docking of two hemichannels (HCs). HCs are hexamers of integral membrane proteins called connexins (Cxs). HCs composed of the same connexin isoform are homomeric and HCs composed of different connexin isoform are heteromeric (Goodenough and Paul, 2009). There are 21 human and 20 mouse genes that encode connexins (Sohl and Willecke, 2004). My study is focussed on Cx40 and Cx43, which are the major connexins expressed in the heart. Cx40 is expressed in the atria and in the ventricular conduction system including the Purkinje-His bundle and the upper and lower bundle branches. Cx43 is the most abundant connexin in the heart and it is expressed in the atria, ventricles, and in the Purkinje fibres and lower bundle branches. As the atria expresses both Cx40 and Cx43, it presents the possibility of forming homomeric or heteromeric HCs (Severs et al., 2008). The possibility of forming heteromeric Cx40-Cx43 HCs have been explored through co-immunoprecipitation and functional studies. Co-immunoprecipitation experiments were performed in vascular smooth muscle cells, which co-express Cx40 and Cx43. Anti-Cx40 antibodies were able to co-immunoprecipitate both Cx40 and Cx43, and vice versa. This would suggest that the interaction between Cx40 and Cx43 may be the formation of heteromeric HCs. To support this further, functional studies were performed on cell-pairs co-expressing Cx40 and Cx43, which showed many new levels of single channel conductances that were not observed when expressing Cx40 or
Cx43 alone. These experiments would suggest that Cx40 and Cx43 may be capable in forming heteromeric HCs (He et al., 1999).

Atrial fibrillation (AF) is a sustained cardiac arrhythmia which affects nearly 2.5 million Americans and is expected to almost triple by 2050. This will increase the burden on the healthcare system (Fuster et al., 2006; Go et al., 2001). Patients with AF are twice as likely to experience death and six times more likely to have embolic stroke compared to those without AF (Wolf et al., 1991). In 70% of cases, AF is secondary to systemic disorders and diseases such as hypertension, diabetes, myocardial infarction, or other cardiomyopathies (Benjamin et al., 1998). For the remainder of cases, AF is the primary illness and is termed “idiopathic” or lone AF. Lone AF can be triggered by changes in genes whose protein products are critical in atrial function.

Mutations in GJA5, the gene for encoding Cx40, were found to be linked to AF. In vitro studies of these mutations showed impaired protein trafficking and/or GJ coupling (Gollob et al., 2006; Sun et al., 2013). These studies provide a glimpse into the importance of proper GJ channel function to maintain proper physiological functions. In 2010, Yang and colleagues discovered two missense mutations in the gene for Cx40 which led to mutations L221I and V85I. Based on the pedigrees of the probands, these mutations were shown to be autosomal dominantly inherited with 100% penetrance as the members of the families who carry either mutation in one of their two Cx40 alleles developed an early onset of AF (Fig. 1.3) (Yang et al., 2010).

Our lab has studied the GJ function for the two Cx40 mutants, L221I and V85I, and found that it was similar to wildtype Cx40 (Sun et al., 2014). This was unusual as most, if not all,
known connexin mutants show some level of impairment of GJ channel function compared to wildtype (Bai, 2014). As these mutations apparently did not alter gap junction function, it was possible that hemichannel function was affected. Experiments with propidium iodide (PI) uptake revealed that these mutants appeared to have an increased PI uptake compared with wildtype Cx40 (Sun et al., 2014), indicating these mutants may have an increase in hemichannel function. Since these are autosomal dominantly inherited mutants, one allele would encode the mutant and the other the wildtype. Therefore, the co-expression of Cx40 mutants with wildtype Cx40 (or Cx43) is possible to occur in vivo. We hypothesize that the co-expression of the AF-linked Cx40 mutants, L221I and V85I, with wildtype Cx40 or Cx43 will show an increased PI uptake compared to cells expressing Cx40 alone or Cx40 co-expressed with Cx43.

My data showed that PI uptake was significantly reduced by the gap junction blockers, flufenamic acid and mefloquine, which indicate that the PI uptake was likely mediated by GJ HCs. In addition, the PI uptake of L221I co-expressed with Cx40 was significantly increased compared to the doubly-expressed wildtype Cx40. However, V85I co-expressed with Cx40 or both Cx40 mutants co-expressed with Cx43 showed no significant difference in PI uptake compared to their wildtype controls.
2.2 Methods

2.2.1 Plasmid construction

Both Cx40 mutants, L221I and V85I, and human Cx40-IRES2-EGFP or Cx43-IRES2-EGFP genes were subcloned into a bidirectional vector (pBI-CMV1) (Fig. 2.1) (Clontech Laboratories Inc., Mountain View, CA) at the following sites:

MCSI: IRES2-EGFP

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sites:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx43</td>
<td>NheI and NotI</td>
</tr>
<tr>
<td>Cx40</td>
<td>NheI and NotI</td>
</tr>
</tbody>
</table>

MCSII: Cx40, L221I, V85I

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sites:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx40</td>
<td>EcoRI and BglII</td>
</tr>
<tr>
<td>L221I</td>
<td>EcoRI and BglII</td>
</tr>
<tr>
<td>V85I</td>
<td>EcoRI and BglII</td>
</tr>
</tbody>
</table>

The human Cx40-IRES-GFP, Cx43-IRES-GFP and Cx26-GFP constructs were created as previously described (Sun et al., 2013; Thomas et al., 2004). The non-fusion GFP-tagged (V85I-IRES-GFP and L221I-IRES-GFP) constructs were generated by the QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA) on the respective template with the following primers and were sequenced to prevent any errors:

V85I Forward 5’-CAGATCATCTTCATCCACGCCCT-3’

Reverse 5’-AGGGCGTGAGATGAAGATGCAG-3’

L221I Forward 5’-CTGTCCTCCCTCATTAGCCTGGCTG-3’

Reverse 5’-CAGCCAGGCTATGGAGGAGGACAG-3’
Figure 2.1 Schematic of bidirectional vector. Two proteins of interest can be expressed simultaneously (Clontech Laboratories, Inc., 2009). In this case, the mutant and wildtype connexins are co-expressed to mimic the predicted expression patterns in the atria.
2.2.2 Cell culture and transfection

HeLa (human cervical carcinoma, American Type Culture Collection, Manassas, VA) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Burlington, ON) containing 4.5 g/L D-(+)-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum and 1% penicillin and streptomycin, in an incubator with 5% CO$_2$ at 37°C. HeLa cells were plated at 40–60% confluence on 35 mm petri dishes 24 hours before transfection. For each transfection, HeLa cells were incubated with 1.0 µg of a cDNA construct and 2 µL of X-tremeGENE HP DNA transfection reagent (Roche, Mississauga, ON) in Opti-MEM I + GlutaMAX-I medium supplemented with HEPES and 2.4 g/L sodium bicarbonate (Invitrogen) for 5 hours. Medium was then changed back to DMEM and cells were used for dye uptake assays approximately 24 hours after transfection.

2.2.3 Dye uptake assay

The dye uptake assay with propidium iodide (PI) was used to assess the hemichannel function of Cx40 and mutants with untagged GFP. HeLa cells were cultured at a low density to allow for isolated, single cells to be transiently transfected as described above. The cells were washed with regular extracellular solution (ECS) (also known as divalent cation-containing ECS, DCC-ECS) (in mM): 142 NaCl, 5.4 KCl, 1.4 MgCl$_2$, 2 CaCl$_2$, 10 HEPES and 25 D-(+)-Glucose. The pH and osmolarity of ECS was adjusted to 7.35 and 298 mOsm, respectively. The cells were then washed with divalent cation free-ECS (DCF-ECS), which contains no Ca$^{2+}$ or Mg$^{2+}$ and 2 mM EGTA to chelate the remaining ambient divalent cations. The cells were incubated in DCF-ECS-containing PI (150 µM) at 37°C
for 15-20 minutes to facilitate PI-uptake. After incubation, the cells were washed three times with DCC-ECS and the percentage of transfected cells (green with GFP) showing PI-uptake was measured under a fluorescent microscope (DMI2E2, Leica). Cells in pairs and clusters were excluded from measurement to avoid errors produced by gap junctions. Negative and positive controls (GFP and Cx26-GFP transfected cells, respectively) and the various incubation conditions (e.g. with hemichannel blockers flufenamic acid [50 µM] or mefloquine [25 µM]) were indicated in each experiment. For each experiment approximately 40–50 cells were counted to obtain a percentage of PI-uptake. The bar graphs were generated with 4–12 transfections.

