Putative Pore-lining Residues and Intracellular Magnesium Influence Connexin50 Unitary Gap Junction Channel Conductance

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Abstract

Gap junction channels (GJs) are oligomers of connexins forming channels linking neighbouring cells. GJs formed by different connexins show distinct unitary channel conductance ($\gamma_j$), transjunctional-voltage dependent gating ($V_j$-gating) properties, and magnesium modulation. We investigate here how pore-lining residues may influence these GJ properties. We mutated putative pore-lining residues in Cx50 GJ (G8E/G46E/V53E individually or in combination) to investigate $\gamma_j$, $V_j$-gating, and magnesium modulation. GJs formed by combination mutants (G8E-G46E, G46E-V53E, and G8E-G46E-V53E) were functional and showed a significantly increased $\gamma_j$ with little change in $V_j$-gating, while G8E-V53E mutants failed to form functional GJs. The $\gamma_j$s of the mutant GJs were reduced up to 30% by inclusion of magnesium in intracellular solution.

Studying the individual mutations revealed that magnesium-dependent reduction of $\gamma_j$ are due to one or combinations of V53E and/or G46E. Increasing negatively charged glutamate residues at 53rd and/or 46th positions of Cx50 increased magnesium-sensitive ion permeation.

**Key words**: Gap junction channel, unitary conductance, Mg$^{2+}$-dependent modulation, patch clamp, electrostatics
Dedication

I would like to dedicate this to my family who, as always, have been a pillar of support. I would also like to thank Daisuke, Kelly, Gloria, Rebecca, Joyce, Madhavi, and all other friends for their constant love and motivation.
Co-Authorship Statement

Electrophysiology work was completed by Swathy Sudhakar. Homology structure models of wildtype and mutant Cx50 channels were generated by Hiroshi Aoyama.
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Abbreviations

ATP  Adenosine triphosphate
CL  Cytoplasmic loop
CT  Carboxyl-terminus
Cx  Connexin
DIC  Differential interference contrast
DMEM  Dulbecco’s modified Eagle’s medium
EGTA  Ethylene glycol-bis(β-aminoethyl ether)-N,N,N’ ,N'-tetraacetic acid
eGFP  Enhanced green fluorescent protein
E1  The first extracellular loop
E2  The second extracellular loop
ECS  Extracellular solution
GJA8  Gap junction alpha 8 gene
GJ  Gap junction channel
Gj,ss  Normalized steady state transjunctonal conductance
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICS  Intracellular solution
Ij  Junctional current
ij  Junction current of a single channel
KCa3.1  Potassium intermediate/small conductance calcium-activated channels

ms  Millisecond

mV  Millivolts

MP26  Membrane protein 26

N2A  Mouse neuroblastoma cells

nS  Nanosiemens

NT  Amino terminus

pIRES  Plasmid containing internal ribosome entry site

pS  Picosiemens

SEM  Standard error of the mean

SCAM  Substituted cysteine accessibility method

TM  Transmembrane domain

Vj  Transjunctional voltage

Vm  Transmembrane voltage

γj  Unitary channel conductance
Chapter 1 – Introduction

1.1 Gap Junction Channels

Gap junction channels (GJs) were first observed using electron microscopy as hexagonal arrays of junction proteins with a gap of 2-3 nm between the coupled plasma membranes of adjacent cells (Larsen 1977; Revel et al. 1967; Robertson 1963). GJs are now known to mediate direct intercellular communication between neighbouring cells (Goodenough 1976; Loewenstein 1966; Rash et al. 1973). GJs may exist as an individual channel or as an aggregate of multiple channels – often forming gap junctional plaques (Johnson et al. 1974; Larsen 1977). GJs link the cytoplasm of coupled cells to allow for permeation of nutrients, metabolic waste products, and signaling molecules, such as IP3, cGMP, or calcium, up to 1 kDa in size (Bruzzone et al. 2001; Campos-de-Carvalho 1988; Larsen 1977; Loewenstein 1976; Verselis et al. 1986).

Depending on the tissue localization, GJs may mediate different biological effects (Spray et al. 1985). For example, in excitable cells such as cardiomyocytes, GJs allow for the swift and efficient propagation of action potentials through the myocardium (Spray et al. 1985). In avascular organs, such as the lens of the eye, GJs allow for the import of nutrients and export of waste products to maintain molecular homeostasis (Spray et al. 1985). Ablation of GJs linking Sertoli cells and Leydig cells in the testis of mice resulted in decreased sperm motility, indicating that GJ-mediated communication is necessary for spermatogenesis (Kidder et al. 2016; Roscoe et al. 2001). Dye coupling experiments of rat visual cortex show an extensive GJ intercellular network to be present shortly after birth to facilitate astrocyte maturation (Binmoller et al. 1992).
Cellular dysfunction can be exacerbated by altering GJ localization patterns, GJ abundance, or altering GJ-mediated communication between cells following an insult to the tissue (Severs 1994a; Severs 1994b). GJs can prevent or propagate the spread of cytotoxic molecules from an affected cell to a larger population (Andrade-Rozental et al. 2000). Therefore, GJs are an important contributor for the development and maintenance of healthy tissues.

1.2 Gap Junction Structure

GJs are formed through the docking of two hemichannels (also known as connexons) from plasma membranes of neighbouring cells (Goodenough 1976; Makowski et al. 1977) (Fig.1-1A). GJs can be categorized as either homotypic or heterotypic depending on the hemichannel composition where docking of identical hemichannels result in the formation of homotypic GJs and the docking of different hemichannels form heterotypic GJs (Swenson et al. 1989; Werner et al. 1989) (Fig.1-1C). It is well documented that not all hemichannels can successfully dock together and form fully functional GJs, which may be a method to curb uncontrolled GJ-mediated intercellular communication (Bruzzone et al. 1993; Harris 2001; Jassim et al. 2016; Karademir et al. 2016; Swenson et al. 1989). Hemichannels can be further deconstructed into 6 oligomerized connexin protein monomers (Ahmad et al. 1999; Goodenough 1974; Sikerwar et al. 1981; Unwin 1987). There are 21 connexins genes identified in the human genome and they are found on different chromosomes depending on the isoform (Fishman et al. 1991; Sohl et al. 2004). Connexins are distributed ubiquitously throughout the body, however specific isoforms may be localized in specific tissue types (Beyer et al. 1989; Beyer et al. 1990). Connexins can be categorized according to their molecular mass, for example Cx36 corresponds to a connexin 36 kDa in size, or into categories (α, β, γ, δ, or ε) based on sequence identity amongst connexin isoforms (Beyer et al. 1990; Eiberger et al. 2001; Sohl et al. 2004).
topology of connexins includes intracellular N- and C- termini (NT and CT respectively), 4 transmembrane (TM1-4) domains, a cytoplasmic loop (CL), and two extracellular loops (E1 and E2) (Finbow et al. 1987; Hertzberg et al. 1988; Yeager et al. 1992; Zimmer et al. 1987) (Fig 1-1B). Hemichannels can be classified as being homomeric if composed of six identical connexins, or heteromeric if connexin composition is varied (Ahmad et al. 1999; Sosinsky 1995) (Fig. 1-1C). GJs formed by different connexin show distinct channel and hemichannel properties (Bevans et al. 1998).
Figure 1.1 Gap junction composition.

A) Gap junction channels (GJs) form through the docking of two hemichannels from plasma membranes of adjacent cells. B) General topology of a connexin monomer with 4 transmembrane domains (TM1-4), 2 extracellular loops (E1 and E2), 1 cytoplasmic loop, and cytosolic amino-terminus (NT) and carboxy-terminus (CT). C) Various types of GJs and hemichannel composition depending on connexin oligomerization.
Connexin genes are transcribed and then translated into proteins, which may begin oligomerization in the rough endoplasmic reticulum before completing the process in the trans-Golgi network however, further intracellular trafficking routes to the plasma membrane depends on the connexin isoform (Laird 2006; Martin et al. 2001a; Martin et al. 2001b). Cx32 and Cx43 traffic to the plasma membrane through the Golgi secretory pathway before insertion into non-junctional areas of the plasma membrane (Lauf et al. 2002; Martin et al. 2001a; Musil et al. 1993). Once vesicles with oligomerized hemichannels fuse with the plasma membrane, hemichannels can move laterally within the plane of the membrane and function as channels themselves, or dock with another hemichannel from a neighbouring cell to form a GJ (Lauf et al. 2002). Obstruction of the Golgi secretory pathway with drugs did not affect Cx26 trafficking or oligomerization, suggesting an alternate trafficking route for Cx26 (George et al. 1999). The unique trafficking properties of Cx26 is thought to be at least partially attributed to the TM1 domain as mutations in this domain disrupt Cx26 trafficking abilities (Martin et al. 2001a). Alterations in Cx32 trafficking have been associated with mutations linked to development of X-linked Charcot-Marie-Tooth disease by trapping the mutant channel within the cytoplasm and abolishing Cx32 mediated intercellular communication (Matsuyama et al. 2001). Turnover of connexins happens within 2-14 hours, possibly to accommodate quick regulation of the amplitude GJ-mediated currents (Laird 2006).

1.3 Gap Junction Regulation

Connexins are differentially modified at every step in their biosynthesis by transcription factors, phosphorylation, domain cleavage (Kistler et al. 1987; Laird et al. 1991; Saez et al. 2003; Wang et al. 1995). Differential modification of connexin isoforms contributes to the unique GJ properties composed of various connexins (Laird et al. 1991; Wang et al. 1995). For
example, glucocorticoids have been observed to upregulate transcription of Cx26 and Cx32 but not that of Cx43 (Ren et al. 1994). Cx43’s multiple phosphorylation sites are imperative in mediating high turn-over rates associated with these GJs and also govern GJ plaque formation (Laird et al. 1991; Musil et al. 1990). It is well documented that lens connexins naturally undergo CT cleavage during their maturation process (Kistler et al. 1987). Chemical and voltage-dependent regulation of GJ function is commonly observed in cells to quickly promote or impede conductance (Bukauskas et al. 1997). Abundance of GJs can be modulated by physiological agents, such as cAMP, calmodulin, steroid hormones, or by chemical inhibitors, such as cyclohexamide or actinomycin D (Cole et al. 1986; Furger et al. 1996; Garfield et al. 1980; Peracchia et al. 1984; Peracchia et al. 1983). Here I will focus on GJ modulation by intracellular protons, divalent cations and voltage changes across GJ.

1.3.1 Chemical regulation

The functional status of GJs at the cell-cell interface can be modulated by the local chemical environment, including changes in intracellular pH, or divalent cation concentrations (Chen et al. 1992; Cole et al. 1986; Ruch et al. 1987; Spray et al. 1981a; Spray et al. 1984; Veenstra 1990; Verselis et al. 1986). High intracellular levels of H+ (or reduced intracellular pH values) promote GJ closure (Noma et al. 1987; Peracchia et al. 1980b; Spray et al. 1986; Spray et al. 1981b). Introduction of positive charges in the CT reduce CO₂ sensitivity of the GJ, implicating CT as a determinant of chemical-gating characteristics (Wang et al. 1997). Chimera studies of the CL domain switched between Cx32 and Cx38, as well as CT truncation experiments of Cx43, affected pH-gating characteristics of each connexin indicating that CL and CT domains play a role in pH-gating (Ek-Vitorin et al. 1996; Morley et al. 1996; Wang et al. 1996). A “ball and chain” model has been proposed as a mechanism for pH-gating in Cx40 and
Cx43 with the CT of both connexins acting as the “ball” to obstruct the GJ pore (Stergiopoulos et al. 1999). Structural elements governing chemical-sensitivity and chemical-gating are thought to lie within the CL and CT domains of various connexin isoforms (Ek-Vitorin et al. 1996; Liu et al. 1993; Peracchia et al. 1985; Peracchia et al. 2000; Wang et al. 1998).

In addition to modulation by protons, GJ channel function can be modulated by divalent cations including calcium (Ca\(^{2+}\)) and magnesium (Mg\(^{2+}\)). Increased intracellular Ca\(^{2+}\) concentrations significantly downregulate gap junctional currents by promoting a closed conformation of GJs (Loewenstein et al. 1978; Loewenstein et al. 1967; Noma et al. 1987; Peracchia et al. 1980a; Wrigley et al. 1984). Calmodulin, in a Ca\(^{2+}\)-dependent manner, decreases permeability of Cx43 to AlexaFluor, a dye that is able to pass through GJs as a marker to observe the extent of GJ coupling, while an inhibitor of calmodulin function was able to maintain levels intercellular dye transfer (Lurtz et al. 2007). Similarly, Ca\(^{2+}\)-regulation of Cx32 hemichannels is thought to occur through Ca\(^{2+}\)-binding sites of negatively charged amino acids in the pore vestibule to block the pore and alter channel conductance (Gomez-Hernandez et al. 2003).

Neuronal signaling is thought to be partially regulated by [Mg\(^{2+}\)]\(_i\), with low [Mg\(^{2+}\)]\(_i\), due to high chelation by ATP, leading to an increase in Cx36-mediated currents over time and high [Mg\(^{2+}\)]\(_i\), due to increased ATP breakdown, promoting GJ closure over time (Palacios-Prado et al. 2013). Palacios-Prado and colleagues have identified the region of Mg\(^{2+}\)-sensitivity to reside within a negatively charged residue E1 and have conferred Mg\(^{2+}\)-sensitivity onto Mg\(^{2+}\)-insensitive connexins with an E1 point mutation (Palacios-Prado et al. 2014). Single channel conductance of human Cx37 was down-regulated in the presence of high [Mg\(^{2+}\)]\(_i\), and gating parameters of Cx37-mediated currents are altered by Mg\(^{2+}\) binding sites within the channel pore (Banach et al. 2000; Ramanan et al. 1999). This model is supported by similar findings in Cx50
using cysteine mutagenesis where several E1 mutations created ion binding sites which could alter conductance (Verselis et al. 2008). The role and mechanism of intracellular $\text{Mg}^{2+}$-$\text{[Mg}^{2+}]_i$-dependent modulation of GJ channel function is largely unknown.

While the mechanism underlying $\text{[Mg}^{2+}]_i$-dependent modulation in GJs is not clear, $\text{[Mg}^{2+}]_i$ modulation in other channels have been studied in further detail. For example, $\text{Mg}^{2+}$ regulates the function of big potassium channels (BK channels), cyclic-GMP activated channels, and potassium intermediate/small conductance calcium-activated (KCa3.1) channels (Colamartino et al. 1991; Jow et al. 1998; Stoneking et al. 2014). Binding of $\text{Mg}^{2+}$ to the $\alpha$ subunit of BK channels enhances channel function by stabilizing the open state (Shi et al. 2001). While intracellular $\text{Mg}^{2+}$-mediated effects do vary in a voltage-dependent manner, the overall effects of physiological $\text{[Mg}^{2+}]_i$ on BK channels is to upregulate channel function by enhancing ion permeation rate (Shi et al. 2001). $\text{Mg}^{2+}$ can bind to BK channels in the open state, consequently decreasing channel closing rates, or in the closed state, effectively increasing channel opening rates, by coupling voltage sensors with voltage gates to alter channel conductance (Chen et al. 2011a; Horrigan et al. 2008). An area of negatively charged residues in the pore vestibule of BK channels is proposed to be ideal for $\text{Mg}^{2+}$-interactions due to electrostatic attractions (Zhang et al. 2006). Cyclic-GMP activated channels in retinal rod cells binds two to three $\text{Mg}^{2+}$ ions within the channel pore to exercise its modulatory effects in a complex voltage-dependent manner (Colamartino et al. 1991). $\text{[Mg}^{2+}]_i$ blocks KCa3.1 channels in a voltage-dependent mechanism by competing with $\text{Ca}^{2+}$ for the activation site to reduce channel opening rates (Stoneking et al. 2014).
1.3.2 Transjunctional Voltage-dependent gating

Another well-characterized GJ function regulation is the closure of GJs dependent on voltage (Bukauskas et al. 2004). GJs span across two plasma membranes and are sensitive to voltage differences between the interiors of the two coupled cells (Bukauskas et al. 1994; Spray et al. 1984; Verselis et al. 1991). This voltage difference, called transjunctural-voltage ($V_j$), induces closure of GJs by a mechanism defined as $V_j$-gating (Oh et al. 2000). The extent of $V_j$-gating of GJs can be analyzed as normalized macroscopic peak-steady state conductance ratios using a two-state Boltzmann fitting (Harris et al. 1981). Some fitting parameters to be observed through this analysis include: $G_{min}$, residual voltage-independent conductance, $V_0$, the voltage at which conductance is halved, and $A$, which is the slope of the fitted curve reflecting $V_j$-sensitivity of the GJ in concern (Harris et al. 1981; Spray et al. 1981a). Through this analysis differences in gating properties depending on connexin composition can be observed, for example Cx50 GJs gating parameters differ remarkably when compared with Cx36 GJs (Tong et al. 2015b).