2.2.4 Statistical analysis

Student’s t-test or one-way ANOVA followed by Newman-Keuls post-test were used to compare two or multiple groups of data, respectively, as specified. Statistical significance is denoted with asterisks (*, p < 0.05; **, p < 0.01; or ***, p < 0.001) on the graphs. The data presented on the graphs are expressed as mean ± standard error of the mean (SEM). Unless specified, all data were obtained from at least three independent experiments.
2.3 Results

2.3.1 Gap junction channel blockers, flufenamic acid and mefloquine, reduce hemichannel mediated propidium iodide uptake

A significant difference in the percentage of HeLa cells showing PI uptake in cells expressing either L221I (75.1 ± 2.9%, N = 12; p < 0.001) or V85I (34.2 ± 3.5%, N = 12; p < 0.001), compared with cells expressing Cx40 (5.0 ± 0.8%, N = 8). Two known gap junction channel blockers, flufenamic acid (FFA) and mefloquine (MFQ), were used to determine whether or not PI uptake was mediated by hemichannels. HeLa cells transiently transfected with L221I bathed in DCF-ECS with either FFA (7.7 ± 2.6%, N = 6; p < 0.001) or MFQ (21.4 ± 4.4%, N = 6; p < 0.001) and PI showed a significant decrease in the percentage of cells showing PI uptake compared to cells expressing L221I (75.1 ± 2.9%, N = 12) without blockers. HeLa cells transiently transfected with V85I bathed in DCF-ECS with either FFA (1.6 ± 0.8%, N = 6; p < 0.001) or MFQ (7.4 ± 2.5%, N = 6; p < 0.001) and PI also showed a significant decrease in the percentage of cells showing PI uptake compared to cells expressing V85I (34.2 ± 3.5%, N = 12) without blockers (Figure 2.2 and 2.3).

These data show that both blockers FFA and MFQ were able to attenuate PI uptake in cells expressing either L221I or V85I. This would suggest that the observed PI uptake seen in cells expressing L221I or V85I were mediated by connexin HCs.
Figure 2.2 Representative fluorescent images of PI uptake of HeLa cells expressing various constructs in different DCF conditions. First column shows cells transfected with genes of interest in green from GFP. Second column shows any visible PI uptake in red. Third column shows the overlay of both first and second columns. Fourth column shows the phase images of the cells. Cells expressing L221I showed prominent PI uptake in DCF. Both gap junction channel blockers, FFA (50 µM) and MFQ (25 µM), reduced PI uptake for HeLa cells expressing L221I. Cells expressing V85I in DCF showed PI uptake. Both FFA and MFQ reduced PI uptake of cells expressing V85I. Scale bars in each series of images represent 20 µm.
Figure 2.3 Graph summarizing PI uptake of HeLa cells expressing L221I or V85I in DCF conditions. The PI uptake of cells expressing L221I in both FFA (7.7 ± 2.6%, N = 6) and MFQ (21.4 ± 4.4%, N = 6) in DCF showed a significant reduction in PI uptake compared to L221I without blockers (75.1 ± 2.9%, N = 12). The PI uptake of cells expressing V85I in both FFA (1.6 ± 0.8%, N = 6) and MFQ (7.4 ± 2.5%, N = 6) in DCF showed a significant reduction in PI uptake compared to V85I without blockers (75.1 ± 2.9%, N = 12). Negative control not shown. The number of transfections (N) are denoted by the numbers on the bars. Statistical significance is denoted by asterisks: ***, p < 0.001.
2.3.2 Co-expression of connexin40 mutant, L221I, and connexin40 showed an increased propidium iodide uptake

Both AF-linked Cx40 mutants, L221I and V85I, are autosomal dominant with one gene allele containing the mutation and the other with wildtype Cx40. To study the effects of the co-expression of mutant and wildtype Cx40 we used a bidirectional vector capable of expressing the mutant, either L221I or V85I, and the wildtype Cx40 with untagged GFP as a reporter (via IRES-GFP plasmid).

Transiently transfected HeLa cells were bathed in DCF-ECS to promote PI uptake. Both Cx40 mutants, L221I (61.1 ± 6.5%, N = 6) and V85I (21.5 ± 5.0%, N = 6) showed PI uptake, with the positive control, Cx26-GFP (95.9 ± 1.7%, N = 9) working as expected. The hemichannel function of Cx40 mutants co-expressed with Cx40 was assessed by using PI uptake (Figure 2.4). When these mutants were co-expressed with Cx40, a significant increase in PI uptake for L221I (9.0 ± 2.1%, N = 7; p < 0.01, Student’s t-test) was seen compared to cells doubly expressing wildtype Cx40 (1.3 ± 0.9%, N = 5), whereas no difference was seen for V85I (3.3 ± 1.4%, N = 8, Student’s t-test). When the PI uptake of cells co-expressing L221I or V85I with wildtype Cx40 are compared to cells expressing mutants alone, significant decreases in PI uptake were observed. PI uptake of cells co-expressing L221 with Cx40 (9.0 ± 2.1%, N = 7) showed a reduced PI uptake compared to cells expressing only L221I (61.1 ± 6.5%, N = 6; p < 0.001, Student’s t-test). The same has also been observed for V85I as the PI uptake of cells co-expressing V85I and Cx40 (3.3 ± 1.4%, N = 8) showed a reduced PI uptake compared to cells expressing V85I alone (21.5 ± 5.0%, N = 6; p < 0.001, Student’s t-test) (Figure 2.5).
Figure 2.4 Representative images of PI uptake of HeLa cells co-expressing Cx40 mutants, L221I or V85I, with wildtype Cx40 in DCF conditions. Column 1 shows cells transfected with different constructs. Names in the color green denote the side of the bidirectional vector expressing GFP via IRES. Column 2 shows cells with PI uptake in red. Column 3 shows the overlay of both first and second columns. Column 4 shows the phase contrast images of HeLa cells. Cx40 mutants, L221 and V85I, co-expressed with wildtype Cx40 showed little PI uptake. Scale bars in each series of images represent 20 µm.
Figure 2.5 Graph summarizing PI uptake of HeLa cells co-expressing Cx40 mutants, L221I or V85I, with wildtype Cx40 in DCF-ECS. Cells co-expressing L221I and Cx40 (9.0 ± 2.1%, N = 7) showed an increase PI uptake compared to cells doubly expressing Cx40 (1.3 ± 0.9%, N = 5). However, no significance increase in PI uptake was observed for cells co-expressing V85I and Cx40 compared to wildtype. A reduced PI uptake was observed in cells co-expressing L221I with Cx40 (9.0 ± 2.1%, N = 7) compared with cells expressing only L221I (61.1 ± 6.5%, N = 6; p < 0.001, Student’s t-test). The PI uptake of cells co-expressing V85I and Cx40 (3.3 ± 1.4%, N = 8) showed a reduced PI uptake compared to cells expressing V85I alone (21.5 ± 5.0%, N = 6; p < 0.001, Student’s t-test). The number of transfections (N) is denoted by the numbers on the bars. Statistical significance is denoted by asterisks: **, p < 0.01.
2.3.3 Connexin40 mutants co-expressed with connexin43 showed similar propidium iodide uptake to co-expressed wildtype connexin40 and connexin43

As the atria expresses both Cx40 and Cx43 it grants the possibility of Cx40 and Cx43 interacting with one another. Thus, it is possible for these AF-linked Cx40 mutants to interact with Cx43 to form heteromeric hemichannels.

For our experiment, hemichannel function of AF-linked Cx40 mutants co-expressed with Cx43 was assessed by using PI uptake in DCF conditions (Figure 2.6A). The positive and negative controls, Cx26 and GFP, respectively, were the same as the previous experiment involving the co-expression of AF-linked Cx40 mutants with wildtype Cx40, since it was performed at the same time. PI uptake for both L221I co-expressed with Cx43 (18.7 ± 2.4%, N = 4) and V85I co-expressed with Cx43 (18.0 ± 5.1%, N = 4) showed no significant difference compared with the wildtype: Cx40 co-expressed with Cx43 (20.3 ± 2.6%, N = 4; p > 0.05, Student’s t-test) (Figure 2.6B).
A

Cx43 + Cx40
Cx43 + L221I
Cx43 + V85I

PI Overlay Phase

B

% cells with PI uptake

Cx26 GFP Cx43 GFP Cx43 GFP Cx43
9 4 4 6 4 0 20 40 60 80 100
L221I V85I + + +

Cx40

% cells with PI uptake

Cx26 GFP Cx43 GFP Cx43 GFP Cx43
9 4 4 6 4 0 20 40 60 80 100
L221I V85I + + +

Cx40
Figure 2.6 PI uptake of HeLa cells co-expressing Cx40 mutants, L221I or V85I, with wildtype Cx43 in DCF conditions. (A) Representative PI uptake images of cells co-expressing Cx40 mutants, L221I or V85I, with Cx43. Little PI uptake was observed. Connexin names in the colour green denote the side of the bidirectional vector expressing GFP via IRES. Column 1 shows GFP fluorescence. Column 2 shows cells with PI uptake in red. Column 3 is an overlay which is the combination of green and red fluorescence. Column 4 displays a phase contrast image of HeLa cells. Scale bars in each series of images are represented as 20 µm. (B) Graph summarizing PI uptake of cells co-expressing Cx40 mutants, L221I or V85I, with Cx43. No significant difference was observed comparing cells co-expressing Cx40 mutants with Cx43 with the wildtype. The number of transfections (N) is denoted by the numbers on the bars.
2.4 Discussion

To verify if PI uptake was mediated through connexin hemichannels, two connexin hemichannel blockers, FFA and MFQ, were used. Both blockers, FFA and MFQ, were shown to be effective in reducing PI uptake of cells expressing either AF-linked Cx40 mutants, L221I or V85I. These data support the idea that PI uptake is mediated through connexin hemichannels. This portion of the study has already been published (Sun et al., 2014).

To assess the hemichannel function of co-expressed AF-linked Cx40 mutants, L221I or V85I, with Cx40 or Cx43, a bidirectional vector with a predicted 1:1 expression ratio was used to express both mutant and wildtype at the same time. This was done in an attempt to mimic native atrial tissue cells in the patients, i.e. the cardiomyocytes of the atria express both Cx40 and Cx43 (Severs et al., 2001). Both of these mutants linked to early onset of AF in the families in an autosomal dominant way and AF co-segregated with both mutant alleles with 100% penetrance, which indicated that one Cx40 mutant allele was sufficient to lead to an early onset of atrial fibrillation as displayed in the pedigree (Figure 1.3) (Yang et al., 2010).

Each mutant was expressed with Cx40 or Cx43 in HeLa cells in the DCF condition, and assessed with PI for dye uptake. HeLa cells were incubated in PI for 15 minutes to allow for saturation because it is thought that hemichannels have two gates; slow loop-gating or fast Vj-gating, and both must be opened in order for PI to pass through (Bukauskas and Verselis, 2004). By assessing the hemichannel function of different heteromeric hemichannels formed by either L221I or V85I with Cx40 or Cx43 with the dye uptake
assay, L221I/Cx40 hemichannels showed an increased permeability to PI which would be an apparent gain-of-function compared to Cx40/Cx40 hemichannels. This partially supports the hypothesis that the co-expression of AF-linked Cx40 mutants with Cx40 or Cx43 will show an increase in hemichannel function.

When the Cx40 mutants, L221I and V85I, were co-expressed with Cx40 or Cx43, reductions in PI uptake compared to L221I or V85I alone were observed. When L221I was co-expressed with Cx40 or Cx43, a significant reduction in PI uptake was observed compared to L221I alone. In addition, when V85I was co-expressed with Cx40, a significant reduction in PI uptake was observed compared to V85I alone. However, there was no difference in PI uptake between V85I alone and when V85I was co-expressed with Cx43. It was previously suggested that Cx40 and Cx43 were able to form heteromeric hemichannels due to co-immunoprecipitation and GJ channel function data (He et al., 1999). The PI uptake data obtained would also suggest that Cx40 and Cx43 may interact with each other, instead of independently, to affect hemichannel function.

Our experiments only used the dye uptake assay in the DCF condition to assess hemichannel function. This condition was successful in assessing hemichannel function when the mutants were expressed alone. However in this case, it may be possible that L221I or V85I co-expressed with Cx40 or Cx43 are more sensitive to other stimuli such as changes in membrane potential, mechanical stimulation, or intracellular Ca^{2+}, which have also been shown to increase hemichannel opening probability (Bennett et al., 2003; De Vuyst et al., 2006; Goodenough and Paul, 2003). It is also possible that multiple conditions need to be employed in order to obtain a robust response.
Even though the hypothesis that the co-expression of Cx40 mutants with wildtype Cx40 or Cx43 would show an increased function compared to wildtype was not fully supported, since only one Cx40 mutant allele was required to cause disease, L221I/Cx40 heteromeric hemichannels still showed an apparent gain-of-function. In previous studies, a gain in hemichannel function has been shown to play a role in inherited diseases with connexin mutations. Cx30 mutants, G11R and A88V, linked to Clouston syndrome, which is a form of ectodermal dysplasia, maintained GJ function with increased hemichannel function in vitro. Under physiological conditions, ATP-release via hemichannels was observed compared to wildtype (Essenfelder et al., 2004). Other mutants in Cx32, linked to X-linked Charcot-Marie-Tooth (CMTX) disease, were also shown to have alterations in hemichannel activity. S85C, a Cx32 mutant, showed hemichannel-mediated large voltage dependent currents unlike the wildtype, and F235C, have leaky hemichannels that is linked to a neuropathy (Abrams et al., 2002; Liang et al., 2005). Cx43 mutations involved in oculodentodigital dysplasia (ODDD), such as G138R, were also found to show increases in hemichannel activities (Dobrowolski et al., 2007). It is evident that mutations that lead to a gain of hemichannel function provide a pathology for disease.

A gain in hemichannel function can provide a number of ways to make the heart prone to arrhythmia. 1) If the hemichannels prematurely opened, Na\(^+\) and K\(^+\) would flow according to their concentration gradients. This would result in membrane depolarization away from the SA node to trigger an ectopic action potential, which may lead to uncoordinated contraction, and subsequently, that area of the atria will enter into a refractory period which would make it insensitive to any incoming action potentials from the SA node (Rudy, 2008). 2) ATP would also be released into the extracellular space. ATP would potentially
bind to purinergic receptors, which subsequently trigger intracellular Ca\(^{2+}\) wave propagations. Prolonged high amounts of Ca\(^{2+}\) may trigger premature apoptosis, which could be a potential factor in the onset of AF (Dale, 2008; Ermak and Davies, 2002). 3) As ions and small molecules are potentially released due to hemichannel opening, critical metabolites such as NAD\(^+\) or ATP (Kar et al., 2012) may also be released which could prove detrimental to the cardiomyocytes. Although the direct mechanism may be unclear, this still suggests that the gain-of-function seen for L221I/Cx40 hemichannels is a prime suspect in the pathogenesis of lone AF.
2.5 References


Clontech Laboratories, Inc. 2009. pBI-CMV1 Vector Information. PR963272. Mountain View, CA, USA. [Information Sheet]


Chapter 3: Functional study of a novel autosomal dominant atrial fibrillation-linked \textit{GJC1}/connexin45 mutant

3.1 Introduction

Gap junction (GJ) channels are responsible for the direct communication between apposed cells. Various ions and small metabolites less than 1 kDa in size are able to pass through these intercellular channels. GJ channels are formed by the head-to-head docking of hemichannels (HC), which are contributed by apposed cells. HCs are hexamers of tetraspanning integral membrane proteins known as connexins (Evans and Martin, 2002; Goodenough and Paul, 2009). There are 21 genes in the human genome and 20 genes in mouse genome that encode these connexins (Sohl and Willecke, 2004).

In the heart, connexin45 (Cx45) is abundantly expressed in the sinoatrial (SA) and atrioventricular (AV) nodes, without other connexins. Cx45 is also expressed in the bundle of His, upper and lower bundle branches, and Purkinje fibres together with Cx40 and Cx43 (Severs et al., 2008). Action potential propagation from the SA node must travel through the atria to get to the AV node and from there it will travel through the ventricular conduction system to the ventricles. It is very likely that Cx45 hemichannels will dock with either Cx40 or Cx43 to form heterotypic GJ channels in order to propagate the action potentials from the SA node to the atria.

The importance of Cx45 has been shown in a number of mouse studies. Cx45 knockout mice are not viable since they die as early as E9.5 due to impairments in the formation of blood vessels and smooth muscle (Kruger et al., 2000). Cx45 has also been shown to be highly expressed throughout the heart during development, but during the maturation,
Cx45 expression is gradually reduced and localized into the SA and AV nodes, and the ventricular conduction system; low levels of Cx45 are detectable in both adult atria and ventricles (Alcolea et al., 1999). Cardiomyocyte-restricted Cx45-knockout mice were shown to be viable but showed a significant increase in AV conduction delay as implicated by its ECG and intracardiac measurements (Frank et al., 2012).