Initially only one gate was proposed for each hemichannel, however further studies support a two gate contingent model with one fast- and one slow-gate for each hemichannel (Bukauskas et al. 1994; Harris et al. 1981; Paulauskas et al. 2012; Paulauskas et al. 2009; Verselis et al. 1991; White et al. 1994a). This model explains that the electric field experienced by each gate is influenced by the state of the other gates (Harris et al. 1981). If one cell is depolarized, channel closure would ensue in the cell with positive potential difference by $V_j$-gating (Harris et al. 1981; Verselis et al. 1991). Since there are two slow gates and two fast gates within a full GJ there are 16 different states the gates may be found in, which may explain the differences between the fully conducting state, sub-conducting state, and the fully closed state as
well as the asymmetry of conductance in heterotypic docking (Paulauskas et al. 2012). Conformational change in one gating domain may alter the other gating domain due to interactions between hemichannels constituting a GJ (Verselis et al. 1991). The two types of V_j-gating characterized after observing multiple sub-conducting states in single channel recordings (Bukauskas et al. 1994; Rubin et al. 1992; Trexler et al. 1996). The fast V_j-gating brings the channel conductance to a sub-conducting residual state in a relatively quick manner (< 2 milliseconds) (Bukauskas et al. 1994; Spray et al. 1981a; Spray et al. 1984; Veenstra 1990). The slow V_j-gating, or loop gating, occurs over a 15-60 milliseconds interval and brings channels to a fully closed state (Bukauskas et al. 1997; Bukauskas et al. 1994; Verselis et al. 1991). GJs contain two mirror symmetrical docked hemichannels where each hemichannel can close rapidly (fast gating) or slowly (slow gating), with both scenarios leading to the closure of the full GJ (Bukauskas et al. 2004; Harris et al. 1981; Spray et al. 1981a). As each hemichannel contains two gates, one for each type of V_j-gating, closure of one gate is sufficient to abolish permeation through the whole GJ, however evidence also points to allosteric interactions between opposed hemichannels to influence gating parameters (Oh et al. 2000; Rubin et al. 1992; White et al. 1994a). Thus GJ gating can be examined by studying the respective hemichannel gating properties (Ebihara et al. 1995). GJs are V_j-sensitive, in some cases, the docked hemichannels initiate gating in the cell with a positive V_j compared to the other cell of the coupled pair while others prefer closure of channels at relatively negative V_js, a property termed “gating polarity” (Bukauskas et al. 2004; Harris et al. 1981). The charge of the first few amino acids in the NT determine the fast gating polarity of Cx26 and Cx32 where opposite gating polarities for Cx26 and Cx32 are thought to arise from differences in charge of these NT amino acids (Verselis et al. 1994). However charge substitutions of TM1/E1 border amino acids reverse gating polarity of
Cx26 and Cx32, suggesting a charge complex between multiple domains acting as a voltage sensor (Verselis et al. 1994). Each gate may operate with different gating polarities, for example the fast gate of Cx50 closes at positive interior voltages and the slow gate closes at negative interior voltages (Bukauskas et al. 2001; Bukauskas et al. 2004) (Fig 1.2).
Figure 1.2 Fast and slow gating model and the gating polarity of each gate in Cx50 GJs.

A) A representation of a GJ spanning two plasma membranes of neighbouring cells. Each hemichannel has one fast gate (green arrow) and one slow gate (yellow arrow). When \( V_j = 0 \text{ mV} \), i.e. there is no potential difference between the two cell interiors, both gates are open. B) When a large \( V_j \) is present, the fast gate closes rapidly to a sub-conducting state on the docked Cx50 hemichannel with a \( + V_j \) (indicative of a positive gating polarity for the fast gate of Cx50 GJ). C) Only occasionally when a large \(-V_j\) is applied, the slow gate closes completely on the docked Cx50 hemichannel with \(-V_j\) (indicative of a negative gating polarity for the slow gate of Cx50 GJ).
Unlike other ion channels with a localized V$_j$-sensor, V$_j$-sensor of GJs is thought to arise from an interaction between domains of docked hemichannels (Rubin et al. 1992; Verselis et al. 1994; White et al. 1994a). While differences in V$_j$-sensor composition may be a reason behind various gating behaviours of different connexins, exact structural determinants governing V$_j$-gating properties of Cx50 remain unknown (Barrio et al. 1991; Tong et al. 2015b; Verselis et al. 1994; Xin et al. 2013). V$_j$-sensor is believed to be located, at least in part, within the NT to initiate conformational changes in connexins in order to rapidly close GJs (Oh et al. 2000; Verselis et al. 1994). A proline residue in TM2 is thought to shift due to voltage differences, moving the NT into the pore leaving only a minute residual conductance, a property of fast-gating (Maeda et al. 2009; Oh et al. 2000; Suchyna et al. 1993). Crystallography models of Cx26 show a physical obstruction of the pore by the NT, supporting the NT as a gating determinant (Oshima et al. 2007; Oshima et al. 2008). NT mutations of Cx37 affected hemichannel gating behaviour but did not interfere with gap junction plaque formation (Kyle et al. 2008).

Mutagenesis experiments replacing Cx43 NT with that of Cx37 showed channels lacking fast-gating properties (Ek Vitorin et al. 2016). Cx26T8M, a non-syndromic hearing loss linked mutation, has been characterized with altered V$_j$-gating which may contribute to disease etiology due aberrations in conductance (Mese et al. 2004). Fast V$_j$-gating associated domains are theorized to be close to the pore entrance on the cytoplasmic side to ensure quick closure of the channel (Verselis et al. 1991). Glutamate residues of Cx40 NT play a prominent role as a V$_j$-sensor (Musa et al. 2004). Moreover a high resolution crystal structure of Cx26 GJ reveals NT as a pore-lining domain, specifically constituting a highly constricted region of the pore, which supports the model of NT as a V$_j$-sensing domain (Maeda et al. 2009). A hydrogen bond network keeps the 6 NT domains of a hemichannel in the open state, but the release of one NT from this
network may be sufficient to facilitate a sub-conducting residual state (Maeda et al. 2009). NT of Cx32 observed with 1H-NMR spectroscopy particularly highlights residue G12 in allowing for unrestricted movement and positioning of the NT within the pore (Kalmatsky et al. 2009). Replacement of Cx50 NT with that of Cx36 resulted in remarkably altered \( V_j \)-gating properties, lending support to the role of NT in gating (Xin et al. 2013; Xin et al. 2010; Xin et al. 2012a).

As such, evidence from electrophysiological studies, structural simulations, and other functional studies strongly support the role of NT as a \( V_j \)-sensor and likely involved in the fast gate (Maeda et al. 2009; Mese et al. 2004; Musa et al. 2004; Oshima et al. 2007; Oshima et al. 2008; Xin et al. 2010).

While fast \( V_j \)-gating is often assigned to NT residues, slow \( V \)-gates are believed to be located within E1 (Trexler et al. 2000; Verselis et al. 1991; Verselis et al. 2008). Structural evidence from Cx26 crystallography model, indicating both NT and E1 as pore-lining domains, supports functional evidence of NT-E1 interactions to initiate gating in Cx26 and Cx32 (Maeda et al. 2009; Verselis et al. 1994). Substituted cysteine accessibility method (SCAM) is a method of mutating one residue at a time into cysteine, after which charged thiol reagents may be added into the solution, and observing differences in channel properties to identify possible pore-lining residues (Kronengold et al. 2003b). SCAM experiments identify NT, TM1, and E1 of Cx46 as being structural components of gating (Kronengold et al. 2012). Tryptophan scanning of Cx32 and Cx43 indicate several residues in TM1 as being intolerant to mutagenesis specifically affecting residues need for voltage-gating interactions. It is believed that E1 and E2 undergo a conformational change, collapsing onto the hemichannel pore to close a hemichannel (Bukauskas et al. 2004; Pfahnl et al. 1999; Tang et al. 2009; Trexler et al. 1996). Cx46E1 residues may be a \( V_j \)-sensor required for loop gating (Tang et al. 2009; Trexler et al. 2000; Verselis et al. 2008).
Movement of pore-lining residues in Cx50E1, F43 and G46 specifically, have been found to be implicated in loop gating (Verselis et al. 2008). Straightening of the latter half of TM1 and primary half of E1 into Cx26 pore in response to application of varying Vj potentials observed through molecular dynamics simulations also lend strong support to the role of E1 in loop gating (Kwon et al. 2012). Similarities between slow Vj-gating and chemical-gating properties of Cx43, Cx43-EGFP, and Cx32 suggest overlapping functions for some structural elements (Bukauskas et al. 2001; Peracchia et al. 1999). For example, Cx37 hemichannels seem to induce channel block via the recruitment of polyvalent cations in a voltage-dependent manner, using both voltage-dependent and chemical-gating (Puljung et al. 2004). In summary, crystal structure studies, SCAM experiments, and electrophysiology evidence implicate TM1 and E1 of various connexins as gating domains (Anumonwo et al. 2001; Ek Vitorin et al. 2016; Kronengold et al. 2003b; Maeda et al. 2009; Shibayama et al. 2006).

An alternative type of voltage-dependent gating is sensitivity to trans-membrane potential (Vm), which like slow-gating brings the channel to a completely closed state (Bukauskas et al. 1997; Bukauskas et al. 1994; Verselis et al. 1991; Weingart et al. 1998). Vm-gating has also been shown to be disrupted using chemical-gating inhibitors such as long chain alkanols or pH suggesting an interaction between chemical and Vm-dependent gating (Bukauskas et al. 2004; Weingart et al. 1998). As Cx26 is the only connexin identified so far with a dependence on Vj as well as Vm, Vm properties are not investigated here (Barrio et al. 1991; Bukauskas et al. 2004; Rubin et al. 1992).

1.4 Single channel conductance and pore-lining domains

GJ properties, such as rate of ion permeation, vary depending on GJ composition (Beyer et al. 1995; Elfgang et al. 1995). The ability of a single GJ to conduct ions, hereby referred to as
unitary conductance or \( \gamma_j \), can vary from a minute 5-10 pS of Cx36 to a robust 333 pS of Cx37, the largest GJ single channel observed thus far (Moreno et al. 2005; Srinivas et al. 1999b; Veenstra et al. 1994b). Single GJ normally display a main open state with a large conductance, a fully closed state with no conductance, and may also display multiple sub-conducting states with residual conductance as is observed with electrophysiological recordings in Cx32, Cx43, Cx50, and others (Moore et al. 1991; Moreno et al. 1991; Tong et al. 2014). It is not fully clear what determines the \( \gamma_j \), but the pore diameter and electrostatic properties are believed to play a role in ion permeation (Oh et al. 1997; Veenstra et al. 1994b). \( \gamma_j \) does not seem to correlate with the estimated pore diameter in all GJs, as Cx37 was permeable only to 8 Å PEG probe despite having around a 300 pS conductance while Cx32 GJ channels, \( \gamma_j \) is 55 pS, could allow a larger 11.2 Å PEG probe to pass (Gong et al. 2001).

Pore-lining domains are more likely to be responsible for determining \( \gamma_j \) properties of a GJ either by altering pore diameter or pore electrostatics (Bevans et al. 1998; Gong et al. 2001; Veenstra et al. 1994b; Weber et al. 2004). X-Linked Charcot-Marie-Tooth Disease linked TM1 mutants in Cx32 showed a decreased permeability to larger PEG molecules, supporting a role of TM1 as a pore-lining domain and as a \( \gamma_j \) determinant (Oh et al. 1997). Exchange of TM1 between Cx32Cx43E1 and Cx46 hemichannels resulted in exchange of conductance and V_j-gating properties from recipient to donor characteristics (Hu et al. 1999). SCAM experiments on hemichannels formed by a Cx32Cx43E1 chimera or Cx46 identified that TM1 residues as pore-lining due to access of thiol reagents from the extracellular space (Zhou et al. 1997). Structural simulation studies of Cx26 propose NT presence in the channel pore supporting the idea that the NT, at least partially, lines the pore (Oshima et al. 2007; Oshima et al. 2011). Oh et al. have identified residues N2, G5, and T8 in the NT to alter \( \gamma_j \) and reactive to thiol reagents supporting
the role of these residues as pore-lining (Oh et al. 2008). SCAM studies of Cx46 found multiple residues in the TM1-E1 border, as well as NT, to be reactive to charged thiol reagents which further altered conductance through changes to pore electrostatics (Kronengold et al. 2012; Kronengold et al. 2003a; Kronengold et al. 2003b). Cysteine mutagenesis of Cx50E1 residues identify F43, G46, and D51 as metal binding sites with metal ions diffusing through an open pore to access these residues (Verselis et al. 2008). Cx26 structural model at 3.5 Å resolution envisions the primary half as NT, latter half of TM1, and the first half of E1 as pore elements, with side chains of pore-lining residues directly facing the pore lumen allowing for interactions with ions conducted through the channel (Maeda et al. 2009). Hence SCAM studies, crystal structure evidence, and functional studies on NT and E1 suggest these domains likely line the pore of GJs (Kronengold et al. 2012; Maeda et al. 2009; Oh et al. 2008). There is an alternative model of a pore-lining TM3 domain modulating conductance, with structural evidence from truncated Cx26 crystallographic model, however the role of TM3 in lining GJ pore remains contested (Milks et al. 1988; Skerrett et al. 2002; Unger et al. 1999).

1.5 Cx50

The lens is an avascular organ exchanging nutrients and waste products with the surrounding aqueous humour through passive diffusion (Goodenough 1979). GJs mediate intercellular communication and facilitates nutrient exchange maintaining lens physiology, keeping cells translucent and ultimately preventing cataract formation (White et al. 1992). While lens epithelial cells contain organelles, lens fiber cells are largely devoid of organelles utilizing glycolysis as the main energy source (Goodenough 1979; White et al. 1992). Lysis of organelle membranes during fiber cell maturation process overloads the ubiquitin-proteosome system with substrates, effectively protecting connexins from degradation (Boswell et al. 2009). Another
method of protection for lenticular connexins from the natural degradation machinery is the
spontaneous cyclization of the NT, which prevents hydrolysis by aminopeptidases (Lyons et al.
2014). Stem cells in the lens equator also replenish lens fiber cells to maintain active cell-cell
communication (White et al. 1994b). While the epithelium contains Cx43, lens fiber GJs seem to
be mainly composed of Cx46 and Cx50 (Beyer et al. 1987; Gruijters et al. 1987; Kistler et al.
1985; Musil et al. 1990; White et al. 1992). Cx50 and Cx46 contribute equally to lens fiber cell
GJ function resulting in a highly voltage-dependent coupling in lens fiber cells as opposed to
those in Cx43-coupled epithelial cells (Donaldson et al. 1995). Localization and functional
studies show that Cx50 does not form functional GJs with Cx43, however it does dock and
function successfully with Cx46 while Cx43/Cx46 heterotypic GJs are also functional (White et
al. 1994b; Wolosin et al. 1997). Cx46 and Cx50 heterotypic GJs do not show significantly
altered gating or single channel properties suggesting highly voltage-sensitive gating and high
unitary conductance as important features of lens fiber homeostasis (Hopperstad et al. 2000).
Cx46/Cx50 heteromeric hemichannels have been documented in vivo further pointing out the
diverse and complex organization of GJs in the lens (Ebihara et al. 1999; Jiang et al. 1996).