Atrial fibrillation is a common cardiac arrhythmia that affects approximately 1% of the Canadian and American population (Fuster et al., 2006). By 2050 there will almost be a tripling in the number of cases of AF, which will increase the burden on the healthcare system (Go et al., 2001). Patients suffering from AF are typically twice as likely to die or six times as likely to experience stroke (Wolf et al., 1991). Based on the ECG parameters of patients with AF, they typically show an erratic pattern instead of a strong P-wave, which suggests uncoordinated activation and ineffective atrial contraction (Wakili et al., 2011). In approximately 70% of the cases, AF is secondary to other systemic disorders or diseases such as diabetes, hypertension, myocardial infarction, or coronary artery diseases (Benjamin et al., 1998). However, in the remaining 30% of cases, AF is a primary disease, which is called idiopathic or lone AF.

A novel Cx45 mutant, M235L, was found in a lone AF patient, which was autosomal dominantly inherited in the patient’s family. This is the first case of a Cx45 mutation being linked to AF. We hypothesize that M235L will show impaired GJ function and impaired coupling compared to wildtype Cx45.

My data indicated that Cx45 M235L GJs showed significantly reduced GJ coupling when expressed alone or heterotypically with wildtype Cx43 (or Cx45) suggesting that the
impairment of GJ function by M235L may be a main factor in predisposing the mutant carriers to AF.

3.2 Methods

3.2.1 Plasmid construction

The human Cx45-IRES-GFP construct was generated by subcloning human Cx45 (in pBluescriptII SK(+) vector [generously provided by Dr. Eric Beyer]) into pIRES2-EGFP between SacI and EcoRI sites. The Cx45 sequence was checked and corrected where necessary. Cx43-IRES-DsRed was generated by subcloning Cx43 from Cx43-IRES-GFP vector into the pIRES-DsRed vector between EcoRI and BamHI sites. Cx45-IRES-DsRed was generated by subcloning Cx45 from Cx45-IRES-GFP vector into pIRES2-DsRed2 vector between SacI and EcoRI sites. The Cx45-IRES-GFP vector was used as a template to generate the untagged individual point mutant, M235L-IRES-GFP, with the QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA) with the following primers:

Forward - 5’-CTTCCTTCTGTATATTGTATGTTACAGGC-3’

Reverse - 5’GCCTGTAACACCATAATATCAGAAGGAAG-3’

All connexin clones were sequenced to confirm the accuracy of the nucleotide sequence with no additional variations being introduced.
3.2.2 Cell culture and transfection

HeLa and N2A (human cervical carcinoma and mouse neuroblastoma cells, respectively, American Type Culture Collection, Manassas, VA) cells were used. Related methodology is described in Chapter 2. In addition, approximately 24 hours after transfection N2A cells were replated on glass coverslips for 2-3 hours prior to electrophysiological recording, and 24 hours after transfection HeLa cells used in the dye uptake assay.

3.2.3 Electrophysiological recording

The $V_j$-gating property of cell pairs expressing either Cx45 or its mutant, M235L, was measured by dual whole-cell voltage-clamp technique as described earlier (Bai et al., 2006; Tong et al., 2014; Xin et al., 2010). The glass coverslips were transferred to a recording chamber on an inverted microscope (Leica DM IRB, Wetzlar, Germany) filled with extracellular fluid (ECF) at room temperature (22 – 25°C). The composition of ECF is (in mM): 135 NaCl, 2 CsCl, 2 CaCl$_2$, 1 MgCl$_2$, 1 BaCl$_2$, 10 HEPES, 5 KCl, 5 D-(+)-glucose, 2 Sodium pyruvate, pH 7.2, ~310 mOsm. GFP-positive and DsRed-positive paired cells were patched by two glass micropipettes (pipette resistance 2 - 4 MΩ) which were filled with intracellular fluid (ICF) containing (in mM): 130 CsCl, 10 EGTA, 0.5 CaCl$_2$, 3 MgATP, 2 Na$_2$ATP, 10 HEPES, pH 7.2, ~290 mOsm. Isolated cell pairs were selected to test $V_j$-gating. One cell of the pair was clamped at 0 mV while the apposed cell was administrated with a series of voltage pulses given by a protocol e.g. Voltage pulses of ±20 mV to ±100 mV in 20 mV increments for 7 seconds per pulse. The junctional currents ($I_j$s) were amplified by two Axopatch 200B amplifiers (Molecular Devices, Sunnyvale, CA).
with a low-pass filter (cut-off frequency 1 kHz) and digitalized at a 10 kHz sampling rate via an ADDA converter (Digidata 1322A, Molecular Devices, Sunnyvale, CA).

3.2.4 Dye uptake assay

Methodology was previously described in Chapter 2. For each experiment approximately 40–50 cells were counted to obtain a percentage of PI-uptake. The bar graphs were generated with 3–9 transfections.

3.2.5 Statistical analysis

Methodology was previously described in Chapter 2. In addition, Fischer’s exact test was used to compare the coupling probability of two groups of data, as specified.
3.3 Results

3.3.1 Homotypic M235L showed impaired gap junction coupling and function

The GJ function of the Cx45 mutant, M235L, was examined through the use of the dual whole-cell patch clamp technique. Cell pairs expressing M235L were found to have no transjunctional current (I_j) in response to a V_j-pulse (Fig. 3.1A), which were different from cell pairs expressing wildtype Cx45. The calculated transjunctional conductance (G_j) of this mutant was zero (n = 17, N = 6, p < 0.001, one-way ANOVA), which was significantly different from that of wildtype Cx45 (G_j = 9.8 ± 3.6 nS, n = 14, N = 8). In addition, the coupling probability seen for M235L (0/17 cell pairs showed coupling) was significantly less compared to wildtype Cx45 (10/14 cell pairs showed coupling, p < 0.001, Fischer’s exact test) (Fig. 3.1). Homotypic Cx45 GJs were very sensitive to changes in V_j as it showed strong gating even at ± 20 mV V_jS (Fig. 3.2).
Figure 3.1 Gap junctional conductance of homotypic M235L and Cx45 gap junction channels. (A) Dual whole-cell patch clamp technique was used to measure transjunctional current ($I_j$) for homotypic cell pairs of Cx45 and M235L. (B) Only 10 out of 14 cell pairs showed coupling for Cx45 cell pairs. A mean $G_j$ of 9.8 ± 3.6 nS (n = 14, N = 8) was observed. No cell pairs showed coupling for the homotypic pair of Cx45 mutant, M235L. M235L also showed significantly reduced $G_j$ (0 nS, n = 17, N = 6, p < 0.001, one-way ANOVA). The number of transfections (N) are shown above or in the bars. Asterisks represent statistical significance: ***, p < 0.001
Figure 3.2 Representative macroscopic gating of homotypic Cx45 gap junction channels. Macroscopic transjunctional currents ($I_j$) from cell pairs of homotypic Cx45 were shown in response to the $V_j$-protocol. Symmetric gating can be observed.
3.3.2 M235L showed impaired heterotypic coupling and function with connexin45

Since M235L is predicted to be co-expressed with wildtype Cx45, this mutant may dock with wildtype Cx45. To study the functional status of M235L/Cx45 GJ channels, the dual whole-cell patch clamp method was used in cell pairs expressing M235L-IRES-GFP in one cell and Cx45-IRES-DsRed in the other cell. M235L/Cx45 cell pairs were found to have no transjunctional current (I_j) in response to a V_j-pulse (Fig. 3.3A), while substantial I_j's were recorded in cell pairs expressing wildtype Cx45-IRES-GFP in one cell and Cx45-IRES-DsRed in the other cell. The calculated transjunctional conductance (G_j) of this M235L/Cx45 cell pair was zero (n = 15, N = 4), which was significantly lower than that of wildtype Cx45 (G_j = 2.4 ± 1.1 nS, n = 15, N = 4, p < 0.01, one-way ANOVA). In addition, the coupling probability seen for M235L/Cx45 (0/15 cell pairs showed coupling) was significantly less compared to wildtype Cx45/Cx45 (8/15 cell pairs showed coupling, p < 0.01, Fischer’s exact test) (Fig. 3.3).
Figure 3.3 Gap junctional conductance of heterotypic M235L/Cx45 gap junction channels. (A) Dual whole-cell patch clamp technique was used to measure transjunctional current ($I_j$) for homotypic and heterotypic cell pairs of Cx45 and M235L/Cx45, respectively. Only 8 out of 15 cell pairs showed coupling for Cx45 cell pairs. A mean $G_j$ of 2.4 ± 1.1 nS ($n = 15, N = 4$) was observed. No cell pairs showed coupling for heterotypic cell pairs of M235L/Cx45. M235L/Cx45 also showed significantly reduced $G_j$ (0 nS, $n = 15, N = 4$, $p < 0.01$, one-way ANOVA). The number of transfections ($N$) are shown above or in the bars. Asterisks represent statistical significance: **, $p < 0.01$. 

![Diagram](image-url)
3.3.3 M235L showed impaired heterotypic gap junction coupling and function with connexin43

In addition to interacting with itself or Cx45, the mutant may form heterotypic GJ channels with Cx43 in the atria. To test this heterotypic GJ function, I measured I$_{js}$ in cell pairs with one cell expressing M235L-IRES-GFP and the other cell expressing Cx43-IRES-DsRed. M235L/Cx43 cell pairs were found to have very little I$_{j}$ in response to a V$_{j}$-pulse (Fig. 3.4A), while much higher I$_{js}$ were recorded in cell pairs expressing wildtype Cx45-IRES-GFP in one cell and Cx43-IRES-DsRed in the other cell. The G$_{j}$ of the M235L/Cx43 cell pair was significantly lower ($0.03 \pm 0.03$ nS, n = 14, N = 6) than Cx45/Cx43 cell pairs ($13.5 \pm 5.2$ nS, n = 13, N = 8, p < 0.01, Student’s t-test). In addition, the coupling probability seen for M235L/Cx43 (2/14 cell pairs showed coupling) was significantly less in comparison to wildtype Cx45/Cx43 (9/13 cell pairs showed coupling, p < 0.01, Fischer’s exact test) (Fig. 3.4). Both Cx45/Cx43 and M235L/Cx43 showed strong asymmetric V$_{j}$-gating, however, M235L/Cx43 has smaller I$_{js}$ compared to Cx45/Cx43 (Fig. 3.5).

3.3.4 M235L expressing cells showed no alteration in propidium iodide uptake

The dye uptake assay was done with PI as the dye of interest in DCF conditions in HeLa cells to assess any potential hemichannel function of this Cx45 mutant. Very little PI uptake was observed for both the wildtype Cx45 and the mutant, M235L (Fig. 3.6A). There was no difference in PI uptake of M235L ($3.1 \pm 1.0\%$, N = 6) compared to Cx45 ($5.7 \pm 2.4\%$, N = 6, p > 0.05, Student’s t-test) (Fig. 3.6B), indicating no apparent difference in this Cx45 mutant, M235L, in PI uptake.
Figure 3.4 Gap junctional conductance of heterotypic M235L/Cx43 gap junction channels. (A) Dual whole-cell patch clamp technique was used to measure transjunctional current ($I_j$) for heterotypic cell pairs of Cx45/Cx43 and M235L/Cx43. (B) Only 9 out of 13 cell pairs showed coupling for Cx45/Cx43 cell pairs. A mean $G_j$ of $13.5 \pm 5.2$ nS ($n = 13$, $N = 8$) was observed. 2 out of 14 cell pairs showed coupling for the heterotypic cell pairs of M235L/Cx43. M235L/Cx43 also showed significantly reduced $G_j$ ($0.03 \pm 0.03$ nS, $n = 14$, $N = 6$, $p < 0.01$, Student’s t-test). The number of transfections (N) are shown above or in the bars. Asterisks represent statistical significance: **, $p < 0.01$
Figure 3.5 Representative macroscopic gating traces of heterotypic Cx45/Cx43 and M235L/Cx43 gap junction channels. Macroscopic transjunctional currents ($I_j$) from cell pairs of heterotypic Cx45/Cx43 and M235L/Cx43 were shown in response to the $V_j$ protocol. Smaller macroscopic gating can be observed in M235L/Cx43 relative to Cx45/Cx43. Asymmetric gating can be observed for both Cx45/Cx43 and M235L/Cx43.
A

GFP

Cx26

Cx45

M235L

PI

Overlay

Phase

B

% cells with PI uptake

0 50 100

ns

A

B

ns

9

3

6

6

Cx26  GFP  Cx45  M235L

% cells with PI uptake

0 50 100

ns

9

3

6

6

Cx26  GFP  Cx45  M235L
Figure 3.6 PI uptake of HeLa cells expressing Cx45 mutant, M235L, in DCF conditions. (A) Representative PI uptake images of cells expressing Cx45 mutant, M235L. Little PI uptake was observed. Column 1 shows GFP fluorescence. Column 2 shows cells with PI uptake in red. Column 3 is an overlay, which is the combination of green and red fluorescence. Column 4 displays a phase contrast image of HeLa cells. Scale bars in each series of images are represented as 20 µm. (B) Graph summarizing PI uptake of cells expressing Cx45 mutant, M235L, wildtype Cx45, the positive control, Cx26, and the negative control, GFP. No significance (ns) was observed comparing cells expressing M235L with the wildtype. The number of transfections (N) is denoted by the numbers on the bars.
3.4 Discussion

This is the first study of a Cx45 mutation linked to AF. Both atria-enriched connexins, Cx40 and Cx43, have been shown to have mutations linked to early onset of AF. (Bai, 2014; Molica et al., 2014; Severs et al., 2008; Thibodeau et al., 2010). It would be interesting to characterize this Cx45 mutant in order to elucidate any potential mechanisms that may predispose the mutant carriers to AF.

The dual whole-cell patch clamp technique was used to study the GJ channel function of the Cx45 mutant, M235L. Cell pairs with homotypic M235L GJs revealed that there was no GJ channel function compared to wildtype Cx45. Since M235L is an autosomal dominant mutation, one gene allele is enough to link disease; the other gene allele would still be wildtype Cx45. It is possible that in the nodal cells of the atria, that M235L can heterotypically dock with Cx45. In studying this, heterotypic M235L/Cx45 GJ channels showed no Gj which would suggest that no functional heterotypic M235L/Cx45 GJ channels were formed. In addition to docking with other Cx45-expressing nodal cells, it may be possible for the nodal cells to dock with the cardiomyocytes of the atria since the action potentials from the SA node must propagate through the atria to trigger contraction. Since the atria co-expresses Cx40 and Cx43 (Severs et al., 2008), it is possible for M235L to dock with either Cx40 and/or Cx43. The heterotypic GJ channel function of M235L and Cx43 was studied, which revealed virtually no conductance similar to the negative controls, and significantly lower than the conductance of Cx45/Cx43 GJ channels. Since Cx45/Cx40 showed no GJ channel function (see Chapter 4) we did not pursue a full functional study of M235L/Cx40 GJs. Future studies may look into the heteromeric interactions between
Cx45 M235L with wildtype Cx45 and the heterotypic GJ channels of M235L with cells co-expressing both Cx40 and Cx43, as all these GJs are predicted to exist in the heart.

The dye uptake assay was used to study the potential hemichannel function of M235L. AF-linked Cx40 mutants, L221I and V85I, which were previously studied, showed an increase in PI uptake. This may be due to a gain in hemichannel function, which could play a role in the pathogenesis of AF (Sun et al., 2014). M235L, however, showed very little PI uptake and was not statistically different from that of Cx45. However, we could not rule out the possibility of M235L hemichannels having an altered function when exposed to other specific stimuli, such as changes in transmembrane potential, mechanical stimulation, or increased intracellular calcium (Bennett et al., 2003; De Vuyst et al., 2006; Goodenough and Paul, 2003).

It is unclear as to how impaired Cx45 GJ channel function can lead to AF since this is the first Cx45 mutant linked to AF. Previous studies regarding AF-linked connexin mutants generally show an impaired GJ channel function, such as the Cx40 mutants studied by Gollob et al., in 2006. One of the mutants, P88S, was a somatic mutation found in the atria of two patients. It showed an impaired GJ channel function compared to wildtype Cx40 (Gollob et al., 2006). Another AF-linked Cx40 mutant, I75F, which is a germline autosomal dominant mutation, has also showed impaired GJ channel function (Sun et al., 2013). It has been implicated that impaired GJ channel function in the atria introduces increased heterogeneity and reduced conduction velocity, which could be substrates for AF (Bai, 2014; Wakili et al., 2011). A similar instance of this may involve the nodal regions of the heart, which may contribute to AF.
Work with Cx45 knockout mice have been performed in previous studies. In general, Cx45 knockouts are not viable between E9.5 and E10.5 due to impaired blood vessel formation and smooth muscle development (Kruger et al., 2000). As such, cardiomyocyte-restricted Cx45 knockout mice were used to study the role of Cx45 in the heart. Mice lacking Cx45 in the heart showed no change in resting heart rate and no spontaneous arrhythmia, however, mice also express Cx30.2 and Cx40 at their nodes, which may compensate for the loss of Cx45 to some degree. Humans, however, do not express the Cx30.2 equivalent, Cx31.9, or Cx40 at their nodes, which may differ from what was observed in mice (Kreuzberg et al., 2009). Mice heterozygous for Cx45 showed strong Cx45 expression in cardiac tissues and they were viable enough to breed, but in Cx40 knockout mice, however, heterozygous expression of Cx45 exacerbated the issues seen in Cx40 knockouts (Frank et al., 2012; Kruger et al., 2006). This suggests that there may be some interplay between Cx40 and Cx45 regarding proper action potential propagation. Cx45 has been demonstrated to be critical in development and may also play a role in the maintaining proper cardiac conduction. As patients with M235L have been shown to develop AF it will be interesting to see directly how the impairment of GJ channel function of Cx45 can lead to AF in humans.