1.5.1 Role

Cx50 was first identified as membrane protein 70, a unique lenticular connexin resistant
to factors affecting Cx43, the only other lens connexin identified at the time (Gruijters et al.
1987; Kistler et al. 1985; White et al. 1992). GJA8, the gene coding for Cx50, is found on
chromosome 1 in humans, a region homologous to mouse chromosome 3 (Church et al. 1995;
Kerscher et al. 1995). Cx50 is found more abundantly in cortical fibers than in the lens nucleus
regardless of lens age, alluding to an exclusive function of Cx50 in maintaining deep cortical
homeostasis (Kistler et al. 1986). Confocal immunofluorescence microscopy has located Cx50 in
the lens epithelium, providing one possible mechanism for coupling between the epithelium and fiber cells (Dahm et al. 1999; White et al. 1998). However there may be other factors mediating epithelial-fiber cell communication as Cx50 knockout mice also display unaltered intercellular coupling between the two regions (White et al. 1998). Using immunocytochemistry, Cx50 has been found to be localized throughout the mammalian retinal visual pathway, suggesting that Muller cells and astrocytes use Cx50-mediated intercellular communication for metabolic regulation or signal propagation (Schutte et al. 1998). Studies of murine Cx50 in neuroblastoma cells highlight the highly $V_j$-sensitive nature and a highly conducting single channel 220 pS of Cx50 GJs (Srinivas et al. 1999a). Cx50 hemichannels gate at positive potentials and are highly sensitive to pH-gating by external acidification, unlike Cx46 (Beahm et al. 2002). Murine Cx50 is first expressed early in the gestation period, at day 9 or day 10, and steady expression continues into adulthood indicating a role for Cx50 in lens development (Zhou et al. 2002). Cx50 mediates second messenger exchange between coupled lens cells to induce mitosis which is necessary for physiological lens cell proliferation (Sellitto et al. 2004). Some mutations in Cx50 result in retention of organelles within lens fiber cells and decreased levels of soluble proteins interfering with the normal differentiation process in lens fiber cells (Berthoud et al. 2013). In vivo studies have also shown that Cx50 and Cx46 mediate different functions in lens development where Cx50 may be responsible for elongation of primary lens fiber cells and Cx46 overlooks the formation of secondary lens fiber cells postnatally (Xia et al. 2006b). Studies suggest that expressing either Cx46 or Cx50 is sufficient to maintain a connected intercellular network for ion exchange (Cheng et al. 2008). Both Cx46 and Cx50 circulate fluid within fiber cells with hydrostatic pressure driving the movement of sodium ions through GJs along which water ions follow to maintain osmotic pressure (Gao et al. 2011). Lenticular hemichannels
regulate calcium homeostasis by opening when exposed to hyposmotic solutions to allow calcium influx and propidium iodide dye uptake (Mandal et al. 2015). GJ mediated intercellular transport in the lens declines with age corresponding with a decrease in levels of Cx46 and Cx50, which may allude to a mechanism causing age-related cataracts (Gao et al. 2013).

1.5.2 Modifications of Cx50 GJs

The CT of Cx50 is cleaved in an age-dependent processing event by a calcium-dependent endogenous protease in older fiber cells within the lens nucleus (Kistler et al. 1987; Kistler et al. 1990; Rao et al. 1990; Voorter et al. 1989). One difference between full-length and truncated Cx50 properties is pH-sensitivity, where full-length Cx50 reversibly uncouples in response to acidification, but truncated Cx50 is not similarly affected, suggesting reduced pH-sensitivity in truncated Cx50 (DeRosa et al. 2006; Lin et al. 1998; Xu et al. 2002). This may be an important mechanism keeping Cx50 functional in deeper and more acidic regions of the lens (Lin et al. 1998; Mathias et al. 1991; Xu et al. 2002). Cx50 is phosphorylated on multiple residues with various protein kinases in a cAMP and calcium-independent manner (Arneson et al. 1995). Phosphorylation by protein kinase A, also in the CT, upregulate Cx50 hemichannel and GJ function in vivo (Liu et al. 2011a).

Fibroblast growth factor and transforming Growth Factor beta-1 and 2 increases gap junctional coupling through the extracellular signal-regulated kinase pathway (Boswell et al. 2010; Le et al. 2001). Cx50 is thought to utilize the mitogen-activated protein kinase signaling pathway to mediate osmotic regulation using sodium ions in postnatal fiber cells (Shakespeare et al. 2009). Cx50 differentially regulates expression of cell-cycle factors, such as ubiquitin ligase Skp2, by either inhibiting or promoting interactions with other factors to oversee cell-cycling and differentiation (Shi et al. 2015). Cx50 binds zonula occludens protein 1, found in large
abundance in the lens, at a PDZ-binding motif in CT to mediate normal Cx50 function as deletion of this motif resulted in a phenotype resembling Cx50 knockouts (Chai et al. 2011; Nielsen et al. 2001; Nielsen et al. 2003). A calmodulin binding site has been identified in CL and CT of Cx50 as the critical domain for calcium-calmodulin-regulated modulation of Cx50 conductance (Chen et al. 2011b; Zhang et al. 2005). Aquaporin-0 enhances Cx50 GJ coupling by promoting cell adhesion (Liu et al. 2011b). In summary Cx50 is modified by a variety of cellular proteins in order to maintain normal lenticular physiology in the absence of which pathophysiology could result.

1.5.3 The role of Cx50 in cataracts

Ablation of Cx50 directly results in development of microphthalmia and cataracts, as well as reduced eye and lens mass, strongly implicating Cx50 in normal lens development and maintaining lens physiology (White et al. 1998). Deletion of GJA8 is associated with impeded lens growth and lens fiber maturation (Rong et al. 2002). Normal levels of Cx43 and Cx46 alone is not sufficient to maintain proper lens physiology, just as Cx43 or Cx46 knockout animals could not be rescued with Cx50 alone, suggesting a non-redundant role for each lenticular connexin (Gao et al. 1998; Gong et al. 1997; White et al. 1998). Replacement of Cx46 gene for GJA8 in animals resulted in impaired postnatal lens development, however the high refractive index of the lens was maintained suggesting some redundant function amongst lenticular connexins (White 2002). A double knockout of Cx43 and Cx50 surprisingly still resulted in normal prenatal lens development, however postnatal intercellular communication was greatly impaired, alluding to the role of lenticular connexins in postnatal lens growth and homeostasis (White et al. 2001). A double knockout of Cx46 and Cx50 led to mice with reduced gamma-
crystallin protein, inner fiber cell swelling, and eventual degradation of inner fiber cells, while peripheral fiber cells were not similarly affected (Xia et al. 2006a).

Disruption of normal structure, trafficking, or function of Cx50 is linked to the development of congenital cataracts through transverse, insertion, or missense mutations (Bakthavachalu et al. 2010; Gao et al. 2010; Ge et al. 2014; Graw et al. 2001; Kumar et al. 2011; Lichtenstein et al. 2009; Schmidt et al. 2008; Shiels et al. 1998; Steele et al. 1998; Willoughby et al. 2003; Yamashita et al. 2002; Zhou et al. 2011). Cx50L7Q, a NT mutation, causes cataracts by altering gating properties supposedly due to the role of NT as a major determinant of Vj-gating (Liska et al. 2008). V44A, V44E, W45S, and G46V are all E1 cataract-linked mutations affecting normal Cx50 function through different mechanisms (Rubinos et al. 2014; Tong et al. 2011; Vanita et al. 2008; Zhu et al. 2014). Cx50V44E and W45S are both dominant negative inhibitors of wildtype Cx50 function although trafficking to the plasma membrane is not altered (Rubinos et al. 2014; Tong et al. 2011; Vanita et al. 2008). Surprisingly, V44A and G46V cause cataracts by forming constitutively open hemichannels and compromising fiber cell viability (Tong et al. 2011; Zhu et al. 2014). Cx50E48K mutation found in E1 is linked to dominant congenital cataracts abolishing GJ coupling and conductance without altering hemichannel function (Banks et al. 2009). Many mutations in Cx50 NT and E1 result in the development of cataracts thus it is imperative to investigate the role of these domains in governing Cx50 properties to understand disease etiology and to treat these conditions (Liska et al. 2008; Tong et al. 2011; Zhu et al. 2014).

Overexpression of Cx50 in oocytes did not affect protein levels of crystallins, Cx46, membrane protein 26 (MP26), or zonula occludens 1, however vesicles containing Cx50 were trapped in the cytoplasm and decreased epithelial cell differentiation and cataractogenesis were
observed (Chung et al. 2007). Type 2 diabetes patients also display cataract phenotype which may be due to lenticular hemichannel degradation or malformation of MP26 causing an overall a cessation of the intercellular network needed to maintain lens homeostasis (Mangenot et al. 2009). Certain polymorphisms in GJA8 have also been linked to a greater susceptibility to the development of age-related cataracts (Liu et al. 2011c). An increase of intercellular communication of metabolites by replacing Cx46 for Cx50 at the GJA8 locus has shown to significantly suppress cataract phenotype, especially in double knock-in mice, preventing the onset of nuclear cataracts suggesting administration of small molecules which increase lens intercellular trafficking as an alternative to surgery (Li et al. 2010).

1.6 Rationale

Crystal structure studies, SCAM experiments, and functional studies indicate NT and E1 as pore-lining domains which may determine $\gamma_j$ and $V_j$-gating properties of GJs, however exact factors governing these channel properties remain largely unknown (Kronengold et al. 2012; Maeda et al. 2009; Oh et al. 2008). Mutations on the residues in the NT domain alter $\gamma_j$ and $V_j$-gating properties (Peracchia et al. 2005; Xin et al. 2012a). Increasing negative charge of proposed pore-lining residue Cx50G46, i.e. G46D or G46E, resulted in increased $\gamma_j$ to as high as ~293 pS in G46E GJ, which is almost equivalent to the largest Cx37 GJ $\gamma_j$, indicating Cx50 GJs are not fully optimized for the rate of ion permeation at individual channels and mutations of putative pore-lining residue to negatively charged glutamate (E) substantially increase $\gamma_j$ (Tong et al. 2014). This exciting result triggered us to look into the pore-lining residue differences
between Cx50 and Cx37, especially on NT and E1 domains. We found key differences in two positions in Cx50, i.e. G8 and V53 residues, which in the corresponding positions of Cx37 they are E8 and E53. It is possible that these putative pore-lining residues in Cx37 are partially responsible for its larger $\gamma_j$, and that mutations of these two residues in Cx50 could make $\gamma_j$ of Cx50 channel even larger, especially in combination with Cx50G46E GJs which already showed a $\gamma_j$ similar to that of Cx37. Exchanging these residues in Cx50 in order to have individual and combination mutations, G8E, G46E, and/or V53E, may further increase the mutant GJ $\gamma_j$.

Additionally, each of these mutants involves an increase in negative charges along the pore, which could significantly change the pore electrostatic properties, which had been shown to be critical not only on $\gamma_j$, but also for other channel properties, such as $V_j$-gating and modulation by magnesium (Palacios-Prado et al. 2013; Tong et al. 2015b; Tong et al. 2014; Xin et al. 2013).

1.7 Hypothesis

We hypothesize that mutations on putative pore-lining residues of Cx50 G8, G46, and V53 into glutamic acid (E) residue individually or in combination affect $\gamma_j$, $V_j$-gating properties and sensitivity to intracellular Mg$^{2+}$.

1.8 Objectives

To test our hypothesis experimentally we have the following objectives:

1) Investigate effects of the Cx50 mutants G8E-G46E, G8E-V53E, G46E-V53E, and G8E-G46E-V53E on $\gamma_j$ and $V_j$-gating.
2) Examine the intracellular Mg\(^{2+}\) sensitivity of the mutant Cx50 mutant GJ properties (\(\gamma_j\) and \(V_j\)-gating)

3) Study mechanisms responsible for the altered channel properties in these Cx50 mutants.

1.9 References


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Chapter 2 - Manuscript

2.1 Introduction

Gap junction channels (GJs) provide a passage for direct intercellular communication between neighbouring cells (Larsen 1977; Loewenstein 1966; Revel et al. 1967). GJs connect the cytoplasm of coupled cells, allowing ions and metabolites up to 1 kDa in size to traverse through the channel to maintain both electric and metabolic homeostasis (Larsen 1977; Loewenstein 1976; Spray et al. 1985; Verselis et al. 1986). One hemichannel from the plasma membrane of each coupled cell dock together to form a GJ (Goodenough 1976; Makowski et al. 1977). GJs may be homotypic, if two identical hemichannels dock together, or heterotypic, if hemichannel composition varies between the hemichannels (Swenson et al. 1989; Werner et al. 1989).

Hemichannels themselves are oligomers of six connexin monomers and can be either homomeric or heteromeric with reference to the composition of identical or different connexins, respectively (Ahmad et al. 1999; Goodenough 1974; Sosinsky 1995; Unwin 1987). There are 21 human connexin isoforms ubiquitously distributed throughout the body although specific isoforms may be expressed in particular tissues (Beyer et al. 1990; Sohl et al. 2004). Connexins are predicted to have the same topology consisting of 4 transmembrane domains (TM1-4), 2 extracellular loops (E1 and E2), a cytoplasmic loop, and intracellular N- and C- termini (NT and CT respectively) (Finbow et al. 1987; Yeager et al. 1992; Zimmer et al. 1987). GJs composed of different connexins vary considerably in properties, such as unitary channel conductance ($\gamma_j$), transjunctonal-voltage-dependent gating ($V_{j-gating}$), or response to chemical regulation. Experimental evidence demonstrated that different GJ properties in different connexins are at
least partially due to the differences in connexin sequences (Brennan et al. 2015; Palacios-Prado et al. 2013; Srinivas et al. 1999b; Tong et al. 2015b; Tong et al. 2014; Veenstra et al. 1994b).