During development, Cx45 is highly expressed throughout the heart. In the adult heart, however, Cx45 expression is reduced and localized in specific regions such as the SA and AV nodes, and the ventricular conduction system. During development, it may be possible for Cx45 and Cx40 (or Cx43) to interact in the atria (Alcolea et al., 1999). As the Cx45 mutant, M235L, showed no transjunctional coupling in homotypic cell pairs or heterotypic cell pairs with Cx43 (and possibly Cx40) it may be possible for M235L to exert a dominant-
negative effect on Cx43 (and Cx40). Other AF-linked mutants such as I75F, showed no transjunctional coupling conductance and exhibited dominant-negative effects on Cx43 (and Cx40) (Sun et al., 2013). Future studies may serve to identify whether or not M235L exhibits dominant-negative effects on Cx43 (and Cx40) and how that may play a role in the development of AF.

To conclude, M235L, a germline autosomal dominant Cx45 mutation, was recently discovered and is the first Cx45 mutation linked to AF. M235L did not show GJ channel function when expressed in cell pairs. No functional couplings were observed when M235L expressing cells paired with wildtype Cx45 or Cx43 expressing cells. These impairments in Cx45 mutant-containing GJs may alter the functional coupling in/near the SA node and possibly in the atria to lead to AF. In addition, it is clear that Cx45 plays a major role in cardiac development and cardiac conduction.
3.5 References


Chapter 4: Engineered connexin40 variant improves docking and function to connexin43 and connexin45

4.1 Introduction

The connexin (Cx) is a tetraspanning integral membrane protein. 12 connexin subunits comprise a single gap junction (GJ) channel and spans both membranes of apposed cells (Goodenough and Paul, 2009). There are 21 genes in the human genome and 20 genes in the mouse genome encoding connexins (Sohl and Willecke, 2004). Connexins oligomerize into a hexamer to form hemichannels (HCs). HCs from apposing cells dock head-to-head by non-covalent interactions, including hydrogen bonds, in order to form full GJ channels. Since many tissues express more than one connexin isoform, it is possible for different connexin isoforms to oligomerize together to form heteromeric HCs; this is in addition to forming homomeric HCs which are composed of the same connexin isoform. It is also possible to have two different homomeric HCs from apposing cells to dock with one another to form homomeric heterotypic GJ channels (Goodenough and Paul, 2009).

In the heart, specifically in the atria, Cx40 and Cx43 are co-expressed, while Cx45 is enriched in the SA and AV nodes. This presents the possibility to form heterotypic GJ channels such as Cx40/Cx43 in the atria, or Cx40/Cx45 and Cx45/Cx43 at the border between atrial and nodal cells (Severs et al., 2008). Previous studies regarding the heterotypic compatibility between Cx40 and Cx43 revealed contradictory conclusions; some studies suggest that Cx40/Cx43 heterotypic GJ channels are functional whereas other studies suggest otherwise (Bruzzone et al., 1993; Lin et al., 2014; Rackauskas et al., 2007; Valiunas et al., 2000). Studies involving the heterotypic compatibility between murine
Cx40 and Cx45 revealed that these connexins were co-localized, and showed functional channels with asymmetric gating, which suggest Cx40 and Cx45 are compatible to form heterotypic GJ channels (Coppen et al., 1998; Elfgang et al., 1995; Rackauskas et al., 2007), however, no study was performed on the heterotypic compatibility between human Cx40 and Cx45.

In 2009, the first high resolution crystal structure of a human Cx26 GJ channel was resolved. Numerous hydrogen bonds (HBs) were revealed at the extracellular docking interface which suggest that HBs play an important role in the docking of HCs to form functional GJ channels (Maeda et al., 2009). The role of HBs in HC docking was also examined by studying the heterotypic docking between Cx26 and Cx32. Disease-linked mutations of Cx32 on the extracellular domain reduced the number of available HBs, which impaired GJ function and plaque formation. This, however, was rescued by introducing structurally complementary mutations in Cx26 which increased the number the HBs. The increase in HBs by introducing these mutations in Cx26 was able to restore GJ function (Gong et al., 2013). It is unknown if the heterotypic docking between human Cx40 and Cx43 or Cx40 and Cx45 are able to form functional heterotypic GJ channels.

Sequence alignment of Cx40, Cx43, Cx45, and Cx26 was performed. Many amino acid residues appeared to be well-conserved in these connexins, in particular at the extracellular domains, which suggests that the Cx26 crystal structure could be used as a template to develop homology models in these cardiac connexins. By using the homology models, the HB sites suspected to be involved in docking were revealed in the extracellular domains, one of which was the amino acid residue at the position 54 on Cx26. On Cx40, the corresponding position is 55. Aspartic acid (D) is present in Cx40, which differs from
asparagine (N) which is found in Cx43 and Cx45 (Bai and Wang, 2014). It is possible that this is a critical residue in the docking between Cx40 and Cx43 as well as Cx40 and Cx45. We hypothesize that Cx40, Cx43, and Cx45 GJ channels possess similar structures at the docking interface as Cx26 and use similar residues for docking. The mutation in the Cx40 E1 domain, D55N, which makes Cx40 similar in the residues found in Cx43 and Cx45, will increase the heterotypic docking of Cx40/Cx43 and Cx40/Cx45.

For this study we generated a Cx40 variant, D55N, based on the model of docking compatibility by using the structure of Cx26 as a template. D55N was used to form heterotypic GJ channels with Cx43 and Cx45, and was able to increase GJ conductance when paired with Cx43 or Cx45 compared to heterotypic GJ channels formed by wildtype Cx40/Cx43 or Cx40/Cx45. This study suggests that the structural model of Cx26 was an excellent template to develop homology models for cardiac GJ channels and was able to help up design a proper variant to increase docking compatibility between different connexins.
4.2 Methods

4.2.1 Plasmid construction

The human Cx40-IRES-GFP construct was created as previously described in Chapter 2. Cx43-IRES-DsRed and Cx45-IRES-DsRed were created as previously described in Chapter 3. The Cx40-IRES-GFP vector was used as a template to generate the untagged individual point mutant, D55N-IRES-GFP, with the QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA) with the following primers:

Forward - 5’-GATTTCCCGGTGAATACGATTCAGCC-3’

Reverse - 5’-GGCTGAATCGTATTACACCGAAATC-3’

All connexin clones were sequenced to confirm the accuracy of the nucleotide sequence with no additional variations being introduced.

4.2.2 Cell culture and transfection

Methodology was previously described in Chapter 2. Approximately 24 hours after transfection N2A cells were replated on glass coverslips for 2-3 hours prior to electrophysiological recording.

4.2.3 Electrophysiological recording

Methodology was previously described in Chapter 3.

4.2.4 Statistical analysis

Methodology was previously described in Chapter 2.
4.3 Results

4.3.1 Heterotypic Cx40 D55N/Cx43 showed an increased gap junctional coupling conductance than Cx40/Cx43

Based on the structural model of Cx26, the variant of Cx40, D55N, was designed and engineered in an attempt to improve heterotypic docking to Cx43. Cell pairs with one cell expressing Cx40 and the other cell expressing Cx43 showed very little transjunctional conductance ($G_j$) ($0.2 \pm 0.2$ nS, $n = 20$, $N = 5$). However, cells expressing the Cx40 variant, D55N, were well-coupled to Cx43 expressing cells with a significantly higher $G_j$ ($7.1 \pm 3.6$ nS, $n = 15$, $N = 4$, $p < 0.05$, Student’s t-test) (Fig. 4.1).

Representative transjunctional currents ($I_{js}$) of Cx40/Cx43 and D55N/Cx43 heterotypic gap junctions in response to a series of $V_j$-pulses show a stark contrast in the current amplitudes. At 10x zoom into the $I_{js}$ Cx40/Cx43 heterotypic GJs, apparent unitary channel currents could be observed (Fig. 4.2). Rapid $V_j$-gating was observed when D55N cells were pulsed with $+V_j$ (or $-V_j$ for the Cx43 expressing cell). When the opposite $V_j$ was applied to D55N, a slower $V_j$-gating was displayed (Fig. 4.2).

Because of the significant increase in $G_j$, this would suggest an improvement in heterotypic docking with Cx43. It appears that the use of Cx26 as a structural model was helpful for us in designing a variant to improve Cx40/Cx43 heterotypic docking.
Figure 4.1 Gap junctional conductance of heterotypic D55N and Cx43 gap junction channels. (A) Dual whole-cell patch clamp technique was used to measure transjunctional current ($I_J$) for heterotypic cell pairs of Cx40/Cx43 and D55N/Cx43. (B) Only 6 out of 20 cell pairs showed coupling for Cx40/Cx43 heterotypic cell pairs. Small $G_J$ ($0.2 \pm 0.2 \text{nS}, n = 20, N = 5$) was observed. 9 out of 15 cell pairs showed coupling for the heterotypic pair of Cx40 variant, D55N, with Cx43. D55N/Cx43 also showed significantly higher $G_J$ ($7.1 \pm 3.6 \text{nS}, n = 15, N = 4, p < 0.05$, Student’s t-test). The number of transfections (N) are shown above or in the bars. Asterisks represent statistical significance: *, $p < 0.05$. 

[Diagram of Figure 4.1]
Figure 4.2 Representative macroscopic gating traces of heterotypic Cx40/Cx43 and D55N/Cx43 gap junction channels. Macroscopic transjunctional currents ($I_j$) from cell pairs of heterotypic Cx40/Cx43 and D55N/Cx43 were shown in response to the $V_j$-protocol. Small macroscopic gating can be observed in Cx40/Cx43. A box zoomed in at 10x shows that Cx40/Cx43 traces are mostly single or multi-channel activities. Asymmetric gating can be observed for D55N/Cx43.
4.3.2 Heterotypic Cx40 D55N/Cx45 showed an increased gap junctional coupling conductance than heterotypic Cx40/Cx45

To study heterotypic docking of human Cx40 and Cx45, we mixed cells expressing Cx40 with cells expressing Cx45 and then studied heterotypic cell pairs with the dual whole-cell patch clamp method. No transjunctional currents were observed in response to a $V_j$-pulse (Fig. 4.3A, n = 20, N = 8), indicating that heterotypic Cx40/Cx45 GJs were not functional possibly due to an incompatibility at the docking interface. The introduction of the Cx40 variant, D55N, was used to determine if it may be responsible for the heterotypic incompatibility. When Cx40 D55N expressing cells were paired with Cx43 expressing cells, $I_j$s were significantly increased in response to the same $V_j$-pulse (Fig 4.3). $G_j$ was plotted, and was significant increased (9.9 ± 3.5 nS, n = 17, N = 6, p < 0.01, Student’s t-test) compared to Cx40/Cx45 cell pairs (Fig. 4.3). Asymmetric $V_j$-gating was also observed as shown in the representative macroscopic trace (Fig. 4.4).
Figure 4.3 Gap junctional conductance of heterotypic D55N and Cx45 gap junction channels. (A) Dual whole-cell patch clamp technique was used to measure transjunctional current ($I_j$) for heterotypic cell pairs of Cx40/Cx45 and D55N/Cx45 (B) Only 2 out of 20 cell pairs showed coupling for Cx40/Cx45 heterotypic cell pairs. No $G_j$ was observed overall. 11 out of 17 cell pairs showed coupling for the heterotypic docking of the Cx40 variant, D55N with Cx45. D55N/Cx45 also showed significantly higher $G_j$ (9.9 ± 3.5 nS, $n = 17$, $N = 6$, $p < 0.01$, Student’s t-test). The number of transfections (N) are shown above or in the bars. Asterisks represent statistical significance: **, $p < 0.01$
Figure 4.4 Representative macroscopic gating trace of heterotypic D55N/Cx45 gap junction channels. Macroscopic transjunctional current ($I_j$) from a cell pair of heterotypic D55N/Cx45 was shown in response to the $V_j$-protocol.
4.4 Discussion

The premise for this study was to use the structural model of Cx26 as a template to predict the contribution of equivalent residues in cardiac connexins responsible for heterotypic docking compatibility. Sequence alignment revealed an odd docking-relevant residue in the Cx40 D55 position as the corresponding position in all other connexins is Asn (N). In the heterotypic docking of Cx40/Cx43 and Cx40/Cx45, a Cx40 variant, D55N, was engineered in an attempt to improve docking compatibility. This position was predicted to be involved in forming a docking HB (Bai and Wang, 2014), of which, HBs in general were shown to be critical in the docking of HCs to form functional GJ channels (Gong et al., 2013; Maeda et al., 2009).

This study revealed that a majority of heterotypic Cx40/Cx43 cell pairs showed no coupling, which was similar to a number of previous studies, which suggested that Cx40/Cx43 GJ channels were non-functional (Bruzzone et al., 1993; Elfgang et al., 1995; Rackauskas et al., 2007).

In the present study on human Cx40/Cx45 heterotypic cell pairs, no functional coupling was observed, which was opposite of what was seen for murine Cx40/Cx45 heterotypic cell pairs (Elfgang et al., 1995; Rackauskas et al., 2007). Two possible reasons exist that may explain this observation. Firstly, sequence alignment of human Cx40 and murine Cx40 revealed a few differences in the NT, TM1, TM3, TM4, CL, and CT. These differences may have an indirect effect on the structure at the docking interface. Sequence alignment of human and murine Cx45 revealed a 97% homology, with differences only in CL and CT, which is unlikely to play an important role in the structure of the docking interface.
Secondly, previous studies used HeLa cells to express their connexins as opposed to N2A cells, which were used in this study (Elfgang et al., 1995; Rackauskas et al., 2007). In our lab, HeLa cells were shown to have low background coupling conductance from time to time (Tong and Bai, unpublished observation). It is possible that the background coupling conductance of HeLa cells contributed to the coupling conductance seen in murine heterotypic Cx40/Cx45. At present we could not rule out other possibilities or experimental conductions for this apparent difference.

For both types of heterotypic GJ channels (Cx40/Cx43 and Cx40/Cx45), the introduction of the Cx40 variant, D55N, increased the GJ conductance (Gj). Overall, these data support the idea that the Cx40 D55 is an important residue for the heterotypic docking compatibility in heterotypic Cx40/Cx43 and Cx40/Cx45 docking.

As the Cx40 variant, D55N, improved heterotypic docking to Cx43, detailed Vj-gating characterization was possible and was shown to be asymmetrical. When the D55N expressing cell of the heterotypic D55N/Cx43 cell pair was pulsed with +Vj, rapid Vj-gating was observed as opposed to being pulsed with -Vj. This was consistent with what was reported regarding the predicted gating polarity of wildtype murine Cx40 (Gonzalez et al., 2007). It is possible that the differences in murine and human Cx40 may lead to a change in Vj-gating polarity, however, the Ij's observed for Cx40/Cx43 are too small to identify its Vj-gating polarity. It is very likely the introduction of D55N did not alter the Vj-gating polarity of Cx40, however, future studies may be directed to identifying the Vj-gating polarity of human Cx40 to determine if that is the case.
The cardiac action potential propagating from the SA node (mainly expressing Cx45) to the atria (mainly expressing Cx40 and Cx43) can occur through heterotypic Cx40/Cx45 (or Cx43/Cx45) GJ channels. In the atria, action potentials could go through heterotypic Cx40/Cx43 and other GJ channels, and then reaching the AV node (mainly expressing Cx45) it propagates through Cx40/Cx45 (or Cx43/Cx45) GJ channels. A number of mutations on Cx40 have led to early onset of atrial fibrillation (Gollob et al., 2006; Sun et al., 2013). Typically, AF-linked mutants displayed an impairment of GJ function which may lead to a reduction in conduction velocity to promote reentry and may become a driver for AF (Wakili et al., 2011). Previous studies performed on Cx40 knockout mice suggested a reduction in conduction velocity, which may have explained the presence of arrhythmia that was found in some of the mice (Bagwe et al., 2005; Hagendorff et al., 1999; Kirchhoff et al., 1998), however, increases in conduction velocity in Cx40 knockout mice were also found (Beauchamp et al., 2006). Human studies regarding the relationship between Cx40 and conduction velocity have also shown that conduction velocity was inversely proportional to Cx40/[Cx40 + Cx43] expression (Kanagaratnam et al., 2002). Regardless, a decrease or increase in conduction velocity may both play roles in the promotion of heterogeneity and arrhythmia (Rohr et al., 1997). With this perspective, it is tempting to speculate that the introduction of D55N may provide more heterotypic compatible GJs for the propagation of action potentials due to the increased heterotypic coupling in Cx40/Cx43 and Cx40/Cx45 GJ channels. It is possible that D55N could play a beneficial role in the heart, however, proper models would be needed to study any effects this mutation will have in vivo.
The structural model of Cx26 appeared to be well-suited to identify critical residues responsible for docking incompatibility such as Cx40/Cx43 and Cx40/Cx45, and to predict and improve the docking ability of other incompatible connexins. It is possible that the combination of modeling, mutagenesis, and experimental study of the functional outcomes of mutant connexins can be done to understand docking compatibility in cardiac or other connexins.
4.5 References