Exact mechanisms governing differences in GJ $\gamma_j$, the rate of ion permeation, are not fully clear. Pore size and electrostatics are thought to be main determinants of $\gamma_j$ in several models (Gong et al. 2001; Oh et al. 1997; Veenstra et al. 1994b). A simple hypothesis believes that GJs have a much larger pore diameter comparing to classical tetrameric or pentameric membrane channels, therefore easier for ion permeation (Hille 2001). Experimental evidence from studying CMTX-linked Cx32 mutant GJs suggest that pore size (estimated by exclusion of different size polyethylene glycol [PEG] molecules) is correlated with the $\gamma_j$ (Oh et al. 1997). However, many studies showed experimental evidence that GJs with high $\gamma_j$ often showed a lower cut-off size PEG or dye molecules (Dong et al. 2006; Ek-Vitorin et al. 2005; Gong et al. 2001; Veenstra et al. 1994b). These studies proposed an important role of electrostatic properties of the GJ pore for the rate of ion permeation, especially those pore-lining residues with their side chains directly facing the channel lumen (Veenstra et al. 1995; Veenstra et al. 1994a; Veenstra et al. 1994b; Wang et al. 1997). Experimental evidence and structural models with hemichannels and GJs support a presence of NT domain within the pore affecting ion permeation (Kronengold et al. 2012; Oh et al. 2008; Oshima 2014; Oshima et al. 2007). In addition to NT, TM1, and E1 domains have also been shown to be pore-lining domains (Hu et al. 1999; Kronengold et al. 2003a; Trexler et al. 2000; Verselis et al. 2008; Zhou et al. 1997). Mutations on these pore-lining residues often lead to altered rate of ion permeation in their GJs and/or hemichannels (Peracchia et al. 2005; Trexler et al. 2000; Verselis et al. 1994; Xin et al. 2012a). A recently resolved high resolution crystal structure model (3.5 Å) of Cx26 GJ also suppose that NT, TM1, and E1 are pore-lining domains (Maeda et al. 2009). These pore-lining domains in different connexins
showed high sequence identity and similarity to those of Cx26 arguing that perhaps they may have similar GJ structures as that of Cx26 and use the same domains to form their GJ pore. Based on this hypothesis, we aligned amino acid sequences of NT, TM1, and E1 domains in Cx50 with those of Cx37, focusing on the key differences in the putative pore-lining residues in these two large γj GJs. We identifies two positions displayed drastic difference between Cx50 and Cx37, i.e. the G8 in the NT and the V53 in the E1 domain of Cx50. At the corresponding positions in Cx37, they both are glutamate residues, E8 and E53. We hypothesize that pore-lining residues E8 and E53 in Cx37 are partially responsible for its larger γj. Exchanging these residues in Cx50 will increase the γj. We recently found that introducing a negatively charged glutamate at the 46th position (G46E) in the E1 domain substantially increase the γj from 200 S to nearly 300 pS (Tong et al. 2014). Here we evaluate the mutations G8E, G46E, and V53E in Cx50 individually or in combination on the resultant GJ γj. Our data on the Cx50 triple mutant indicate that nature-designed GJs may not be utilizing the full ion permeation capacity. Due to the fact that that these putative pore-lining residues could also play a role in Vj-gating and magnesium modulation, we studied these properties together with their effects on γj.

Understanding of factors regulating ion permeation, gating, and modulation by magnesium in Cx50 GJs is a crucial step in elucidating reasons for natural differences in GJ properties of various connexins.

2.2 Materials and Methods

Construction of Cx50 and mutants

Mouse Cx50 cDNA was subcloned into a pIRES2-EGFP vector as previously described (Tong et al. 2015b; Tong et al. 2014). Double and triple mutations (G8E-G46E, G8E-V53E, G46E-V53E, G8E-G46E-V53E) and individual point mutations (G8E, G46E, V53E) were
generated using the Cx50 or respective single/double mutant vector as a template with a Quick-
Change site directed mutagenesis kit (Stratagene, La Jolla, CA) (Tong et al. 2015b). Primers for
G8E and G46E are as described previously (Tong et al. 2014; Xin et al. 2010) and primers for
V53E are as follows:

Cx50V53E
Forward: 5’ G CAA TCT GAT TTT GAA TGC AAC ACC CAG 3’
Reverse: 5’ CTG GGT GTT GCA TTC AAA ATC AGA TTG C 3’

Cell Culture and transient transfection

GJ-deficient mouse neuroblastoma cells (N2A) purchased from American Type Culture
Collection (ATCC, Manassas, VA) were grown in Dulbecco’s modified Eagle medium (DMEM)
containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Tong et al.
2015b; Tong et al. 2014). In preparation for transfection, cells were plated in 35 mm dishes and
confluency was maintained around 50-70% until transfection (Tong et al. 2015b; Tong et al.
2014). 1 μg of either Cx50 or a mutant vector was transfected with 2 μL X-tremeGENE HP
DNA Transfection Reagent (Roche Applied Sciences, Indianapolis, IN) (Tong et al. 2015b;
Tong et al. 2014). Cells were cultured for 24 hours after transfection and re-plated on to glass
coverslips before performing patch clamp recording.

Patch clamp recording

Dual whole-cell patch clamp was then used to measure \( V_{ij} \)-gating and \( \gamma_i \) properties of
isolated cell pairs expressing either homotypic wildtype Cx50 or mutants (Bai et al. 2014; Tong
et al. 2015b; Tong et al. 2014; Xin et al. 2010). Glass coverslips with transfected cells were
transferred to the recording chamber of the microscope (Leica DM IRB, Wetzlar, Germany) and
submersed in extracellular solution (ECS) at room temperature. ECS composition is (in mM): 140 NaCl, 2 CsCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, 4 KCl, 5 D-glucose, 2 Na pyruvate at pH 7.4 and 320 mOsm. Isolated cell pairs fluorescing green were patched with two glass micropipettes (resistance 2-4 MΩ) filled with intracellular fluid solution (ICS) (Tong et al. 2015b; Tong et al. 2014; Xin et al. 2010). Mg²⁺-free ICS contains (in mM): 130 CsCl, 10 EGTA, 0.5 CaCl₂, 4 Na₂ATP, 10 HEPES at pH 7.2 and 295 mOsm. Mg²⁺-containing ICS composition is as follows (in mM): 130 CsCl, 10 EGTA, 0.5 CaCl₂, 2 Na₂ATP, 3 MgATP, 10 HEPES at pH 7.2 and 295 mOsm. One cell of the cell pair was clamped at 0 mV while the opposing cell was subject to voltage clamp protocols ranging from ±20 mV to ±100 mV in 20 mV increments (Tong et al. 2014; Xin et al. 2013; Xin et al. 2010; Xin et al. 2012b). The recorded junctional currents were amplified with Axopatch 200B amplifiers with low-pass (cut-off frequency 1 kHz) and subsequently digitalized at 10 kHz sampling rate via an ADDA converter (Digidata 1322A, Molecular devices, Sunnyvale, CA) (Tong et al. 2015b; Tong et al. 2014).

Homology structure modeling

High sequence identity (49%) was identified overall between Cx26 and Cx50 after alignment of mouse Cx50 and Cx26 sequence for homology structure models (Tong et al. 2015b; Tong et al. 2014). The crystal structure of Cx26 (2ZW3) was used as a template for homology structure model of Cx50 (Maeda et al. 2009). If a mutation caused an abnormal inter-atomic contact, adjustments were made manually in the COOT and also by CNS energy refinement programs. The model was inspected manually to assess structural validity as described previously (Tong et al. 2014). Adaptive Poisson-Boltzmann Solver (APBS)(Baker et al. 2001) and PDB2PQR server (http://nbcr-222.ucsd.edu/pdb2pqr_1.8/) were used to calculate electrostatic potentials of all atoms in the protein (Tong et al. 2014). APBS parameters used were
set as described by Maeda et al. PyMOL software was used for structure presentations (Tong et al. 2015b; Tong et al. 2014).

**Data Analysis**

Boltzmann fitting of $G_{j,ss}$-$V_j$ relationship curves was used to determine changes in $V_j$-gating properties (Harris et al. 1981). Only cell pairs expressing a junctional conductance of 5 nS or less were selected for Boltzmann fitting analysis (Wilders et al. 1992). For each selected trace, the normalized steady-state conductance ($G_{j,ss}$) was determined using ratios of steady state to peak current (Spray et al. 1981a). $G_{j,ss}$ of negative and positive $V_j$s were plotted independently with the two-state Boltzmann equation (Spray et al. 1981a):

$$G_{j,ss} = \frac{G_{\text{max}} - G_{\text{min}}}{1 + e^{A(V_j - V_0)}} + G_{\text{min}}$$

$V_0$ is the voltage at which the conductance is reduced by half ($(G_{\text{max}}-G_{\text{min}})/2$), $G_{\text{min}}$ is the residual conductance, $G_{\text{max}}$ is the maximum normalized conductance, and $A$ describes the slope of the fitted curve (Spray et al. 1981a). Parameters were then compared using one way ANOVA followed by Dunnett’s Multiple Comparison Test (Tong et al. 2014; Xin et al. 2013; Xin et al. 2010). To compare parameters between Mg$^{2+}$-free and Mg$^{2+}$-containing ICS, two way ANOVA and Bonferroni’s posttest was used.

Unitary channel current values ($i_j$s) were obtained using cell pairs with one or two operational channels (Tong et al. 2015b; Tong et al. 2014). $i_j$ amplitudes were measured directly using Clampfit9 (Molecular Devices, Synnyvale, CA, USA) after digital filtering using notch (cut-off frequency 60 Hz) filters and plotted at corresponding $V_j$s (Tong et al. 2015b; Tong et al. 2014). Slope of the linear regression fitted to $i_j$-$V_j$ plots is defined as slope unitary conductance.
(γj) as previously described (Tong et al. 2015b; Tong et al. 2014). Slope conductance values of mutants using the same ICS were compared using one way ANOVA and Dunnett’s Multiple Comparison Test and slope conductance values between Mg2+-free and Mg2+-containing ICS were compared using two way ANOVA followed by Bonferroni’s posttest.

2.3 Results

**Characterizing unitary channel properties of the Cx50 double and triple mutants using Mg2+-free ICS**

To study unitary single channel conductance (γj) in double or triple Cx50 mutants, we expressed each of the mutants in N2A cells and selected cell pairs with one or two functional homotypic GJs only. Representative unitary channel currents (ijs) of G8E-G46E (Fig 2.1A), G46E-V53E (Fig 2.2A), and G8E-G46E-V53E GJs (Fig 2.3A) in response to various Vj pulses (20, 40, 60, and 80 mV) are shown using the magnesium-free intracellular solution. All mutants show a main open state and one or more subconducting states (open arrows) during Vj protocols (Fig 2.1A, 2.2A, 2.3A). Slope γjs of the main open state were obtained from linear regression analysis of ij-Vj plots of G8E-G46E (310 ± 9 pS, n = 8) (Fig 2.1B), G46E-V53E (278 ± 7 pS, n = 5) (Fig 2.2B), and G8E-G46E-V53E (334 ± 10 pS, n = 5) (Fig 2.3B). All γjs are significantly greater (p<0.001) than that of wildtype Cx50 (230 ± 2 pS, n = 9). G8E-V53E mutants did not form functional GJs (n = 15) from 3 transfections. A bar graph is used to compare average main γj and statistical significance of the mutants comparing to wildtype Cx50 (Fig 2.4). All functional double mutants and the triple mutant (G8E-G46E, G46E-V53E, and G8E-G46E-V53E) showed an increase in γj, suggesting that an introduction of glutamic acid (E) residues at these positions
facilitate ion permeation in individual channels. In fact, cells expressing triple mutant GJs formed the highest conducting single channels (334 pS) of any known GJs.
Figure 2.1 Unitary channel conductance of G8E-G46E is significantly higher compared to that of wildtype Cx50 in Mg²⁺-free intracellular solution (ICS).

A) Representative Is of G8E-G46E and wildtype Cx50 in response to indicated Vs. Subconducting states are indicated with an open arrow and the fully close state is shown with a black arrow in both the mutant and Cx50. B) Linear regression of Is-Vs plots of G8E-G46E (open circles, black line) and Cx50 (grey circles, grey line) was used to obtain slope γ. G8E-G46E γ is significantly greater (p<0.001) than that of Cx50.
Figure 2.2 Unitary channel conductance of G46E-V53E is significantly higher compared to that of wildtype Cx50 in Mg\textsuperscript{2+}-free ICS.

A) Representative $i_j$s of G46E-V53E in response to indicated $V_j$s. Long lived sub-conducting states are indicated with an open arrow. B) Linear regression of $i_j$-$V_j$ plots of G46E-V53E (open circles, black line) was used to obtain slope $\gamma_j$. G46E-V53E $\gamma_j$ is significantly greater ($p<0.001$) than that of Cx50 (grey line).
Figure 2.3 Unitary channel conductance of G8E-G46E-V53E is significantly higher compared to that of wildtype Cx50 in Mg\(^{2+}\)-free ICS.

A) Representative \(i_{j}\)s of G8E-G46E-V53E in response to indicated \(V_{j}\)s. Long lived subconducting states are indicated with an open arrow. B) Linear regression of \(i_{j}-V_{j}\) plots of G8E-G46E-V53E (open circles, black line) was used to obtain slope \(\gamma_{j}\). G8E-G46E-V53E \(\gamma_{j}\) is significantly larger than that of Cx50 (grey line).
Figure 2.4. The average main conductance levels of unitary channel conductance of gap junction channels formed by G8E-G46E, G46E-V53E, and G8E-G46E-V53E in Mg$^{2+}$-free ICS.

The main $\gamma_j$ of each mutant is shown in comparison to that of Cx50 with statistical significance calculated with one way ANOVA followed by Dunnett’s Multiple Comparison Test. The number of asterisks indicate the statistical difference level (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).
G8E-G46E, G46E-V53E, and G8E-G46E-V53E showed a similar $V_j$-gating properties as Cx50 in Mg$^{2+}$-free ICS

Increasing positive and negative $V_j$ pulses across homotypic mutant GJs resulted in mirror symmetrical deactivation of macroscopic junctional currents (Fig 2.5A). The ratio of steady state to peak conductance was obtained ($G_{j,ss}$) and was plotted against each $V_j$. $G_{j,ss}$-$V_j$ plots of G8E-G46E, G46E-V53E, and G8E-G46E-V53E were well fitted to a two-state Boltzmann equation for each $V_j$ polarity (Fig 2.5B, smooth black lines). The Boltzmann fitting parameters for each of these Cx50 mutants, including $G_{\text{min}}, V_0$, and $A$ values, were similar to those of Cx50 (Fig. 2.5B), except the $V_0$ of G8E-G46E channels at $-V_j$ polarity was lower than that of Cx50 (p<0.05) (Table 1).
Figure 2.5 Macroscopic $V_j$-gating properties of G8E-G46E, G46E-V53E, and G8E-G46E-V53E gap junction channels in Mg$^{2+}$-free ICS.

A) $V_j$-pulses ranging from ±20 to ± 100 mV in 20 mV increments were applied to one cell of the cell pair expressing constructs and macroscopic currents recorded from the other cell are shown. For comparison, a Cx50 macroscopic current trace is also shown. B) Normalized $G_{j,ss}$ of G8E-G46E, G46E-V53E, and G8E-G46E-V53E (all open circles) at different $V_j$s were plotted. Smooth black lines represent two-state Boltzmann fitting curves of averaged data from G8E-G46E (n = 4), G46E-V53E (n = 5), and G8E-G46E-V53E (n = 5). Dashed grey lines are Boltzmann fitting curves of $G_{j,ss}$-$V_j$ plots of Cx50 (n = 6) channels.
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**Table 1. Boltzmann fitting parameters for Cx50 and combination mutants in Mg\(^{2+}\)-free ICS.**

Data are presented as mean ± SEM and \( V_0 \) are absolute values. Dunnett’s Multiple Comparison Test to compare the Boltzmann fitting parameters of the mutants against those of wildtype Cx50 with the same \( V_j \) polarity. The number of asterisks indicate the statistical difference level (* \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \)).
Single channel analysis of combination mutants in Mg$^{2+}$-containing ICS

To examine if [Mg$^{2+}$]$_i$ has an impact on $\gamma_j$ of mutant Cx50 GJs, we studied cell pairs expressing homotypic GJs with only one or two operational channels using Mg$^{2+}$-containing ICS (3 mM MgATP). Representative $i_j$s of G8E-G46E (Fig 2.6A), G46E-V53E (Fig 2.7A), and G8E-G46E-V53E (Fig 2.8A) in response to various $V_j$ pulses (20, 40, 60, and 80 mV) are shown below. These mutant GJs show a main open state as well as one or more sub-conducting states (open arrows) during $V_j$ pulse (Fig 2.6A, 2.7A, 2.8A). Slope $\gamma_j$s of the main open state were obtained from linear regressions of $i_j$-$V_j$ plots of G8E-G46E (200 ± 4 pS, n = 5) (Fig 2.6B), G46E-V53E (186 ± 4 pS, n = 5) (Fig 2.7B), and G8E-G46E-V53E (241 ± 8 pS, n = 5) (Fig 2.8B). The $\gamma_j$ of G8E-G46E-V53E GJ is significantly larger (P<0.001) and the $\gamma_j$ of G46E-V53E GJ is significantly lower (p<0.05) than that of wildtype Cx50 (207 ± 0.8 pS, n = 7), while the $\gamma_j$ of G8E-G46E GJ is not different from that of wildtype Cx50 (Fig 2.9A). No $i_j$s were observed in cell pairs expressing G8E-V53E indicating that it is unable to form functional GJ channels (n = 22) in 7 transfections. A bar graph is constructed to compare the average main $\gamma_j$s of each mutant in Mg$^{2+}$-free and Mg$^{2+}$-containing ICS (Fig 2.9B). A small but consistent reduction in $\gamma_j$ was observed for wildtype Cx50 GJ when Mg$^{2+}$-containing ICS was used, while the mutant Cx50 GJ showed a much larger and significant reduction in $\gamma_j$ in the presence of Mg$^{2+}$-containing ICS (Fig 2.9B). These results indicate that: 1) these Cx50 mutants displayed significantly larger $\gamma_j$s than that of Cx50 in Mg$^{2+}$-free ICS; 2) when in the presence of Mg$^{2+}$-containing ICS, only the triple mutant showed a moderate increase in GJ $\gamma_j$, while the $\gamma_j$s of the two double mutants were either lower than that of Cx50 (G46E-V53E) or the same (G8E-G46E).
Figure 2.6 Unitary channel conductance of G8E-G46E is similar to that of wildtype Cx50 in Mg\(^{2+}\)-containing intracellular solutions (ICS).