Chapter 5: General discussion

5.1 Connexins in atrial fibrillation

The present thesis looked into the functional aspects of three cardiac mutants linked to atrial fibrillation (AF), Cx40 L221I, V85I, and Cx45 M235L. The Cx40 mutants, L221I and V85I, were co-expressed with Cx40 (or Cx43) in an attempt to mimic native atrial tissue expression (Severs et al., 2008), and the cells co-expressing mutant and wildtype had their hemichannel (HC) function assessed by the dye uptake assay. The Cx45 mutant, M235L, was the first Cx45 mutant linked to AF, and its gap junction (GJ) function and HC function were studied. For the Cx40 mutants, only L221I-Cx40 expressing cells showed more uptake of propidium iodide (PI) compared to wildtype, which suggests that gain of HC function may link these mutants to AF. In the case for Cx45 M235L, homotypic GJ (M235L/M235L) and heterotypic GJ (M235L/Cx45 and M235L/Cx43) functions were significantly reduced, but no alteration in PI uptake was observed under our experimental conditions. Impairment of Cx45 GJ function could be linked to AF in the mutant carriers, similar to several other AF-linked Cx40 and Cx43 mutants.

AF is the most common sustained cardiac arrhythmia. AF is usually a secondary disease to systemic diseases or disorders such as hypertension, diabetes, myocardial infarction, or cardiomyopathies (Benjamin et al., 1998). In a smaller number of cases, AF without other cardiovascular diseases is called idiopathic or lone AF (Fuster et al., 2006). AF is characterized by irregular atrial action potential propagation, mainly caused by reentrant circuits in clinical cases. If the conduction velocity of action potentials is decreased, this
could allow reentrant circuits to form. Normally, the conduction velocity is too fast to have reentry in the atria due to the refractory period (Wakili et al., 2011).

As cells co-expressing Cx40 and L221I showed more PI uptake compared to cells co-expressing two wildtype Cx40, this could be attributed to the gain-of-function seen in L221I hemichannels. When dye uptake was performed on cells expressing L221I or V85I alone, significant increases in PI uptake was observed compared to cells only expressing wildtype Cx40 (Sun et al., 2014). In addition, no apparent impairments in GJ function were seen for both mutants, which suggest that the increase in HC may be the principal contributor to AF observed in the mutant carriers (Sun et al., 2014). A number of mechanisms may exist that could make the cells expressing these mutants more vulnerable to stress factors, which could lead to cell death and promoting reentry in the atria of the mutant carriers.

The AF-linked Cx45 mutant, M235L, showed GJ impairment with no obvious alteration in PI-uptake compared to wildtype Cx45. Since this is the first AF-linked Cx45 mutant, no comparisons could be made with other AF-linked Cx45 mutants, however there are Cx40 mutants which are similar. Mutations in Cx40 have been linked to AF (Bai, 2014; Molica et al., 2014), and Cx40 knockout mice have also been shown to have increased vulnerability to arrhythmia (Kirchhoff et al., 1998), or humans with altered Cx40 / [Cx40 + Cx43] ratios in the atria (Kanagaratnam et al., 2002). A number of AF-linked Cx40 mutants, such as I75F or P88S, showed impairment in GJ function, which may be the major contributor to trigger AF (Gollo et al., 2006; Sun et al., 2013). Impairment in GJ function may reduce coupling conductance which may lead to a reduction of conduction velocity, a predicted prerequisite for AF (Wakili et al., 2011). Due to the localized expression in the adult
myocardium, it is possible that this reduction in coupling conductance could be seen near the SA or AV nodes of the right atrium.

A summary of what was observed for the AF-linked connexin mutants, L221I, V85I, and M235L, has been compiled in addition to known AF-linked connexin mutants that were previously studied (Table 5.1).

5.2 Heterotypic docking and cardiac function

This study also examined the heterotypic docking compatibility between cardiac connexins, specifically those of Cx40/Cx43 and Cx40/Cx45 (Severs et al., 2008). Combining the sequence alignment of Cx40, Cx43, Cx45, and Cx26, and the crystal structure of Cx26, we identified a potential critical residue involved in GJ docking (Bai and Wang, 2014; Maeda et al., 2009). Mutating this Cx40 residue, D55N was found to be sufficient to make it capable of docking to Cx43 and Cx45.

Since the atria co-expresses Cx40 and Cx43, with Cx45 enriched in the SA and AV nodes, heterotypic interactions are a possibility for these connexins (Severs et al., 2008). I predict that Cx45 in the SA node may form most functional GJ channels with Cx43 and not with Cx40 due to docking incompatibility. Atrial cardiomyocytes may form homotypic GJs, heteromeric heterotypic GJs and homomeric heterotypic Cx40/Cx43 GJs. The latter, and possibly heteromeric heterotypic GJs, may not couple well, but can be improved by the introduction of Cx40 D55N. Perhaps it is possible to use this variant to increase atrial coupling and reduce heterogeneity as a novel treatment for AF.
Table 5.1 Functional status of atrial fibrillation-linked connexin mutants in Cx40, Cx43, and Cx45

<table>
<thead>
<tr>
<th>Connexin</th>
<th>AF-linked mutants</th>
<th>GJ function?</th>
<th>HC function?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx40</td>
<td>G38D, Q49X, I75F, P88S, A96S, M163V, L229M, L221I, V85I</td>
<td>Impairment</td>
<td>Normal (some are untested)</td>
</tr>
<tr>
<td>Cx43</td>
<td>G60S, G138R</td>
<td>Impairment</td>
<td>Increased</td>
</tr>
<tr>
<td>Cx45</td>
<td>M235L</td>
<td>Impairment</td>
<td>Normal</td>
</tr>
</tbody>
</table>
5.3 Limitations and future studies

A majority of this study relied on the dual whole-cell patch clamp method to determine the GJ function of the many mutants and cell pairs examined. Due to the potential of having leak current this may underestimate the amount of $I_j$ that passes through GJ channels. In order to circumvent this I selected cell pairs with high plasma membrane resistance (>500 MΩ, or leak current smaller than 20 pA with a 10 mV $V_j$-pulse) to improve patch clamp quality (Van Rijen et al., 1998). I also used Dextran Texas Red (3 kDa), which is a GJ impermeable dye, to confirm cell pairs with high Gj were not due to cytoplasmic bridges.

The use of the bidirectional vector was important in the study of interactions of co-expressing wildtype Cx40 (or Cx43) with AF-linked Cx40 mutants, L221I or V85I. It is predicted to be expressed at a 1:1 ratio during biosynthesis. However, we are currently unaware if these mutants could trigger early degradation pathways to reduce their apparent steady-state expression levels. A good future experiment may include a Western blot to confirm the 1:1 expression pattern of this vector at a steady-state expression.

Future studies regarding the Cx45 mutant, M235L, may include assessing any potential dominant-negative effects the mutant may have heteromeric interaction with Cx45, Cx40, and Cx43, which could also contribute to the pathogenesis of AF.
5.4 References


Curriculum Vitae

Name: Willy Garwei Ye

Post-secondary Education and Degrees:
Western University
London, Ontario, Canada
2009-2013 HBMSc

Western University
London, Ontario, Canada
2013-2015 MSc (Expected Fall 2015)

Honors and Awards:
The Western Scholarship of Excellence ($2000)
2009

UWO In-Course Scholarships Year II ($700)
2010

Aiming for the Top Tuition Scholarships ($14000)
2009-2012

Dean’s Honor List
2010-2013

Presentations:
Fourth-year Thesis Poster Presentation
April 2013

Physiology and Pharmacology Research Day Poster Presentation
November 2014

London Health Research Day Poster Presentation
April 2015

Related Work Experience
Teaching Assistant
Western University
2013-2014

Publications:


Jassim A, Aoyama H, Ye WG, Chen H, Bai D. 2015. Engineered connexin40 variants increased docking and function of heterotypic connexin40/connexin43 gap junction channels. (submitted)