A) Representative i\(j\)s of G8E-G46E and wildtype Cx50 in response to indicated V\(j\)s. Long lived sub-conducting states are indicated with an open arrow and the fully close state is shown with a black arrow in both the mutant and Cx50. B) Linear regression of i\(j\)-V\(j\) plots of G8E-G46E (open circles, black line) and Cx50 (grey circles, grey line) was used to obtain slope \(\gamma_j\). G8E-G46E \(\gamma_j\) is similar to that of Cx50.
Figure 2.7 Unitary channel conductance of G46E-V53E is significantly reduced compared to that of wildtype Cx50 in Mg²⁺-containing ICS.

A) Representative $i_j$'s of G46E-V53E in response to indicated $V_j$'s. Long lived sub-conducting states are indicated with an open arrow and the fully closed state is indicated with a black arrow.

B) Linear regression of $i_j$-$V_j$ plots of G46E-V53E (open circles, black line) was used to obtain slope $\gamma_j$. G46E-V53E $\gamma_j$ is significantly reduced (p<0.05) compared to that of Cx50 (grey line).
Figure 2.8 Unitary channel conductance of G8E-G46E-V53E is significantly higher compared to that of wildtype Cx50 in Mg\textsuperscript{2+}-containing ICS.

A) Representative $i_j$s of G8E-G46E-V53E in response to indicated $V_j$s. Long lived sub-conducting states are indicated with an open arrow and the fully closed state is indicated with a black arrow.

B) Linear regression of $i_j$-$V_j$ plots of G8E-G46E-V53E (open circles, black line) was used to obtain slope $\gamma_j$. G8E-G46E-V53E $\gamma_j$ is significantly larger ($p<0.001$) than that of Cx50 (grey line).
Figure 2.9 The average main conductance levels of unitary channel conductance of gap junction channels formed by G8E-G46E, G46E-V53E, and G8E-G46E-V53E.

A) The main $\gamma_j$ of each mutant is shown in comparison to that of Cx50. B) The main $\gamma_j$ of each mutant is shown in comparison between Mg$^{2+}$-free and Mg$^{2+}$-containing ICS with statistical significance. The number of asterisks indicate the statistical difference level (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).
G8E-G46E, G46E-V53E, and G8E-G46E-V53E showed similar V\textsubscript{j}-gating properties as Cx50 in Mg\textsuperscript{2+}-containing ICS and in Mg\textsuperscript{2+}-free ICS

Identical V\textsubscript{j} pulses were used to induce macroscopic junctional currents of Cx50 mutant GJs (Fig 2.10A). The ratio of steady state to peak conductance (G\textsubscript{j,ss}) was obtained and plotted against each V\textsubscript{j}. G\textsubscript{j,ss}-V\textsubscript{j} plots of G8E-G46E, G46E-V53E, G8E-G46E-V53E (open circles) were well fitted to two-state Boltzmann equations (Fig 2.10B, smooth black lines). Boltzmann fitting parameters of G8E-G46E were not significantly different from those of Cx50, however G46E-V53E G\textsubscript{min} and V\textsubscript{0}, as well as G8E-G46E-V53E G\textsubscript{min} and A were slightly altered (Table 2). G\textsubscript{j,ss}-V\textsubscript{j} plots of G8E-G46E, G46E-V53E, G8E-G46E-V53E in Mg\textsuperscript{2+}-free ICS were also plotted for comparison and they were generally similar to G\textsubscript{j,ss}-V\textsubscript{j} plots of corresponding mutants in Mg\textsuperscript{2+}-containing ICS (Fig 2.10B, black dashed lines). G46E-V53E V\textsubscript{0} in Mg\textsuperscript{2+}-containing ICS is significantly greater than G46E-V53E V\textsubscript{0} in Mg\textsuperscript{2+}-free ICS (Table 2). Adding [Mg\textsuperscript{2+}]\textsubscript{i} did not alter V\textsubscript{j}-gating properties in most of the mutant GJs, unlike the significant differences observed in γ\textsubscript{j}.
Figure 2.10 Macroscopic $V_j$-gating properties of G8E-G46E, G46E-V53E, and G8E-G46E-V53E gap junction channels in Mg$^{2+}$-containing ICS.

A) $V_j$-pulses ranging from ±20 to ±100 mV in 20 mV increments were applied to one cell of the cell pair expressing constructs and macroscopic currents recorded from the other cell are shown. For comparison, a Cx50 macroscopic current trace is also shown. B) Normalized $G_{j,ss}$ of G8E-G46E, G46E-V53E, and G8E-G46E-V53E (all open circles) at different $V_j$s were plotted. Smooth black lines represent two-state Boltzmann fitting curves of averaged data from G8E-G46E (n = 4), G46E-V53E (n = 4), and G8E-G46E-V53E (n = 5). Dashed grey lines are Boltzmann curves of $G_{j,ss}-V_j$ plots of Cx50 (n = 6) channels. Smooth grey lines are Boltzmann fitting curves of $G_{j,ss}-V_j$ plots of the corresponding mutants in Mg$^{2+}$-free ICS.
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**Table 2. Boltzmann fitting parameters for Cx50 and combination mutants in Mg$^{2+}$-containing ICS.**

Data are presented as mean ± SEM and $V_0$ are absolute values. Dunnett’s Multiple Comparison Test to compare the Boltzmann fitting parameters of the mutants against those of wildtype Cx50 with the same $V_j$ polarity. Bonferonni’s posttest was used to compare the Boltzmann fitting parameters of each mutant to itself in Mg$^{2+}$-containing ICS as compared to Mg$^{2+}$-free ICS at the same $V_j$ polarity. The number of asterisks indicate the statistical difference level (* p<0.05, ** p<0.01, *** p<0.001) in comparison to wildtype Cx50 and († p<0.05, †† p<0.01, ††† p<0.001) in comparison to the corresponding mutant in Mg$^{2+}$-free ICS.
Single channel analysis of individual mutants in Mg$^{2+}$-free and Mg$^{2+}$-containing ICS

In order to investigate the specific residues involved in Mg$^{2+}$-regulated changes in $\gamma_j$, we studied cell pairs expressing point mutations G8E, G46E, or V53E in Mg$^{2+}$-free and Mg$^{2+}$-containing ICS. Representative $i_j$s of G8E, G46E, and V53E in response to 60mV $V_j$ pulse in Mg$^{2+}$-free and Mg$^{2+}$-containing ICS are shown (Fig 2.11A and Fig. 2.11B respectively). Each of these mutant GJs show a main open state and one or more sub-conducting state (open arrows) during the $V_j$ pulse (Fig 2.11A and B). Unitary main open state current amplitude was measured for each of these mutant GJs during $V_j$ pulses of 20-80 mV. Slope $\gamma_j$s of the main open state in Mg$^{2+}$-free ICS were obtained from linear regression analysis of $i_j$-$V_j$ plots (Fig 2.11C) of G8E (228 ± 4 pS, n = 5), G46E (311 ± 14 pS, n = 4), and V53E (236 ± 2 pS, n = 4). G46E $\gamma_j$ is significantly greater (p<0.001) than that of wildtype Cx50 (230 ± 2 pS, n = 9). Slope $\gamma_j$s of the main open state in Mg$^{2+}$-containing ICS was obtained from linear regression analysis of $i_j$-$V_j$ plots (Fig 2.11D) of G8E (265 ± 7 pS, n = 5), G46E (275 ± 7 pS, n = 4), and V53E (194 ± 4 pS, n = 6). G8E and G46E $\gamma_j$s are significantly increased (p<0.001) than that of wildtype Cx50 (207 ± 0.8 pS, n = 7). A bar graph is constructed to compare average main $\gamma_j$ and statistical significance of mutants in Mg$^{2+}$-free ICS (Fig 2.12A) and Mg$^{2+}$-containing ICS (Fig. 2.12B) with wildtype Cx50. A bar graph is also made to compare average main $\gamma_j$ and statistical significance of mutants in Mg$^{2+}$-free ICS with those in Mg$^{2+}$-containing ICS (Fig 2.12C). G8E $\gamma_j$ significantly increases in Mg$^{2+}$-containing ICS, while Cx50, G46E, and V53E $\gamma_j$s significantly decrease in Mg$^{2+}$-containing ICS. Mg$^{2+}$-dependent reduction of Cx50 GJ $\gamma_j$ may require the V53E mutation to exert maximal effects as cell pairs expressing either of these mutations result in decreasing $\gamma_j$. 

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Figure 2.11 Unitary channel conductance of wildtype Cx50, G8E, G46E, and V53E in Mg$^{2+}$-free and Mg$^{2+}$-containing ICS.

A) Representative i$_j$s of wildtype Cx50 and mutants in response to indicated V$_j$s in Mg$^{2+}$-free ICS. Sub-conducting states are indicated with an open arrow and the fully close state is shown with a black arrow in mutants and Cx50. B) Representative i$_j$s of wildtype Cx50 and mutants in response to indicated V$_j$s in Mg$^{2+}$-containing ICS. Sub-conducting states are indicated with an open arrow in mutants and Cx50. C) Linear regression of i$_j$-V$_j$ plots of G8E (open circles, black line), G46E (open circles, dotted black line), V53E (open circles, dashed black line) and Cx50 (grey line) was used to obtain slope $\gamma_j$ in Mg$^{2+}$-free ICS. G46E $\gamma_j$ is larger than Cx50, G8E and V53E $\gamma_j$ is similar to Cx50. D) Linear regression of i$_j$-V$_j$ plots of G8E (open circles, black line), G46E (open circles, dotted black line), V53E (open circles, dashed black line), and Cx50 (grey line) was used to obtain slope $\gamma_j$ in Mg$^{2+}$-containing ICS. G8E $\gamma_j$ is larger than Cx50, G46E and V53E $\gamma_j$ is similar to Cx50.
Figure 2.12 The average main conductance levels of unitary channel conductance of gap junction channels formed by G8E, G46E, and V53E in Mg²⁺-free and Mg²⁺-containing ICS.

A) The main $\gamma_j$ of each mutant in Mg²⁺-free ICS is shown in comparison to that of Cx50 with statistical significance calculated with one way ANOVA followed by Dunnett’s Multiple Comparison Test. The number of asterisks indicate the statistical difference level (* p<0.05, ** p<0.01, *** p<0.001). B) The main $\gamma_j$ of each mutant in Mg²⁺-containing ICS is shown in comparison to that of Cx50 with statistical significance calculated with one way ANOVA followed by Dunnett’s Multiple Comparison Test. The number of asterisks indicate the statistical difference level (* p<0.05, ** p<0.01, *** p<0.001). C) The main $\gamma_j$ of each mutant in Mg²⁺-free ICS is shown in comparison to the corresponding mutant in Mg²⁺-containing ICS with statistical significance calculated with two way ANOVA followed by Bonferroni’s post-test. The number of asterisks indicate the statistical difference level (* p<0.05, ** p<0.01, *** p<0.001).
G8E, G46E, and V53E showed similar $V_j$-gating properties as Cx50 in Mg$^{2+}$-free ICS

Increasing positive and negative $V_j$ pulses resulted in mirror symmetrical $V_j$-dependent deactivation of macroscopic junctional currents in Mg$^{2+}$-free ICS (Fig 2.13A). The normalized steady-state conductance ($G_{j,ss}$) to $V_j$ plots of G8E, G46E, and V53E were well fitted to Boltzmann equations (open circles, smooth black lines) and were similar to that of Cx50 (dashed grey line) (Fig 2.13B). Boltzmann fitting parameters were generally not significantly differently from those of Cx50 in Mg$^{2+}$-free ICS, except the $V_0$ of G8E GJs in $-V_j$ polarity ($p<0.05$) (Table 3). Surprisingly, point mutations failed to significantly alter $V_j$-gating properties in comparison to wildtype Cx50 in Mg$^{2+}$-free ICS.
A

B

Cx50

G8E

G46E

V53E

1S 0.2 nA

-100 -50 0 50 100

Vj (mV)

G

G

G

G

G

G

G
Figure 2.13 Macroscopic Vj-gating properties of G8E, G46E, and V53E gap junction channels in Mg^{2+}-free ICS.

A) Vj-pulses ranging from $\pm 20$ to $\pm 100$ mV in 20 mV increments were applied to one cell of the cell pair expressing constructs and macroscopic currents recorded from the other cell are shown. For comparison, a Cx50 macroscopic current traces are also shown. B) Normalized $G_{j,ss}$ of G8E, G46E, and V53E (all open circles) at different Vj's were plotted. Smooth black lines represent two-state Boltzmann fitting curves of averaged data from G8E (n = 8), G46E (n = 4), and V53E (n = 5). Dashed grey lines are Boltzmann fitting curves of $G_{j,ss}$-Vj plots of Cx50 (n = 6) channels.
<table>
<thead>
<tr>
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<th>$V_j$ polarity</th>
<th>$G_{\text{min}}$</th>
<th>$V_0$ (mV)</th>
<th>$A$</th>
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<tr>
<td>Cx50</td>
<td>+</td>
<td>0.15 ± 0.01</td>
<td>30.4 ± 1.1</td>
<td>0.13 ± 0.01</td>
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<td>(n = 6)</td>
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<tr>
<td></td>
<td>-</td>
<td>0.18 ± 0.02</td>
<td>33.0 ± 1.8</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>G8E</td>
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<td>0.14 ± 0.02</td>
<td>28.1 ± 1.4</td>
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<td>(n = 8)</td>
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<tr>
<td></td>
<td>-</td>
<td>0.14 ± 0.02</td>
<td>26.5 ± 1.5*</td>
<td>0.11 ± 0.02</td>
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<tr>
<td>G46E</td>
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<td>0.14 ± 0.03</td>
<td>32.4 ± 2.8</td>
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<tr>
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<td>0.13 ± 0.02</td>
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<tr>
<td>V53E</td>
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<td>(n = 5)</td>
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<td>0.16 ± 0.04</td>
<td>32.6 ± 3.3</td>
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**Table 3. Boltzmann fitting parameters for Cx50 and individual mutants in Mg$^{2+}$-free ICS.**

Data are presented as mean ± SEM and $V_0$ are absolute values. Dunnett’s Multiple Comparison Test to compare the Boltzmann fitting parameters of the mutants against those of wildtype Cx50 with the same $V_j$ polarity. The number of asterisks indicate the statistical difference level (* $p<0.05$).
G8E, G46E, and V53E showed similar \( V_j \)-gating properties as Cx50 in Mg\(^{2+}\)-free and Mg\(^{2+}\)-containing ICS

Increasing positive and negative \( V_j \) pulses resulted in mirror symmetrical \( V_j \)-dependent deactivation of macroscopic junctional currents Mg\(^{2+}\)-containing ICS (Fig 2.14A). The normalized steady-state conductance (\( G_{j,ss} \)) to \( V_j \) plots of G8E, G46E, and V53E were well fitted to Boltzmann equations (open circles, smooth black lines) and were similar to that of Cx50 (dashed grey line) (Fig 2.14B). Boltzmann fitting parameters of G8E \( V_0 \), as well as V53E \( V_0 \) and \( A \) were slightly altered in Mg\(^{2+}\)-containing ICS (Table 4). V53E \( A \) in Mg\(^{2+}\)-containing ICS is also significantly different from V53E \( A \) in Mg\(^{2+}\)-free ICS (Table 4). Individual point mutations G8E, G46E, and V53E failed to alter \( V_j \)-gating properties upon increasing [Mg\(^{2+}\)]\(_i\) even though \( \gamma_j \) was significantly altered.
Figure 2.14 Macroscopic $V_j$-gating properties of G8E, G46E, and V53E gap junction channels in Mg$^{2+}$-containing ICS.

A) $V_j$-pulses ranging from $\pm$20 to $\pm$100 mV in 20 mV increments were applied to one cell of the cell pair expressing constructs and macroscopic currents recorded from the other cell are shown. For comparison, a Cx50 macroscopic current trace is also shown. B) Normalized $G_{j,ss}$ of G8E, G46E, and V53E (all open circles) at different $V_j$s were plotted. Smooth black lines represent two-state Boltzmann fitting curves of averaged data from G8E (n = 5), G46E (n = 5), and V53E (n = 4). Dashed grey lines are Boltzmann fitting curves of $G_{j,ss}$-$V_j$ plots of Cx50 (n = 6) channels. Smooth black lines are Boltzmann fitting curves of $G_{j,ss}$-$V_j$ plots of each corresponding mutant in Mg$^{2+}$-free ICS.
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<th>$V_0$ (mV)</th>
<th>$A$</th>
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<td>Cx50</td>
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<td>0.16 ± 0.02</td>
<td>33.0 ± 1.8</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>(n = 6)</td>
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<td>0.13 ± 0.03</td>
<td>33.3 ± 2.3</td>
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<td>G8E</td>
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<td>0.12 ± 0.02</td>
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<td>22.7 ± 0.9***</td>
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<td>0.14 ± 0.01</td>
<td>29.4 ± 1.0</td>
<td>0.13 ± 0.02</td>
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<tr>
<td>(n = 5)</td>
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<td>0.11 ± 0.02</td>
<td>28.3 ± 1.3</td>
<td>0.12 ± 0.01</td>
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<tr>
<td>V53E</td>
<td>+</td>
<td>0.15 ± 0.02</td>
<td>27.4 ± 1.8</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>(n = 4)</td>
<td>-</td>
<td>0.14 ± 0.02</td>
<td>25.4 ± 1.6**</td>
<td>0.20 ± 0.05*††</td>
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</tbody>
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Table 4 Boltzmann fitting parameters for Cx50 and individual mutants in Mg\(^{2+}\)-containing ICS.

Data are presented as mean ± SEM, and $V_0$ are absolute values. Dunnett’s Multiple Comparison Test to compare the Boltzmann fitting parameters of the mutants against those of wildtype Cx50 with the same Vj polarity. The number of asterisks indicate the statistical difference level (* p<0.05, ** p<0.01, *** p<0.001) in comparison to wildtype Cx50 and († p<0.05, †† p<0.01, ††† p<0.001) in comparison to the corresponding mutant in Mg\(^{2+}\)-free ICS.
Increasing intracellular Mg$^{2+}$ concentrations generally decreased unitary channel conductance of combination and individual mutations.

To summarize our data on Cx50 mutant GJ $\gamma$s, I created a bar graph to include each of these functional mutant $\gamma$s in Mg$^{2+}$-free and Mg$^{2+}$-containing ICS (Fig. 2.15). Combination mutants G8E-G46E, G46E-V53E, and G8E-G46E-V53E main open state $\gamma_j$ is decreased in Mg$^{2+}$-containing ICS (Fig. 2.15). G46E and V53E main open state $\gamma_j$ were also decreased in Mg$^{2+}$-containing ICS (Fig. 2.15). Only G8E main open state $\gamma_j$ increased in Mg$^{2+}$-containing ICS (Fig. 2.15). Our results suggest that G46E and/or V53E mutations may dictate Mg$^{2+}$-dependent reduction in $\gamma_j$. 
Figure 2.15 The average main conductance levels of unitary channel conductance of gap
junction channels formed by combination and individual mutations obtained in Mg$^{2+}$-
containing ICS and Mg$^{2+}$-free ICS.

The main $\gamma_j$ of each mutant is shown in comparison between Mg$^{2+}$-free and Mg$^{2+}$-containing ICS
with statistical significance calculated with two way ANOVA followed by Bonferroni’s post-
test. The number of asterisks indicate the statistical difference level (* $p<0.05$, ** $p<0.01$, ***
$p<0.001$).
Homology structures and electrostatic properties of combination and individual mutant channels

As there is a high overall level of sequence identity (57%) between mouse Cx50 and human Cx26, Cx50 homology structure model was created using Cx26 crystal structure as a template. The Cx50 homology structure model is shown below and is enlarged to highlight the location of the three putative pore-lining residues we studied (Fig 2.16). The homology structures of individual mutants, double mutants (G8E-G46E and G46E-V53E) and triple mutant (G8E-G46E-V53E) are also shown (Fig 2.16). The minimum pore diameters were measured at each of the three residue positions before and after the mutation. Each of these mutants is predicted to reduce the pore diameter. From the mutant structure models, G46E and V53E mutant GJs have their mutated residue side chains facing directly into the pore lumen while in the case of G8E GJ, the mutated residue appears to not interact directly with the channel lumen (Fig 2.16). G46 is located in a particularly narrow region of the pore with an estimated minimum diameter of 20.3 Å and the G46E mutation leads to a large reduction in diameter (12.6 Å) and V53 is in a position with wider pore diameter (27 Å) and V53E mutation moderately reduces the diameter to 21.3 Å. G8 is located in the first half of NT which forms a funnel structure of the pore with a diameter of 27 Å, which is predicted to be reduced to 25.8 Å following the G8E mutation (Maeda et al. 2009). Similar diameter reductions in each of these three positions are also observed in double/triple mutants (data not shown).
Figure 2.16 Homology models of Cx50, individual (G8E, G46E, V53E), double (G8E-G46E, G46E-V53E), triple (G8E-G46E-V53E) mutations.

A) A side view of Cx50 homology model structural model (cartoon view, only four subunits of twelve are displayed). An enlarged portion of wildtype Cx50 proposed pore-lining residues are shown as spheres before any mutation. The minimum diameters are shown at G8, G46, and V53 positions. B) An enlarged view of Cx50 as well as combination (G8E-G46E, G46E-V53E, G8E-G46E-V53E) and individual (G8E, G46E, and V53E) mutant channels are shown. The side chains of the mutant residues are represented as spheres after mutations as indicated. The minimum diameters are shown for individual mutants G8E, G46E, and V53E.
Pore diameters for all of our mutants (single, double, or triple mutants) are predicted to decrease, while many of these mutants showed an increase in $\gamma_j$, indicating that the diameter reductions in these mutant GJ channels are unlikely to be a rate limiting factor for ion permeation. As the permeating ions carry positive or negative charge, which will be influenced by the electrostatic potentials in the pore, we then calculated pore surface electrostatic potentials for each of the mutant GJs. As shown in Fig 2.17, each of the individual and combination mutants increased negative surface potentials in different part of the pore comparing to that of Cx50 GJ. Considering the pore diameters of these mutants decreased at their respective positions, the introduction of negatively charged glutamate at three locations are predicted to have differential influence to the pore electrostatic potentials, which could be an important factor for facilitating ion permeation in this cation-preferred Cx50 GJs (Srinivas et al. 1999a; Tong et al. 2015b; Tong et al. 2014). It is not clear how Mg$^{2+}$ modulates these mutant GJs, we propose that pre-existing and introduced negatively charged residues may form one or more Mg$^{2+}$ binding site(s), which could decrease the $\gamma_j$ and possibly also the probability of channel opening as documented earlier for Cx43, Cx26, Cx32, Cx36, Cx45, and Cx47 GJs (Palacios-Prado et al. 2014; Zonta et al. 2014).
Figure 2.17 Pore surface electrostatic potentials in the Cx50 mutants.

A side view of cut open Cx50 and mutant GJ channels is shown to show pore surface electrostatic potentials (calculated with APBS program) using dielectric constraints of 2 (protein) and 20 (solutions) (Baker et al. 2001).
2.4 Discussion

We have shown here that mutating two or more putative pore-lining residues to glutamate in Cx50, specifically, G8E-G46E, G46E-V53E, and G8E-G46E-V53E, substantially increases $\gamma_j$ and Mg$^{2+}$-dependent modulation of Cx50 and mutant GJs. The $\gamma_j$ of the triple mutant reached 334 pS, similar to the largest GJ, Cx37, in terms of unitary channel conductance. Our homology structure models suggest that pore surface electrostatic potentials is a dictating factor for ion permeation of Cx50 and the Cx50 mutant GJs. For the first time, we demonstrated that intracellular Mg$^{2+}$ can regulate ion permeation at individual channel levels ($\gamma_j$) in Cx50 and the mutants. Our results indicate that Cx50 GJ is far from optimized for ion permeation and $\gamma_j$ can be modulated by intracellular Mg$^{2+}$. Our study is consistent with many previous studies in that residues in NT and E1 domains of Cx50 and other connexins are likely lining GJ pore, and changes in residues in these domains could affect single channel conductance and cation-dependent modulation.

2.4.1 Factors determining $\gamma_j$ and $V_j$-gating

Critical factors determining ion permeation properties of GJs are not yet fully known. Cx50 single channels have one of the largest conductance, with a $\gamma_j$ of 200 pS, and display a cation-preference (Gong et al. 2001; Srinivas et al. 1999a; Tong et al. 2014). Previous studies on Cx50 highlight the importance of electrostatic environment of putative pore-lining residue G46 rather than pore diameter as a major determinant of $\gamma_j$ in Cx50 (Tong et al. 2015b; Tong et al. 2014). Our study validates this idea and in fact applies it to several proposed pore-lining residues as substituting a small and non-polar glycine or valine residues, in G8, G46, or V53, with a large and negatively charged glutamic acid residue (E), while a simple theoretic prediction that each of these mutations would decrease pore diameter due to a much larger side chain, all consistently
display increased $\gamma_j$. The effect of mutating a single residue in one connexin is magnified twelve times when we consider a full GJ with 12 connexins. Thus introducing a double mutation adds 24 (2 x 12) negative charges to a full GJ, and a triple mutation would lead to an addition of 36 (3 x 12) new negative charges. Negative charges along Cx50 GJ pore may recruit and facilitate the conductance of ions through the channel as Cx50 is known to be a cation-preferring GJ (Srinivas et al. 1999a; Tong et al. 2014). Cx37, the largest conducting single channel of 333 pS, is believed to accommodate such a great ion flow due a ring of acidic residues near the pore opening (Traub et al. 1998; Veenstra et al. 1994b). A similar mechanism has been confirmed in other channels where negative electrostatic potential facilitates big potassium (BK) channel conductance and nicotinic acetylcholine receptor conductance by promoting local cation concentrations and increasing channel conductance (Brelidze et al. 2003; Geng et al. 2011; Imoto et al. 1988). Furthermore, our prediction that increasing the quantity of negative charges of putative pore-lining residues would elevate $\gamma_j$ more than the actions of individual mutations was consistent with our results as G8E-G46E, G46E-V53E, and G8E-G46E-V53E GJs have a larger $\gamma_j$ than wildtype Cx50 or G8E, G46E, and V53E GJs. In fact, G8E-G46E and G8E-G46E-V53E single channels were similar to Cx37 with G8E-G46E-V53E mutants displaying one of the largest $\gamma_j$ (Traub et al. 1998; Veenstra et al. 1994b). Introduction of positive charges in either NT or E1 alters Cx50 GJ conductance (Peracchia et al. 2005; Tong et al. 2014). Consistent with our findings, $\gamma_j$ has been increased by mutations of residues in NT and E1 individually to glutamate or aspartate (both are negatively charged) (Tong et al. 2014; Xin et al. 2013). We are the first to prove that increasing negative charges in multiple proposed pore-lining domains at once may increase $\gamma_j$ without a foreseeable saturation point.
G46E single channels showed an increase in $\gamma_j$ while G8E and V53E were unaltered in Mg$^{2+}$-free ICS. This phenomenon may be partially attributed to differences in surface electrostatic potential (Fig 2.17). G46E GJs are predicted to have increased negative surface charge density, visualized as red regions in the homology models, than G8E or V53E GJs. Additionally, G8E is an NT mutation whose side chain is predicted to face away from the channel lumen, which may explain the lack of significant functional differences in GJ properties of this mutant (Fig 2.16). While V53E side chain is thought to face into the pore, it does not facilitate increased ion permeation (Fig 2.15 and Fig 2.16). This may be due to the pertinence of some proposed pore-lining residues in determining conductance properties more than others.

G8E mutation increases the net negative charge of Cx50 GJs but does not increase ion permeation possibly due to a saturation limit in the relationship between negative charges in NT and $\gamma_j$ (Xin et al. 2013). A previous study on Cx50 E1 residues indicates that some amino acids may be more relevant than others in dictating $\gamma_j$ owing to side chain properties and location (Tong et al. 2015b). This may perhaps occur due to the location of G46 in a narrower section of the pore (20.3 Å) compared to V53 (27 Å) as illustrated by our homology model (Fig 2.16B).

Conversely in Mg$^{2+}$-containing ICS, G8E and G46E GJs display a significantly larger $\gamma_j$ as compared to wildtype or V53E GJs (Fig 2.14B). This could result from the NT mutation increasing local cation concentrations at the mouth of the pore to facilitate increased conductance while the V53E mutations may be located deeper within the pore, unable to affect conductance to the same degree. Our results for G8E $\gamma_j$ in Mg$^{2+}$-containing ICS (265 pS) is moderately higher than a previous study (222 pS) (Xin et al. 2012a), we do not know the reasons, but it could be due to the differences in solution making, osmolarity, and/or pH values for both intracellular and
extracellular solutions (Noma et al. 1987; Srinivas et al. 1999a; Tong et al. 2015b; Tong et al. 2014).

Substantial increase in the $\gamma$s of the double and triple mutants indicate that the resistance of the channel is reduced, possibly at the channel portions near those mutated residues. Local resistance change will change the distribution of $V_j$ at different part of the channel, which would alter the $V_j$-gating sensor’s ability to ‘sense’ $V_j$ changes. Surprisingly the combination mutations did not alter $V_j$-gating parameters when compared to wildtype Cx50 to a great extent. $V_j$-gating is activated as the GJ senses differences in potential between the cytoplasm of coupled cells (Spray et al. 1984; Verselis et al. 1991). As charged amino acids in Cx50 NT and E1, as well as other connexins, are believed to determine $V_j$-gating properties we predicted the mutations to negatively charged glutamate will result in significantly different $V_j$-gating parameters in mutant GJs (Peracchia et al. 2005; Tong et al. 2014; Xin et al. 2012a). These results may be due to the individual locations investigated in the study and their relative contribution to determining $V_j$-gating. G8E has previously been reported to not change gating parameters to the extent of other NT amino acids (Xin et al. 2010). Charged mutations in Cx50G46 reveal that some mutants, for example a larger positively charged lysine residue, virtually eliminated $V_j$-gating of Cx50 GJs (Tong et al. 2014). Investigation of several Cx50 E1 residues also finds that some positions are more pertinent than others in determining Cx50 $V_j$-gating properties which may explain the lack of change in gating parameters with respect to V53E mutations (Tong et al. 2015b). Another consideration is that combination mutations distributes negative charges along the length of the full GJ and possibly maintains the $V_j$-sensor in its original state. This is a preliminary suggestion and further work on combining mutations known to affect Cx50 $V_j$-sensor may elucidate a more thorough mechanism. Overall, our data supports the importance of pore electrostatics as a rate
limiting factor in determining $\gamma_j$ in Cx50, and possibly other cation-preferring GJs, with the location of pore-lining residues’ side chains contributing to pore properties.

2.4.3 Magnesium-dependent modulation

We report here that by inclusion of 3 mM MgATP in our patch pipette solution (the estimated free $[\text{Mg}^{2+}]_i$ ranges between 0.3 – 1.0 mM depending on [ATP] and [ADP] according to web-based program Maxchelator - http://maxchelator.stanford.edu/webmaxc/webmaxcS.htm) decreased the $\gamma_j$ of Cx50 mutants G8E-G46E, G46E-V53E, and G8E-G46E-V53E (Fig 2.9). While wildtype Cx50 exhibits only a 10% reduction in $\gamma_j$ with response to increasing $[\text{Mg}^{2+}]_i$, G8E-G46E, G46E-V53E, and G8E-G46E-V53E showed a significant modulation of $\gamma_j$ in response to increasing $[\text{Mg}^{2+}]_i$ (Fig 2.9). In order to pinpoint the contribution of each individual mutation to the observed Mg$^{2+}$-dependent modulation, we studied G8E, G46E, and V53E mutant GJs and observed the most significant reduction of $\gamma_j$ in V53E GJs, as well as a moderate reduction in G46E GJs, when $[\text{Mg}^{2+}]_i$ was increased (Fig 2.15). Charged E1 residues are commonly ascribed to being divalent cation binding sites in various hemichannels and GJs, which may explain the lack of $[\text{Mg}^{2+}]_i$ sensitivity of GJ channels composed of NT mutant G8E (Ebihara et al. 2003; Palacios-Prado et al. 2014; Zonta et al. 2014). As mentioned previously, our homology structure predicts the side chain of G8E to face away from the pore lumen which may impede interactions with Mg$^{2+}$ (Fig 2.16B). Early studies on Cx37 conductance and its variance with differing $[\text{Mg}^{2+}]_i$ propose many possible mechanisms by which Cx37 $\gamma_j$ could be altered including specific binding sites, channel blockade, or even a change in ion-selectivity (Banach et al. 2000). A more recent study, discussing Cx36 GJs, is that putative pore-lining E1 domain contains an Mg$^{2+}$-sensor which can induce structural changes to alter macroscopic GJ conductance (Palacios-Prado et al. 2013). Further studies of Cx36 Mg$^{2+}$-dependent modulation
pinpoint putative E1 pore-lining residue D47, as a critical location in sensing changes in [Mg\textsuperscript{2+}]\textsubscript{i} (Palacios-Prado \textit{et al.} 2014). This is the first study to address Mg\textsuperscript{2+}-dependent modulation of Cx50 γ\textsubscript{j}. Negative charges in proposed pore-lining Cx50E1 mutants, including G46E and V53E, may form a ring of negative charges within the channel lumen which act synergistically to bind Mg\textsuperscript{2+} and subsequently alter GJ activity via pore occlusion, such as the case with Ca\textsuperscript{2+}-dependent regulation of Cx32 hemichannels or Mg\textsuperscript{2+}-dependent modulation of Cx36 macroscopic currents (Gomez-Hernandez \textit{et al.} 2003; Palacios-Prado \textit{et al.} 2014). A similar mechanism is observed with respect to calcium interactions with Cx26 GJs, specifically with gamma carboxylated E1 residue D47 (Zonta \textit{et al.} 2014). A mechanism similar to the intersubunit interactions between negatively (D50) and neutral (Q48) residue in the presence of Ca\textsuperscript{2+} proposed to reduce the open probability of both Cx26 hemichannels and GJs, may explain the modulatory effects of Mg\textsuperscript{2+}-binding (Lopez \textit{et al.} 2013; Sanchez \textit{et al.} 2013; Zonta \textit{et al.} 2014). While many factors regarding Mg\textsuperscript{2+}-dependent regulation of γ\textsubscript{j} in Cx50 GJs remain unknown, the mechanism of Mg\textsuperscript{2+}-regulation of BK channels have been investigated in more detail and may provide clues to understand Mg\textsuperscript{2+}-dependent behaviour of GJs.

BK channels are modulated by intracellular [Mg\textsuperscript{2+}] in a calcium and voltage-dependent manner to binding sites along the channel pore (Morales \textit{et al.} 1996; Shi \textit{et al.} 2002; Zhang \textit{et al.} 1995; Zhang \textit{et al.} 2001). The binding sites are generally thought to be negatively charged residues, such as glutamic acid (E), which use electrostatics to facilitate interactions with Mg\textsuperscript{2+} ions (Lingle 2008; Shi \textit{et al.} 2002; Xia \textit{et al.} 2002; Yang \textit{et al.} 2006; Zhang \textit{et al.} 2006). The S4 segment, a voltage sensor for BK channels, is postulated to interact with Mg\textsuperscript{2+}-binding sites in order to coordinate the Mg\textsuperscript{2+}-regulated state of BK channel opening (Hu \textit{et al.} 2003). Such a mechanism may explain the 30% Mg\textsuperscript{2+}-dependent reduction of γ\textsubscript{j} in G8E-G46E, G46E-V53E,
and G8E-G46E-V53E GJs. As the NT domain is thought to contain V\textsubscript{j}-sensor elements of GJ channels and considering the fact that the mutant GJs do not show altered V\textsubscript{j}-gating properties, it can be assumed that the Cx50 V\textsubscript{j}-sensor is unaffected by these mutations. Then, a coordination of interactions between a Cx50 V\textsubscript{j}-sensor within NT and negatively-charged Mg\textsuperscript{2+}-binding sites within E1, possibly including G46E and/or V53E, could be a mechanism for Mg\textsuperscript{2+}-dependent modulation of Cx50 GJs. In summary, introducing negative charges into putative pore-lining residues, in addition to increasing γ\textsubscript{j}, may have created Mg\textsuperscript{2+}-binding sites to alter Cx50 structure and result in decreased γ\textsubscript{j}.

2.4.4 Disease-linked mutations in NT/E1

As NT and E1 domains are highly conserved between various connexins, mutations in these domains of many connexins are often linked to disease (Beyer \textit{et al.} 2013; Laird 2014; Liska \textit{et al.} 2008; Shao \textit{et al.} 2012; Wang \textit{et al.} 2015). A multitude of cataract-linked mutations are found in the NT and E1 domain, with varying effects on GJ channel function. L7Q, a Cx50 NT mutation, causes cataracts semi-dominantly and alters gating properties as the NT is a major determinant of V\textsubscript{j}-gating properties (Liska \textit{et al.} 2008). Surprisingly V44A and G46V cause cataracts by forming constitutively open hemichannels and compromising fiber cell viability (Tong \textit{et al.} 2011; Zhu \textit{et al.} 2014). Cx50G46R mutation, adding a positive arginine residue into E1, is another cataract-linked mutation but the mechanism causing the disease is not known (Ge \textit{et al.} 2014). Cx50E48K mutation found in E1 is linked to dominant congenital cataracts abolishing GJ coupling and conductance without altering hemichannel function (Banks \textit{et al.} 2009). Mg\textsuperscript{2+} deficiency is another link to development of cataracts as intracellular cation concentrations are altered, furthering the progression of the disease (Agarwal \textit{et al.} 2013). There are several cataract-linked mutations in Cx50NT and E1 domains which affect various GJ
properties thus understanding the mechanism determining $\gamma$, $V_j$-gating, and chemical modulation amongst other properties is an important step in elucidating etiology of pathophysiology, specifically in positions investigated in this report such as G46V and G46R.

Cx26, Cx32, and Cx43 are also intolerant to mutations in NT and E1 domains and contribute to various disease states. Charcot-Marie-Tooth X-linked disease is often attributed to loss-of-function mutations of Cx32 NT (Abrams et al. 2001). Severe skin hyperkeratosis is one pathological manifestation of Cx43G8V mutation with increased hemichannel activity (Wang et al. 2015). ODDD-linked Cx43 NT mutants are thought to exhibit dominant negative effects on function by disrupting normal NT structure (Shao et al. 2012). A duplication of Cx43F52, an E1 residue, is linked to ODDD, however exact mechanisms are unknown (Laird 2014; Paznekas et al. 2003). Cx26G45E, equivalent of Cx50G46, is a keratitis-ichthyosis-deafness syndrome-causing mutation owing to decreased extracellular calcium sensitivity and consequent increased hemichannel activity (Mese et al. 2011). In summary, while it is known that intact NT and E1 domains are required for normal functioning, their specific contribution to connexin function and regulation and disease pathology remains unknown.

2.4.5 Structure function studies of Cx50 NT/E1

Cx50D3N reversed Cx50’s gating polarity from positive to negative, increased chemical gating sensitivity, and increased speed of current deactivation asserting the importance of the NT determining gating properties (Peracchia et al. 2005; Srinivas et al. 2005). Substituted mutagenesis experiments of Cx36 NT exchanged with that of Cx50 as a chimera or specific point mutations, N9R and D3E, dramatically affected $V_j$-gating properties and unitary conductance as compared to that of wildtype Cx50 (Xin et al. 2010; Xin et al. 2012a). Heterotypic studies of the chimera and point mutations reveal a complicated gating mechanism which cannot simply be
predicted by the summation of individual hemichannel behaviour (Xin et al. 2012b). The NT is also thought to be involved in monovalent ion regulation of Cx50 hemichannels (Srinivas et al. 2006). Sensitivity of Cx50 hemichannels to most extracellular monovalent cations reside in amino acids in the first half of NT while the binding site for potassium has been proposed to be in E1, possibly by cations traversing through the channel pore to bind residues in pore-lining domains (Srinivas et al. 2006). This study further supports that Cx50 NT is important in determining $\gamma_j$, $V_j$-gating, and cation-sensitivity.

Amino acid substitutions in Cx50 E1 are often the cause of cataracts via changes to voltage sensitivity or altered permeation rate, identifying this domain as a major structural element determining gating and conductance properties (DeRosa et al. 2007; Graw et al. 2001; Steele et al. 1998). Charge-related substitutions in Cx50G46 dramatically alter unitary conductance and $V_j$-gating properties, where negative amino acid substitutions increase unitary conductance and positive amino acids substitutions diminish conductance (Tong et al. 2014). Cx26G45, equivalent to Cx50G46, is a pore-lining residue in a narrow region of Cx26 GJ pore according to electron crystallography model, suggestive of this amino acids’ direct involvement of the ion permeation pathway (Maeda et al. 2009; Tong et al. 2014). Studying homology models of human Cx50 and locations of cataract-related mutations further highlight the pore-forming nature of this residue (Sarkar et al. 2014). Scanning mutagenesis experiments identify F43, G46, and D51 residues in Cx50E1 to be metal binding sites, pore-lining, and also involved in gating (Verselis et al. 2008). In summary there are a few structure-function studies of Cx50 addressing structural determinants of unitary conductance and $V_j$-gating, however exact factors controlling these properties remain unknown. Additionally as the mechanism of Mg$^{2+}$-modulation in Cx50 GJs have not yet been addressed at all and this is the first study to investigate Mg$^{2+}$-dependent
regulation of Cx50 GJs. Future studies investigating the impact of \([\text{Mg}^{2+}]_i\) on open probability or stability of closed single channel states would clarify specific effects of intracellular \(\text{Mg}^{2+}\).
2.5 References


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Chapter 3 – Discussion

3.1 Overall Study

This study explored the electrophysiological consequences of increasing negatively charged putative pore-lining residues in Cx50 and effects of magnesium-dependent modulation on Cx50 and mutant GJs. Surface electrostatics are proposed to be an important factor determining $\gamma_j$ of various GJs (Tong et al. 2014). Previous studies on putative pore-lining residue G46 in Cx50 specifically highlight the role of side chain charge as a positively charged amino acid substitution decreased conductance and negatively charged amino acid substitutions increased conductance (Tong et al. 2015b; Tong et al. 2014). We show here that the number of negative residues may be the limiting factor in determining $\gamma_j$ of Cx50 GJs as double (G8E-G46E and G46E-V53E) and triple (G8E-G46E-V53E) mutations all significantly increased the $\gamma_j$ of mutant GJs as compared to wildtype Cx50 (Chapter 2, Fig 2.4). Proposed pore-lining NT and E1 domains are thought to contain structural determinants of $V_j$ gates determined by structural simulations and functional studies (Maeda et al. 2009; Oshima et al. 2007; Oshima et al. 2008; Xin et al. 2010). Surprisingly, neither individual nor combination mutations induced significant changes in $V_j$-gating properties (Chapter 2, Table 1-3). A possible explanation is that these residues may not be the main determinants of gating parameters, and other amino acids within NT and E1 are more pertinent in this respect (Tong et al. 2015b; Xin et al. 2010). Additionally, introducing negative charge into putative pore-lining residues significantly increases $\text{Mg}^{2+}$-sensitivity of Cx50 GJs (Chapter 2, Fig 2.15). Investigating the role of individual mutations (G8E, G46E, and V53E) reveals the possible contribution of G46E and V53E residues in
mediating Mg$^{2+}$-dependent modulation of $\gamma_j$. Thus we show the importance of pore electrostatics in determining both $\gamma_j$ and Mg$^{2+}$-sensitivity of Cx50 GJs.

3.2 Determining factors of $\gamma_j$ and $V_j$-gating properties

$\gamma_j$ and $V_j$-gating parameters are believed to vary depending on connexin composition as marked differences were observed between properties of Cx32 and Cx43 or Cx36 and Cx50 (Eghbali et al. 1990; Tong et al. 2015b; Xin et al. 2010). A wide range in $\gamma_j$ may be an important factor in mediating differentiation processes in a conductance dependent manner (Veenstra et al. 1992). Pore-lining domains are most likely implicated in regulating channel conductance by altering pore diameter, using electrostatic forces to facilitate ion permeation, or a combination of both but the exact mechanism regulating GJ currents is largely unknown (Gong et al. 2001; Tong et al. 2014; Xin et al. 2010). While pore diameter may be an important factor in determining conductance of some connexins, functional studies of disease-linked mutations and permeation of PEG probes of different sizes reveal a lack of correlation between pore size and rate of ion permeation in several other connexins (Gong et al. 2001; Oh et al. 1997). Cx37, the largest conducting single channel, may perhaps accommodate a high rate of ion permeation due to a ring of negative charges along the pore which increases local cation concentrations to improve ion permeation rate (Veenstra et al. 1994b). Studies on Cx50 identify that $\gamma_j$ may rely more on pore electrostatics rather than pore diameter specifically at position G46 (Tong et al. 2015b; Tong et al. 2014). Our results further support this finding as combination mutations (G8E-G46E, G46E-V53E, and G8E-G46E-V53E), with a greater number of negative charges which correlates to increased negative charge density along the pore as indicated by our homology structure models, significantly increase $\gamma_j$. Cx50 is a cation-prefering GJ and combination mutations with increased negative charges may increase cation concentrations near the pore to mediate a higher
rate of ion permeation (Srinivas et al. 1999a; Tong et al. 2014; Veenstra et al. 1994b). Furthermore, the glutamic acid residues we introduced have a larger side chain size than both the original glycine (G8 and G46) and valine (V53) residues, but $\gamma_j$ was still increased in combination mutant GJs which is evidence that electrostatics rather than pore diameter is a determinant of conductance in Cx50 GJs. Preliminary data on Cx50G46A and Cx50G46L did not show altered Cx50 $\gamma_j$, perhaps due to the relative minor changes in pore diameter which is not enough to form a steric barrier to ion permeation. Our investigation of engineered negatively charged putative pore-lining mutations indicate a possible reason for increased $\gamma_j$ after the application of negative thiol reagents (Kronengold et al. 2003b). Additionally, Cx37 has a glutamic acid residue in position 53, so our study also suggests a possible role of this amino acid on determining Cx37 $\gamma_j$. As Cx50V53E did not alter $\gamma_j$ as an individual mutation but was able to induce a significant increase in $\gamma_j$ as combination mutations, it may be proposed that interactions between amino acid residues are necessary to accommodate increased ion permeation (Banach et al. 2000; Sanchez et al. 2013). A question put forth for future considerations is if $\gamma_j$ can be saturated, i.e. if we continue adding negatively charged residues to pore-lining areas of cation-preferring GJs, can increases to $\gamma_j$ persist or is there a upper-limit? Another factor to investigate is if anion-preferring GJs can be similarly regulated. G46E GJs successfully increased $\gamma_j$ as compared to wildtype Cx50 in Mg$^{2+}$-free ICS validating position G46 in Cx50 as a more pertinent residue regulating $\gamma_j$ (Tong et al. 2015b; Tong et al. 2014). G8E GJs did not alter $\gamma_j$ which may serve to validate our homology structure model visualizing G8E side chain facing away from the pore lumen (Chapter 2, Fig 2.16B).

We originally predicted $V_j$-gating properties to be drastically altered with the combination mutations, however our results show that gating parameters were largely unaffected.
(Chapter 2, Table 1). The $V_j$-sensor is believed to be located across the NT and E1 domains as both are pore-forming structures (Maeda et al. 2009). By constructing combination mutations we may have distributed the negative charges along the GJ, which does not significantly impair gating sensitivity, even though point mutations in other positions within NT and E1 domains of Cx50 do change $V_j$-gating properties (Peracchia et al. 2005; Tong et al. 2015b; Tong et al. 2014; Xin et al. 2010). Another factor to consider is that negatively charged substitutions may not alter gating parameters as much as positively charged mutations, as exemplified by previous studies on Cx50G46 residue (Tong et al. 2014). Preliminary studies of human Cx50G46R, a cataract-linked mutation, showed that this mutation rendered GJs non-functional. Unpublished data indicate no junctional currents were observed in cell pairs expressing this construct providing support for the idea that substitutions of large, positively charged residues such as arginine (R) result in dramatically altered Cx50 GJ properties. Studies on Cx50, as well as other connexins, also support the importance of particular amino acids over others in determining GJ properties (Musa et al. 2004; Tong et al. 2015a; Tong et al. 2015b; Xin et al. 2013). Thus, the amino acid residues investigated in this study may not be directly involved in determining $V_j$-gating properties or the negative mutations studied may not be sufficient enough to induce changes in gating parameters although $\gamma_j$ is significantly increased. Another mechanism for modulating $\gamma_j$ was explored by studying the electrophysiological response of wildtype Cx50 and mutant GJs to increases in $[\text{Mg}^{2+}]_i$.

3.3 Magnesium-dependent modulation of Cx50 mutant GJ channels

GJ properties are regulated by second messengers, phosphorylation, and intracellular cations to attenuate function (Loewenstein et al. 1978; Noma et al. 1987; Spray et al. 1981b; Spray et al. 1984). Intracellular Mg$^{2+}$ ($[\text{Mg}^{2+}]_i$) seems to decrease permeability of coupled
junctional membranes similar to mechanisms by which intracellular calcium abolishes permeability (Loewenstein et al. 1967). The uncoupling induced by increased \([\text{Mg}^{2+}]_i\) may be a protective mechanism to prevent the spread of cellular injury (Loewenstein et al. 1967). Cx37 GJs experience a voltage-dependent \(\text{Mg}^{2+}\) modulation of macroscopic currents (Banach et al. 2000; Ramanan et al. 1999). \([\text{Mg}^{2+}]_i\) has recently come to light as an important regulator of macroscopic Cx36 GJ conductance as a means of controlling synaptic plasticity (Palacios-Prado et al. 2013). Putative pore-lining, and negatively charged, aspartic amino acid (D47) in Cx36E1 determined to be \(\text{Mg}^{2+}\)-binding site as mutations to this residue abolished \(\text{Mg}^{2+}\)-sensitivity and substitution of aspartate into \(\text{Mg}^{2+}\)-insensitive Cx43 conferred \(\text{Mg}^{2+}\) sensing properties (Palacios-Prado et al. 2014). However, the impact of \([\text{Mg}^{2+}]_i\) on regulating \(\gamma_j\) has not been explored in most connexins. We engineered several negatively charged mutations in proposed pore-lining domains in order to investigate \(\text{Mg}^{2+}\)-sensitivity of Cx50 GJs with respect to \(\gamma_j\). Indeed our results agree with previous studies as \(\gamma_j\) of combination mutations (G8E-G46E, G46E-V53E, and G8E-G46E-V53E) were significantly reduced in the presence of elevated \([\text{Mg}^{2+}]_i\) (Chapter 2, Fig 2.9). Studying individual mutations (G8E, G46E, and V53E) reveal a role of the E1 mutations G46E and V53E as possible \(\text{Mg}^{2+}\)-sensors (Chapter 2, Fig 2.9). Our study provides further evidence for the role of negatively charged putative E1 pore-lining residues in playing a role in \(\text{Mg}^{2+}\)-dependent regulation of \(\gamma_j\) in Cx50 GJs.

\([\text{Mg}^{2+}]_i\)-mediated regulation of other channels have been studied in more detail and may provide a clue into the mechanism of \(\text{Mg}^{2+}\)-dependent modulation of GJs. For example, \(\text{Mg}^{2+}\) influences the function of BK channels, cyclic-GMP activated channels, and potassium intermediate/small conductance calcium-activated (KCa3.1) channels by regulating ion permeation rate (Colamartino et al. 1991; Jow et al. 1998; Stoneking et al. 2014). \(\alpha\) subunit of
BK channels binds Mg\(^{2+}\) to stabilize the channel’s open state and consequently upregulate function (Shi et al. 2001). Mg\(^{2+}\) can differentially regulate BK channels as binding to the open state decreases channel closing rates, or binding to the closed state to effectively increase channel opening rates (Chen et al. 2011a). Negatively charged pore-lining residues of BK channels are thought to be ideal for Mg\(^{2+}\)-interactions due to electrostatics and thus the negatively charged Cx50 mutations may be key in Mg\(^{2+}\)-dependent regulation (Zhang et al. 2006). Cyclic-GMP activated channels in retinal rod cells binds Mg\(^{2+}\) at pore-lining binding sites to exercise its modulatory effects on channel conductance (Colamartino et al. 1991). \([\text{Mg}^{2+}]_i\) blocks KCa3.1 channels and reduces channel opening rate by competing with Ca\(^{2+}\) for the activation site (Stoneking et al. 2014). Mg\(^{2+}\) may regulate GJs via allosteric binding sites to enhance interactions with other regulatory factors as seen in KCa3.1 channels to modulate overall channel function (Golowasch et al. 1986). Another mechanism of Mg\(^{2+}\)-dependent modulation, in addition to Mg\(^{2+}\) binding to sites, is a screening effect by Mg\(^{2+}\) altering the electrostatic profile of the channel (McLaughlin et al. 1971). Multiple negatively charged sites along the channel pore bind divalent cations and subsequently change the potential of the channel pore to alter membrane depolarization state (Blaustein et al. 1968). In summary, by studying Mg\(^{2+}\)-modulation of other channels we may understand the mechanism behind Cx50 GJ modulation. This regulation may occur through binding sites to directly alter conductance, allosteric binding sites, or through screening effects (Golowasch et al. 1986; McLaughlin et al. 1971; Shi et al. 2001).

Low free \([\text{Mg}^{2+}]_i\) has been shown here to alter wildtype Cx50 and several mutant GJ channels with increased negative charge density. Internal Mg\(^{2+}\) is usually bound to ATP in an inverse relationship with an increase in \([\text{Mg}^{2+}]_i\) caused by ATP dissociation and a decrease due
to ATP binding Mg$^{2+}$ (Grubbs 2002; Luthi et al. 1999). Intracellular Mg$^{2+}$ levels in the lens, usually higher (89 mg/ lens g), show a marked decrease with age (30 mg/ lens g) and is also decreased in cataract tissue (0.71 mg/lens g) (Swanson et al. 1971). As Mg$^{2+}$ is an important cofactor for many important enzymes, including the Na$^+$/K$^+$ ATPase pump, Mg$^{2+}$-deficiency is associated with several disease such as cataracts, glaucoma, and diabetic retinopathy (Agarwal et al. 2013). Aberrant GJ-mediated intercellular communication may possibly be curbed with an introduction of Mg$^{2+}$, as a dietary supplementation of Mg$^{2+}$, along with vitamins A, C, and E, have recently been shown to alleviate Cx26-mediated hearing loss (Green et al. 2016; Thatcher et al. 2014). More research is imperative in understanding the mechanism of Mg$^{2+}$-dependent modulation of Cx50 GJs as Mg$^{2+}$ dysregulation is implicated in lenticular diseases (Agarwal et al. 2013; Sun et al. 2000).

3.4 Physiological and pathological role of divalent cations

Divalent cations play important roles in maintaining healthy physiology in various tissue types as enzyme cofactors, channel regulators, plasma membrane regulation, and more (Gaburjakova et al. 2016; Kasuya et al. 2016; Mao et al. 2015; Quiroga-Roger et al. 2015). Calcium and magnesium are both required to maintain intestinal membrane permeability and integrity (Cassidy et al. 1967). Alkaline earth metal cations modify single channel currents of cardiac ryanodine receptors indicating the importance of these divalent cations in a normal physiological setting (Kasuya et al. 2016). Calcium is preferentially associated, with other divalent cations to a minor extent, with cholesterol rich regions to alter cholesterol organization in the plasma membrane (Mao et al. 2015). Manganese, magnesium, and cobalt enhance and regulate activity of the human ribokinase enzyme (Quiroga-Roger et al. 2015).
effects regulate calcium association of calmodulin and by extension calmodulin function (Ababou et al. 2015).

Increases in calcium is associated with neuropathological conditions and sickle cell-like erythrocyte phenotype, amongst other pathophysiological manifestations (Alvarez-Ferradas et al. 2015; Eaton et al. 1978). Negative residues within the binding site for zinc of β-amyloid enhance zinc-dependent DNA association possibly contributing to Alzheimer’s etiology (Khmeleva et al. 2015). Zinc is involved in differential expression of apoptotic factors in prostate cancer cells, highlighting the widespread roles of zinc (Sztalmachova et al. 2012). Copper ions trigger intracellular accumulation of human superoxide dismutase, mimicking ALS-like symptoms (Li et al. 2013). Increased exposure to nickel can alter intracellular calcium homeostasis and lead to consequences relating to pulmonary diseases (Cortijo et al. 2010). Thus magnesium, along with other divalent cations, are vital in maintaining physiological conditions as they comprise a multitude of roles within the cell and dysregulation of cations can contribute to various disease states.

3.5 Limitations and Future Studies

Our results may have identified the effects of pore electrostatics on $\gamma_j$ and $[\text{Mg}^{2+}]_i$-dependent regulation of Cx50 GJs. However we do address some limitations of this study. Due to a lack of an atomic structure model for Cx50, the residues chosen for mutagenesis are an estimate of pore-lining positions. Cx26 is the only connexin of which there is a highly resolved (3.5 Å) crystal structure model. GJ properties, including $\gamma_j$ and $V_j$-gating, are thought to be conserved within similar domains due to high sequence identity, however there are differences between connexins which may modify predicted main chain structures. This would mean that structural modifications are not necessarily effects of pore-lining mutations we had introduced.
To address this very important limitation, a high resolution crystal structure of Cx50 would be the best way to validate our functional data with a structural model. By altering GJ properties with the mutations, we could provide support for the pore-lining nature of G8, G46, and V53 in Cx50. Performing electrophysiological experiments on other putative pore-lining residues may help confirm their location as pore-lining. We identified that the triple mutation (G8E-G46E-V53E) was most competent in increasing Cx50 $\gamma_j$ among the studied mutants. Our study strongly validates the importance of electrostatics in determining $\gamma_j$ and it may be interesting to investigate an extension of these results by mutating several putative pore-lining residues at once. As mentioned previously, another question to address is if there is an upper limit to $\gamma_j$ or if we are able to continually increase $\gamma_j$. Generating positively charged putative pore-lining Cx50 residues have already been shown to reduce $\gamma_j$, the effects of multiple positive charges on $\gamma_j$ is yet to be determined. Pore electrostatics seem to play an important role in determining Cx50 $\gamma_j$, however more work needs to be completed to understand the mechanistic significance of residue charge on GJ properties.

Our study identifies single mutations (G46E and V53E) as possible Mg$^{2+}$ binding sites. A structure of Cx50 and Mg$^{2+}$ in binding sites, such as one recently resolved for Cx26, would be strong evidence for G46 and V53 as binding areas (Zonta et al. 2014). Similar to Palacios-Prado and colleagues’ studies on magnesium sensitivity of Cx36, it would be interesting to examine the effects of magnesium on macroscopic currents over time similar to the decrease macroscopic currents due to higher [Mg$^{2+}$], in question within minutes (Palacios-Prado et al. 2014; Palacios-Prado et al. 2013). Investigating a greater range of intracellular [Mg$^{2+}$] and asymmetric application of intracellular Mg$^{2+}$may elicit further details affinity of Mg$^{2+}$-binding to have the
modulatory effects. While charges of residues seem to dictate $\gamma_j$ and modulation by divalent cations in Cx50, most specifics of the mechanism underlying this regulation remains unknown.

### 3.6 Summary

Here we have shown the importance of increasing negatively charged residues on Cx50 GJ properties including $\gamma_j$ and Mg$^{2+}$-sensitivity by mutating several putative pore-lining residues (G8E, G46E, and V53E). Combination mutations (G8E-G46E, G46E-V53E, and G8E-G46E-V53E) all significantly increased $\gamma_j$ with G8E-G46E-V53E GJs displaying the largest $\gamma_j$ recorded thus far. Mutations introducing negatively charged glutamate residues into Cx50 have already been shown to increase Cx50 $\gamma_j$, but we show here that increasing negative charge with multiple mutations are even more effective in increasing $\gamma_j$. GJs with high $\gamma_j$, such as Cx37 GJ, is predicted to have rings of negatively charged amino acids within the pore which could play a role in facilitating increased ion permeation. Further studies investigating the saturation limit of Cx50 $\gamma_j$ as well as the effects of multiple positive charges in putative pore-lining domains may be pivotal in understanding the full implication of pore electrostatics on GJ properties. Investigating Mg$^{2+}$-sensitivity properties of the mutant GJs in this study identified a significant reduction in $\gamma_j$ with increased [Mg$^{2+}$]. Studying individual point mutations to identify specific residues responsible for this property ascertain G46E and/or V53E mutations as possible Mg$^{2+}$ binding sites. While Mg$^{2+}$-dependent modulation of Cx36 has been explored in detail, this is the first study to address the Mg$^{2+}$-sensitivity of Cx50 GJs. More work regarding the range of [Mg$^{2+}$]i able to induce $\gamma_j$ changes as well as structural models of Mg$^{2+}$ binding sites would enhance our understanding of a mechanism underlying Mg$^{2+}$-dependent modulation.
3.7 References


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